COMPARISON OF COMMERCIAL DNA EXTRACTION KIT PROTOCOLS FOR THE EXTRACTION OF DNA FROM BONES PREPARED USING THE NOVEL THERALIN™ METHOD

by

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Dedicated to the victims of crime who
remain unidentified

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List of Abbreviations

- DNA = Deoxyribonucleic Acid
- STR = Short Tandem Repeat
- NamUs = National Missing and Unidentified Persons System
- EDTA = Ethylenediaminetetraacetate
- PCR = Polymerase Chain Reaction
- DTT = Dithiothreitol
- PCI = Phenol-Chloroform-Isoamyl alcohol
- qPCR = Quantitative Polymerase Chain Reaction
- Ct = Cycle Threshold
- LA = Large Autosomal
- SA = Small Autosomal
- EPG = Electropherogram
- RFU = Relative Fluorescent Units
- MPS = Massively Parallel Sequencing
- SNP = Single Nucleotide Polymorphism
- IGG = Investigative Genetic Genealogy

Abstract

The extraction of DNA from bones to produce high quality samples is extremely important in cases involving unidentified human remains and victims of mass disasters, and many times in these cases, only human bone is available as a source of DNA that can be used to identify the individual. A novel method of preparing bones for extraction using Theralin™, a precipitating fixative that demineralizes bone, has been developed by researchers at George Mason University. This novel method essentially transforms the bone into a gum-like state that allows for the bone material to be cut into smaller pieces and then digested using lysis buffers. Research has not yet been performed to determine an ideal method of DNA extraction from Theralin-prepared bone, and this study seeks to answer that question. In this study, four different methods of DNA extraction using commercial extraction kits were carried out on bone samples that were prepared using the Theralin method. These extraction methods were the Qiagen QIAamp® DNA Investigator kit protocol with MinElute columns, the PrepFiler™ BTA Forensic DNA Extraction kit protocol with PrepFiler magnetic beads, a combination method using the Qiagen lysis buffers with the PrepFiler magnetic beads, and a combination method using the PrepFiler BTA lysis buffers with the Qiagen MinElute columns. Following extraction with these four methods, the DNA extracts were quantified using the Quantifiler™ Trio DNA Quant kit, and the quantification values of the extracts were compared to determine which method provided the highest amount of DNA. The extracts were amplified using the GlobalFiler™ PCR Amplification kit and genotyped using the Applied Biosystems 3500 Genetic Analyzer with GeneMapper™ ID-X v1.6 software. The STR profiles were compared to the known profiles of the bone samples and analyzed for allelic dropout and signal strength metrics to determine the quality of each DNA extract. The results of this study provided insight into the usefulness and

efficiency of the Theralin method of bone preparation, combined with a commercially available DNA extraction technique, to give quality DNA extracts that can be used for downstream applications.

Introduction

The extraction and purification of deoxyribonucleic acid (DNA) from forensic samples is the essential first step in the DNA analysis process. In this step, DNA is isolated from the rest of the cell material in the sample, and this DNA is then used for quantification, amplification, and genotyping. The quantity of the DNA that is obtained, in conjunction with the quality of that DNA, will impact the success of short tandem repeat (STR) genotyping, which is utilized across forensic DNA labs for identification and databasing purposes. The extraction of DNA from challenging samples such as bone is vital in forensic cases that involve unidentified human remains, missing persons, or mass disaster victims (1).

The National Missing and Unidentified Persons System (NamUs) reports that there are up to 600,000 missing persons cases reported every year (2). Their database shows that 4,400 unidentified individuals are found each year (2). At the time of writing this paper, they report that there are 14,534 open cases involving unidentified individuals, and 23,833 open cases involving missing persons (3). It is critical that these cases are solved and that the families of these victims' experience peace. In these cases, the bones that are available for testing have usually been exposed to different environmental conditions for extended periods of time (1). The extraction of DNA from bone is not, at this time, a one size fits all approach. Different extraction methods are ideal for different types of bone samples; therefore, it's important to research a variety of methods of DNA extraction from these samples and create a higher possibility of the successful utilization of DNA analysis for identification of victims.

A new method of preparing bones for DNA extraction has recently been developed that involves the use of Theralin™. In a paper published by Mueller, et. al., Theralin is described as:

> A precipitating fixative that contains: (i) permeation enhancers that decrease the tissue penetration rate, (ii) reversible cross-linkers that stably cross-link biomolecules during tissue preservation while allowing full biomolecule recovery during extraction, (iii) kinase and phosphatase inhibitors that prohibit ex vivo alterations of protein phosphorylation, and (iv) an osmotically balanced buffer and a carboxylic acid that maintain tissue morphology during the fixation process. (4).

Theralin was originally developed to be used in the medical field, specifically in cancer research. Researchers discovered that, in addition to the properties that Theralin has as a tissue preservative, it can also cause the decalcification of bone (4). In an early study, scientists found more quantifiable DNA in Theralin-fixed bone samples than in formalin-fixed bone samples (4). In the earlier study, traditional forensic DNA extraction protocols were not tested; instead, whole cell lysates were printed onto nitrocellulose slides to measure the amount of DNA present (4). The scientists were able to obtain DNA from these bones using this method, and researchers at George Mason University realized that this method could potentially be used for forensic purposes.

When Theralin is used for forensic purposes, its intended use is to demineralize the bone material prior to the digestion of the material and DNA extraction. Demineralization of bone material is essential when extracting DNA from bones. Booncharoen, et. al. writes that bones are primarily composed of osteocytes and an extracellular matrix, which is made of hydroxyapatite (5). Hydroxyapatite is composed of calcium and phosphate and is the reason why bones have a solid structure (5). The

demineralization step removes the calcium and phosphate ions, which softens the bone and allows for the DNA to be accessible during extraction. The demineralization of bone pieces using Theralin causes them to go from their normal, hard structure to a gum-like state that can be cut with a sterile scalpel.

There are other methods of preparing bones for extraction that have been developed for forensic purposes. Harrel and Hughes-Stamm write that often, ethylenediaminetetraacetate (EDTA) is used at high concentrations to chelate mineral ions and demineralize the bones (6). Typically, this step takes place after the bones have been crushed into powder. Hofreiter, et. al report that EDTA can be detrimental to the recovery of DNA, as it retains some DNA from the demineralization process (7). Not only can EDTA retain valuable DNA, but it also has a limit to the amount of bone powder that can be demineralized at one time (7). Theralin has the potential to increase the recovery of DNA with fewer limits to the amount of bone that can be used. In addition to the potential increased recovery, Theralin also uses whole pieces of bone, as opposed to bone powder. Most protocols that are written for bone DNA extractions involve powdering the bone prior to demineralization (6). This is destructive to the bone sample and can introduce contamination (6). The powderization also uses physical force to crush the bone, which can cause the DNA present in the sample to degrade. The Theralin demineralization process creates an easier preparation method and provides less opportunity for both contamination and degradation.

The Theralin method of bone preparation is unique in that the bone can be demineralized and digested using only the Theralin itself and the buffers that are included in commercial extraction kits. After the bone material has been cut, it is incubated with the lysis buffers that are included in the commercial kits, per the protocol for each kit. The lysis buffers and Proteinase K

work together to complete the digestion of the bone material. The lysis buffers cause the breakdown of both the cell and nuclear membranes, which allows for the release of the DNA. Proteinase K works to dissolve the cellular proteins in the sample (8). After these incubations and subsequent spikes with Proteinase K, much of the bone material has essentially dissolved into liquid with a complete digestion. This process is significantly different from the lengthy one that requires powderization of the bone and the use of a complicated demineralization buffer. This is a time-consuming process, and the demineralization buffer usually requires multiple ingredients that would need to be purchased or made, in addition to the reagents that are included in commercial extraction kits. The difference between Theralin and other methods of bone preparation is intriguing enough to warrant further research to determine optimal methods that can be used to extract DNA from the bone material.

In this study, four different extraction methods were tested on a bone sample from Texas State University. The bone sample used for this project had been exposed to the hot and humid environment in San Marcos, Texas for 304 days before being buried in a shallow grave. The following commercial extraction kits were chosen because of their ability to be used on challenging samples, such as bone. The first extraction method tested was the Qiagen QIAamp® DNA Investigator kit (Qiagen, Germantown, MD), utilizing only the Qiagen buffers and protocol. Second, the PrepFiler™ BTA Forensic DNA Extraction kit (ThermoFisher Scientific, Waltham, MA) was also tested, utilizing only the PrepFiler buffers and protocol. Then, two methods of extraction were tested that combined the Qiagen and PrepFiler BTA protocols. The first method used the Qiagen lysis buffers; Buffer ATL and Buffer AL, with the PrepFiler magnetic beads and the

corresponding PrepFiler BTA protocol (9,10). The second combination method tested utilized the PrepFiler lysis solution, which contains PrepFiler BTA Lysis Buffer, 1.0M dithiothreitol (DTT), and Proteinase K, and the PrepFiler Lysis buffer, with the Qiagen MinElute columns and the corresponding Qiagen protocol (10,9). Following extraction, the DNA was quantified using the Quantifiler™ Trio DNA Quantification Kit (ThermoFisher Scientific, Waltham, MA), and the quantification values and calculated degradation indices were compared. The DNA extracts were amplified using the GlobalFiler™ PCR Amplification kit (ThermoFisher Scientific, Waltham, MA) and genotyped using the Applied Biosystems 3500 Genetic Analyzer (ThermoFisher Scientific, Waltham, MA) and GeneMapper™ ID-X v1.6 software.

The PrepFiler BTA Forensic DNA Extraction kit was specifically created to be used for extracting DNA from bones and teeth. According to the Product Overview, this kit has a shorter processing time and calls for only a couple of tube transfers, which gives the user less chances to contaminate the sample (11). PrepFiler Magnetic Particles, which are silica coated beads, are used in the purification step of the kit's protocol. The DNA that is released during the lysis step is bound to the magnetic beads, purified with the kit's two wash buffers (PrepFiler Wash Buffer A and PrepFiler Wash Buffer B), and then eluted from the Magnetic beads with the Elution buffer (10). The Qiagen QIAamp DNA Investigator Kit has also been created for use with bone samples. It's been optimized to purify DNA and produce quality STR profiles (12). The Qiagen MinElute columns, which are spin columns containing a silica matrix, are used in the purification step of the kit's protocol (12). The columns work in a similar manner to the PrepFiler magnetic beads: the DNA is bound to the silica matrix in the column, washed with the two wash buffers provided with the kit and with ethanol, and eluted with Buffer ATE (9,12).

The research question that this study seeks to answer is: Of the four extraction methods tested, which method is the most effective to produce DNA extracts of a sufficient quantity and quality that can be used for downstream applications such as STR genotyping? It is hypothesized that at least one of the extraction methods will provide high quantities of quality DNA that produce STR profiles after amplification and genotyping.

The initial goal of this project was to determine if any of these extraction methods using commercial extraction kits, in conjunction with the Theralin demineralization process, give sufficient quantities of DNA for STR genotyping. Preliminary studies that have been performed on bone samples prepared with Theralin show that typically, very minute quantities of DNA are obtained (M. Wilson, personal communication, September 19, 2023). These preliminary studies have used only the Qiagen QIAamp DNA Investigator kit protocol with MinElute columns (M. Wilson and G. Williams, personal communication, 2023). Additional extraction methods that use commercial DNA extraction kit protocols were compared in this study to determine if the quantity of recovered DNA from Theralin-demineralized bone samples could be improved. The quantity of DNA that was sufficient for downstream processing, specifically STR genotyping, was determined following the analysis of the STR profiles that were developed in this project. The donor of the bone sample has a DNA profile that is known to the researcher, and the profiles that were developed in this project could be compared to the known profile. The profiles were analyzed for quality by calculating a signal strength metric, and by looking at allelic dropout.

Literature Review

Researchers in forensic science who are interested in finding methods to extract DNA from bones have long taken cues from biologists who study ancient DNA. One of the first methods of extraction from ancient bones that has influenced forensic science is the Rohland method (13). The buffers and techniques that are used in the Rohland method were determined after a comparative study done by Hofreiter and Rohland (13,14). In this comparative study, researchers compared published extraction methods with commercial DNA extraction kits to determine the optimal lysis buffers and purification methods to use to obtain quality DNA from powdered bone samples (14). The results of this study showed that Proteinase K and EDTA were the only reagents that, when added to the digestion buffer, had a beneficial effect on the amount of DNA that was recovered (14). This study compared the use of chaotropic salts or nonchaotropic salts to bind the DNA to silica, and they found that a modified silica method with guanidinium thiocyanate (GuSCN) produced quality results (14). The results of this comparative study were significant in determining ideal methods for extracting and purifying DNA from powdered bone samples, as many of the methods had never been compared before.

Based on the results of the comparative study, the Rohland method was developed (13). The Rohland protocol that was published in 2007 establishes the use of a lysis buffer that contains only EDTA and Proteinase K (13). The protocol calls for the addition of a silica suspension, which is added to the sample with a binding buffer composed of GuSCN, sodium chloride (NaCl), and Tris (13). The silica suspension must have a pH of 4.0 for the DNA to bind successfully, so HCl can be added to the sample until the 4.0 pH is reached (13). The sample is washed with wash buffer composed of ethanol, NaCl, Tris, and EDTA, and then eluted with TE

buffer (13). The authors of this protocol state that the method can be used for ancient and modern bone samples (13).

Another method of extraction from ancient bones was the Dabney method, published in a study in which the mitochondrial genome of a Middle Pleistocene cave bear was sequenced (15). This study establishes that DNA fragmentation occurs over time, so the DNA fragments that are being recovered during extraction are very small (15). The size of the fragments was taken into consideration when developing this method, which was based on the previous method developed by Rohland (13,15). The Dabney method uses an extraction buffer that contains EDTA and Proteinase K to lyse the cells in the bone samples (15). Following lysis, the DNA is purified using silica-based particles, when the DNA particles are bound to the silica-based column with a binding buffer composed of sodium chloride, guanidine thiocyanate, and sodium acetate (15). An ethanol wash buffer is used for the desalting of the bound DNA fragments (15). The Dabney protocol is significant because it showed that it is possible to successfully extract short fragments of DNA from ancient bones.

In 2018, after the success of the Dabney method in extracting shorter fragments of DNA, the Rohland protocol was updated (16). The lysis buffer in this updated protocol consists of EDTA, Proteinase K, Tween 20 (a detergent), and water (16). The Dabney method directly influenced the choice of binding buffer, due to the researchers seeking to recover shorter fragments of DNA from the bones than what had been recovered in the previous protocol (15,16). The updated Rohland method establishes two binding buffers that are chosen based on the size of fragments to be recovered, and both binding buffers contain guanidine hydrochloride and Tween 20 (16). To purify the DNA, either silica spin columns or silica-coated magnetic beads are utilized, and it was found that when the silica-coated magnetic beads were used for

extraction, shorter DNA fragments (≤35 base pairs), were recovered than when the silica spin columns were used (16).

Many studies have also been performed that compare different methods of DNA extraction from bone samples. One study that was performed by Hasap, et al. on both fresh bone samples and casework samples compared the PrepFiler BTA kit to a method that used modified organic extraction with silica columns and the QIAamp DNA Investigator kit protocol for purification (17). This research found that when DNA was extracted from fresh bone with the PrepFiler BTA kit, the DNA that was obtained had a median concentration of 59.5ng/µl (17). The median concentration of DNA obtained from the Phenol-Chloroform-Isoamyl alcohol (PCI)-silica method was only 0.620ng/µl (17). The results of the STR genotyping that was performed on casework samples using the Qiagen Investigator IDplex Plus kit showed that the PrepFiler BTA kit produced a median of 30 alleles, while the modified PCI-silica based method produced a median of 8 alleles. (17). This study concluded that the PrepFiler BTA kit is better suited for casework samples (17).

In the study done by Harrel and Hughes-Stamm, instead of a powdered bone sample, the use of whole bone chips for DNA extraction was evaluated (6). The purpose of this study was to develop a method of extracting DNA from bones that did not require the time-consuming, expensive, and potentially contamination-inducing step of powdering the bone (6). The bones chosen for this project had been exposed to different conditions; some were burned, some decomposed, and some embalmed (6). The researchers extracted DNA from the bone chips using the PrepFiler BTA Forensic DNA Extraction kit with the Automate Express (ThermoFisher Scientific, Waltham, MA), which is an instrument that allowed for the automated use of the PrepFiler kit (6). This method was compared to the manual use of the PrepFiler BTA Forensic

DNA Extraction kit and the Qiagen MinElute columns (6). The results of this study showed that more DNA was obtained when the manual extraction methods were used on the powdered bone samples, but these results weren't statistically significant (6). They also found that the bone chips yielded DNA that was more degraded than the DNA from powdered bone (6). The researchers were able to develop full STR profiles from both the whole bone chips and the powdered bone (6). These findings are important to the current study because when bone is prepared using Theralin, the DNA is extracted from pieces of whole bone, not powdered bone.

Parson, et.al performed a study on the development of complete mitochondrial genomes, instead of only the control region of mitochondrial DNA (mtDNA), from hair shaft samples using massively parallel sequencing (MPS) (18). While this study was not performed on bone samples, one of the extraction protocols that was tested for this study involved the use of the Qiagen lysis buffers with the PrepFiler Magnetic Particles (18). This extraction protocol was used to recover DNA from three different hair samples, and extracts from two of these samples produced high mtDNA quantification values, allowing them to be used for the downstream processing in this study (18). The results of this study that used a combination extraction method of PrepFiler Magnetic Particles with Qiagen lysis buffers influenced the decision about which extraction methods to use on bones that were prepared using Theralin.

Research studies that have examined the extraction of DNA from bone samples have pointed out the difficult nature of utilizing bone samples that have been exposed to different environmental elements. Latham and Miller published a paper that describes that exposure to environmental factors will impact the way the bones are preserved, which will then impact the quality of DNA obtained from those bones (1). These factors can include temperature, moisture, microorganism activity, soil composition, and pH of the environment (1). The authors state that

even more than the age of the bone sample, the environment that it is exposed to will greatly impact the degradation of the DNA molecules (1). This information is important because it helps scientists to understand the conditions that human remains are exposed to before they are eventually found and relocated to a secure, indoor location. The challenging bone samples that are utilized in this project have been exposed to different environmental elements, in an effort to mimic real-life casework.

The papers discussed in this review were important in developing the current research project. The findings of Rohland and Dabney show that silica-based methods are ideal for extracting DNA from bone (13,15,16). In the current project, the PrepFiler Magnetic Beads that are silica-coated and the Qiagen MinElute columns that are silica-based were used for the purification of the DNA samples. These papers have also shown that comparisons between the PrepFiler BTA Forensic DNA Extraction kit and the Qiagen methods of extraction are common, and that there is an opportunity to further optimize these protocols for use with whole bone chips. Many previous studies involve testing extraction protocols that combine methods, and it was decided to combine commercial extraction kits in this research to determine if one of the combination methods would yield more ideal results than the standard extraction protocol for each kit. The current study seeks to offer insight into possible improvements to methods used on whole bone chips that have been prepared using Theralin.

Materials

- Qiagen QIAamp® DNA Investigator Kit
- PrepFiler™ BTA Forensic DNA Extraction Kit
- Quantifiler™ Trio DNA Quantification Kit
- GlobalFiler™ PCR Amplification Kit

- GeneMapper™ ID-X software
- Quant Studio™ 5 Real-Time PCR System
- ThermoFisher Scientific Thermomixer
- Applied Biosystems 3500 Genetic Analyzer
- 1.0M DTT
- 96% ethanol
- 99.5% isopropanol
- Hi-Di Formamide
- ThermoFisher Scientific 20 µl, 50 µl, 200 µl, and 100 µl ART pipette tips
- 1.5mL microcentrifuge tubes
- 2.0mL microcentrifuge tubes
- 0.5mL PCR tubes
- Applied Biosystems MicroAmp Optical 96-Well Reaction Plate with Barcode
- Applied Biosystems MicroAmp Optical Adhesive Film Kit
- GraphPad Prism
- Excel
- Kimwipes
- Sterile scalpels
- PPE: gloves, lab coat, hairnet, face mask

Methods

Samples:

The bone sample that was used in this project was donated by Texas State University. This bone belonged to an individual who was donated to the Willed Body Donation Program at the Forensic Anthropology Center at Texas State University, and the body naturally decomposed at the Forensic Anthropology Research Facility (19). This specific individual, Donor 2013.037, died on July 13th, 2013, and was kept in cold storage until their body was moved to the Forensic Anthropology Research Facility (19). The individual decomposed on the surface of the ground for 304 days and was then buried in a shallow grave on May 16th, 2014 (19). On March 22nd, 2015, after 310 days, the individual was exhumed and stored in a unit at the Forensic Science Research Facility until it was transported to the lab on January 19th, 2016 (19). At the time of this research project, the individual from whom this bone belonged to had been deceased for 10 years and 3 months. The specific bone that was utilized for this project was the left tibia. A reference blood card was provided with the bone sample and an STR profile of the donor was developed by GMU researchers in 2021.

Preparation with Theralin – Demineralization:

The preparation of the bones with Theralin for this project was completed by Professor Georgia Williams (G. Williams, personal communication, September 11, 2023). The bone sample, 2013.037, was cut into four pieces that each weighed approximately 225mg. These four pieces of bone were placed into tubes, and 4mL of Theralin was added to each tube. A piece of bone in Theralin can be seen in **Figure 1.** The tubes were rocked at 55rpm for 4 hours. The four pieces of bone that each weighed 225mg were separated into two individual sets, totaling eight bone samples, each weighing approximately 100mg. There were three additional washes with

4mL of Theralin, each for four hours at 55rpm. The first set of bones (Sample 1, 2.1, 3.1, and 4.1) was removed from the rocker after one night of Theralin washes. The second set of bones (Sample 1.2, 2.2, 3.2, and 4.2) was removed from the rocker after 19 days. The second set of bones spent longer in Theralin because it had not fully demineralized after one overnight period. More Theralin needed to be manufactured for use in this project, and once that Theralin was obtained, the samples were fully demineralized and then digested further for extraction. When the samples were removed from the rocker, they were cut into 10 pieces of equal size, as seen in **Figure 2.** The 10 pieces of bone material, weighing approximately 100mg for each sample, were then rinsed first with 3mL of nuclease free water, followed by a second rinse with 2mL of nuclease free water. Each sample, consisting of 10 pieces of bone material, was stored in a 1.5mL microcentrifuge tube until digestion and extraction began.

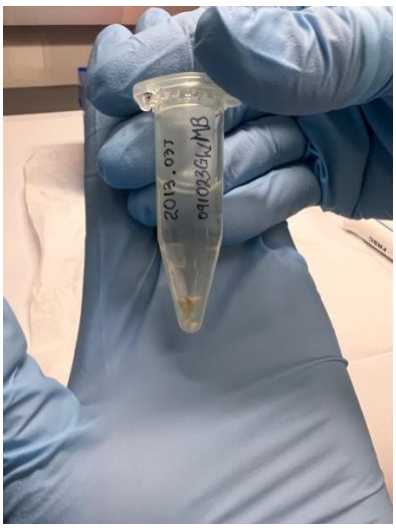

Figure 1. A bone sample in the tube containing Theralin.

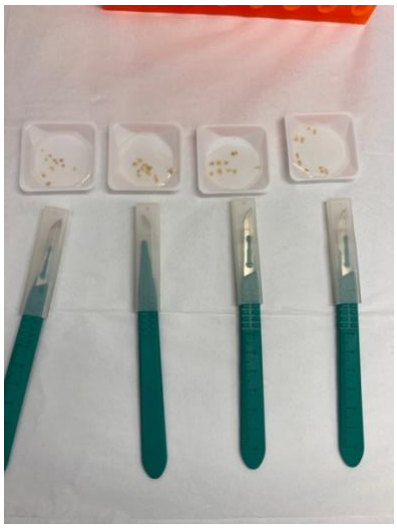

Figure 2. The bone samples from extraction set 2 after they were cut into 10 small pieces.

Extraction Methods

Four different methods of DNA extraction were tested in this project. The first method (referred to as Method 1) involved the use of the Qiagen QIAamp DNA Investigator kit protocol (9). DNA from samples 1 and 1.2 was extracted using this protocol. The lysis protocol includes

an overnight incubation of the samples with Buffer ATL and Proteinase K, followed by the addition of Buffer AL (9). This method was performed with several modifications to the protocol that were discovered in preliminary research done with the Theralin-treated bones (G. Williams, personal communication, August 14, 2023). These modifications were that the initial overnight incubation, done at 56ºC, also included shaking at 1100rpm, which is not written in the protocol. An additional modification to this protocol is that after the initial overnight incubation, samples were removed from the incubator and spiked with 20 µl of Proteinase K. Following this spike, the samples were placed back on the incubator at 56ºC and 1100rpm for 1 hour. This was repeated two more times for each sample. The spikes of Proteinase K assist with the full digestion of the bone samples before continuing with the extraction protocol. When the lysis is complete, ethanol is added to the samples to assist with binding to the columns, and they are transferred to the MinElute columns (9). In the columns, the samples are washed 3 times with Buffer AW1, Buffer AW2, and ethanol (9). Buffer ATE is added to the columns for the elution of the samples (9). In this study, the DNA was eluted into 30 µl of Buffer ATE. The rest of the protocol was performed following manufacturers' recommendations (9).

The second method of extraction was performed on Samples 2.1 and 2.2. This method (referred to as Method 2) used the Qiagen lysis buffers that are included in the QIAamp DNA Investigator kit, which are Buffer ATL and Buffer AL (9). The lysis protocol for these samples, to include full digestion of the bone material, was the same as is written above. Following the lysis, the PrepFiler Magnetic beads were added to the lysate. The PrepFiler protocol calls for 99.5% isopropanol to be used to help with binding (10). This protocol also uses two different wash buffers, Wash Buffer A and Wash Buffer B, to wash the Magnetic Particles once the DNA is bound to them (10). The PrepFiler Elution buffer is used in the final step to elute the DNA into

50 µl (10). The PrepFiler BTA protocol was followed according to manufacturers' recommendations after the addition of the Magnetic Particles (10).

The third method of extraction (referred to as Method 3) was performed on Samples 3.1 and 3.2. These samples underwent DNA extraction using only the PrepFiler BTA Forensic DNA Extraction kit. This includes lysis with the PrepFiler lysis solution, composed of PrepFiler BTA Lysis Buffer, 1.0M DTT, and Proteinase K (10). There were modifications made to this protocol as well. Once the PrepFiler lysis solution is added to the samples, there is a 2-hour incubation at 56ºC and 1100rpm (10). Following this incubation period, the samples were spiked with 7 µl of Proteinase K and then placed back on the incubator for 1 hour at 56ºC and 1100rpm. This was repeated two additional times. These Proteinase K spikes to digest the samples were also performed on the samples that were extracted using Qiagen lysis buffers, as is written above. Another modification that was made to this protocol is that 100mg of bone was used for each extraction. The protocol suggests the use of only 50mg of bone. This was done for the sake of consistency, so that each extraction method began with the same amount of bone sample. The rest of the PrepFiler BTA Forensic DNA Extraction kit protocol was followed according to manufacturers' recommendations (10).

The final method of extraction was performed on Samples 4.1 and 4.2. In this method (referred to as Method 4), the PrepFiler lysis protocol was followed, with the necessary modifications as written in the paragraph above. Following the addition of the PrepFiler Lysis Buffer, ethanol was added to the sample, per the Qiagen protocol (9). The samples were then transferred to the MinElute column, where the QIAamp DNA Investigator Kit protocol was followed according to manufacturers' recommendations (9). The DNA was eluted into 30 µl of Buffer ATE.

Sample Name	Extraction Set	Method of Extraction
B1	1	Qiagen QIAamp DNA Investigator Kit Protocol only (Method 1)
B1.2	2	Qiagen QIAamp DNA Investigator Kit Protocol only (Method 1)
B2.1	1	Qiagen Lysis Protocol w/ PrepFiler Magnetic Beads (Method 2)
B2.2	2	Qiagen Lysis Protocol w/ PrepFiler Magnetic Beads (Method 2)
B3.1	1	PrepFiler BTA Forensic DNA Extraction Kit Protocol only (Method 3)
B3.2	2	PrepFiler BTA Forensic DNA Extraction Kit Protocol only (Method 3)
B4.1	1	PrepFiler BTA Lysis Protocol w/Qiagen MinElute Columns (Method 4)
B4.2	2	PrepFiler BTA Lysis Protocol w/Qiagen MinElute Columns (Method 4)

Table 1. The method of extraction that each sample was subjected to in this study. Samples B1, B2.1, B3.1, and B4.1 were exposed to Theralin for one overnight period. Samples B1.2, B2.2, B3.2, and B4.2 were exposed to Theralin for 19 days.

There were reagent blanks associated with each sample in this study. The reagent blanks were subjected to the specific method of extraction for that sample, quantification, amplification, and genotyping.

Quantification

The samples were quantified using the Quantifiler Trio DNA Quantification Kit. All samples were quantified in triplicate. Five standards were made and included in the quantification run to generate the standard curve. The standards were diluted to 50ng/µl, 5ng/µl, 0.5ng/µl, 0.05ng/µl, and 0.005ng/µl using the Quantifiler THP DNA Dilution Buffer and the Quantifiler THP DNA Standard (20). There were 10 µl of Quantifiler THP PCR Reaction Mix

and 8 µl of Quantifiler Trio Primer Mix added to each well of the reaction plate (20). There were 2 µl of sample extract, 2 µl of each standard, and 2 µl of Quantifiler THP DNA Dilution Buffer, which served as the negative control, added to the correct well of the reaction plate, in accordance with the plate map. The quantification run was carried out on the QuantStudio™ 5 Real-Time PCR instrument (ThermoFisher Scientific, Waltham, MA).

Amplification and Genotyping

The samples were amplified using the GlobalFiler PCR Amplification kit and the Veriti Thermal Cycler (ThermoFisher Scientific, Waltham, MA). The amplification was set up using 0.5mL PCR tubes. There were 7.5 µl of GlobalFiler Master Mix and 2.5 µl of GlobalFiler Primer Mix added to each tube (21). There was 15 µl of each sample added directly to each tube, and the target amount of each sample was 1ng, in accordance with the recommendations for this kit (21). However, this target amount did vary due to the obtained quantification results. A positive control and negative control were also included in the amplification run; the positive control was DNA Control 007, which is included with the GlobalFiler kit, and the negative control was TE buffer. The amplification was performed in a 29-cycle run on the Veriti Thermal Cycler. Following amplification, capillary electrophoresis was set up using 9.6 µl of Hi-Di Formamide and 0.4 µl of GlobalFiler GeneScan 600 LIZ Size Standard in each well to denature the DNA and to provide a size standard for each fragment (21). Added to the plate, in accordance with the plate map, was 1 µl of allelic ladder or 1 µl of sample. Samples were genotyped using the Applied Biosystems 3500 Genetic Analyzer and GeneMapper ID-X v1.6 software. The profiles that were developed were compared with the known profile of the donor.

Data Analysis and Interpretation

Quantification Data

The Quantifiler Trio kit targets 3 different human loci; small autosomal (SA) DNA (fragments of DNA that are approximately 80 base pairs long), large autosomal (LA) DNA (fragments of DNA that are approximately 214 base pairs long), and male DNA (20). Quantification values for all three targets were obtained in this study through quantitative PCR (qPCR). qPCR uses a reporter dye bound to the 5' end of the target sequence and a quencher dye bound to the 3' end of the target sequence that will be polymerized (22). As the quencher dye moves away from the reporter dye, a fluorescent signal is released (22). The fluorescent signal is detected, and the software determines the cycle threshold (C_t) value for that sample, which is the number of PCR cycles it takes for the fluorescent signal to cross a certain value (22). The C_t values for each sample are then compared to the generated standard curve to produce the quantification values for each sample.

Table 2, seen below, gives the three target DNA concentrations for each method of extraction. Each sample was quantified in triplicate. Samples B1 and B4.1, which were extracted using Method 1 and Method 4, respectively, showed the highest quantification values. These values are highlighted in **Table 2**. Sample B1 had the highest LA concentration, while Sample B4.1 had the highest SA and male DNA concentrations. These results were not replicated for both methods in the second set of extractions. Sample B1.2, extracted using Method 1, had very low LA concentration values. However, Sample B4.2, extracted using Method 4, did have the highest LA, SA, and male DNA concentrations for the second set of extractions. Sample B3.2, extracted using Method 3, showed two replicates that resulted in 0ng/µl of large autosomal DNA.

This value of 0ng/µl means that no DNA was detected by the qPCR assay. Sample B3.2 also produced replicates with the lowest small autosomal and male DNA quantification results.

It can be seen in **Table 2** that the samples with the highest LA and SA concentration values (Samples B1 and B4.1) also have the highest male DNA concentration values. The DNA in both of these samples was purified using Qiagen MinElute columns, and this correlation could mean that the columns were able to purify all human-specific loci more successfully than the PrepFiler Magnetic Beads. **Table 2** exhibits the degradation index (DI) values for each sample. These values were calculated using the following formula:

Degradation index = small autosomal concentration ÷ large autosomal concentration

The results of the DI calculations were too variable to warrant further statistical analysis.

Quantification Results for All Samples						
Method of Extraction	**Extraction Set**	**Sample Name**	**Large Autosomal Concentration (ng/µl)**	**Small Autosomal Concentration (ng/µl)**	**Male DNA Concentration (ng/µl)**	**Degradation Index Values**
Method 1: Qiagen Protocol Only	**1**	B1	0.0954	0.1038	0.1126	1.088
	1	B1	0.0904	0.1233	0.1260	1.363
	1	B1	0.0916	0.1232	0.1194	1.345
	2	B1.2	0.0006	0.0053	0.0043	8.809
	2	B1.2	0.0001	0.0056	0.0050	39.266
	2	B1.2	0.0005	0.0051	0.0048	10.813
Method 2: Qiagen lysis protocol w/PrepFiler Magnetic Beads	**1**	B2.1	0.0010	0.0084	0.0105	8.406
	1	B2.1	0.0011	0.0093	0.0116	8.664
	1	B2.1	0.0010	0.0074	0.0094	7.625
	2	B2.2	0.0016	0.0049	0.0052	3.101
	2	B2.2	0.0016	0.0052	0.0058	3.230
	2	B2.2	0.0014	0.0043	0.0048	3.055
Method 3: PrepFiler BTA Forensic DNA Extraction protocol only	**1**	B3.1	0.0014	0.0246	0.0331	17.957
	1	B3.1	0.0027	0.0220	0.0307	8.01
	1	B3.1	0.0022	0.0227	0.0306	10.454
	2	B3.2	0.0001	0.0017	0.0023	26.046
	2	B3.2	0.0000	0.0019	0.0018	0.000
	2	B3.2	0.0000	0.0017	0.0017	0.000

Method 4: PrepFiler BTA lysis protocol w/ Qiagen MinElute columns	**1**	B4.1	0.0496	0.2133	0.2372	4.297
	1	B4.1	0.0534	0.2369	0.2659	4.433
	1	B4.1	0.0522	0.2227	0.2590	4.27
	2	B4.2	0.0033	0.0273	0.0203	8.160
	2	B4.2	0.0038	0.0278	0.0195	7.225
	2	B4.2	0.0016	0.0225	0.0191	14.060

Table 2. Quantification results for all samples and methods of extraction, including calculated degradation index values.

Statistical Analysis of Quantification Data

The data that was collected through DNA quantification showed that there was a difference in the quantities of DNA obtained in Set 1 and Set 2. Samples in Set 2 were exposed to Theralin for 19 days and gave lower quantification values than samples in Set 1, which were exposed to Theralin for one overnight period. Due to this observable result, a two-way ANOVA test was performed in GraphPad Prism to determine if variation within the data was significant (23). This two-way ANOVA test accounted for the difference in the length of time spent in Theralin between sets. **Table 3** shows the results for the two-way ANOVA test that was performed on the large autosomal quantification values. The results in **Table 3** demonstrate that there was significant variation among the data for LA quantification values. The alpha value for this test was 0.05, and with P values less than 0.0001, these results are significant. There is similar variation within the extraction sets, between extraction methods, and in the interaction between extraction set and extraction method.

Two-Way ANOVA - Large Autosomal Values		
Source of Variation	**% of total variation**	**P value**
Interaction	34.66	<0.0001
Extraction Set	30.36	<0.0001
Method	34.88	<0.0001

Table 3. The results of the two-way ANOVA test performed on the large autosomal quantification values.

Table 4 shows the results for the two-way ANOVA test that was performed on the SA quantification values. These results also display significant variation in the data, this time for SA quantification values. The P values were less than the alpha level of 0.05, and again there was seen to be variation within the extraction sets, extraction methods, and in the interaction between extraction set and extraction method. The highest amount of variation was seen within the extraction methods.

Two-Way ANOVA - Small Autosomal Values		
Source of Variation	**% of total variation**	**P value**
Interaction	27.29	<0.0001
Extraction Set	31.54	<0.0001
Method	40.75	<0.0001

Table 4. The results of the two-way ANOVA test performed on the small autosomal quantification values.

Based on the results of the ANOVA test that revealed significant variation, post hoc statistical tests needed to be completed to determine where the variation was coming from. The Sidak's Multiple Comparisons test was performed using GraphPad Prism on both the LA and SA quantification values (23). There were two versions of this Multiple Comparisons test; the first compared the means of all methods in a set to each other. The second test compared the means of individual methods across sets; for example, Method 1 in Set 1 was compared to Method 1 in Set 2. **Figure 3** shows the results of the Sidak's Multiple Comparisons test performed on the mean LA quantification values of methods between extraction sets. The results of this test showed that

there was a significant difference between Method 1 in Sets 1 and 2 and Method 4 in Sets 1 and 2. This significance is supported by the results that were reported earlier: samples B1 and B4.1, which were extracted using Method 1 and Method 4 in Set 1, had the highest large autosomal quantification values. However, this was not repeated in the second set of extraction. The graph in **Figure 3** shows that the means of the large autosomal quantification values for Methods 1 and 4 in Set 1 were significantly higher than the means of the large autosomal quantification values for Methods 1 and 4 in Set 2.

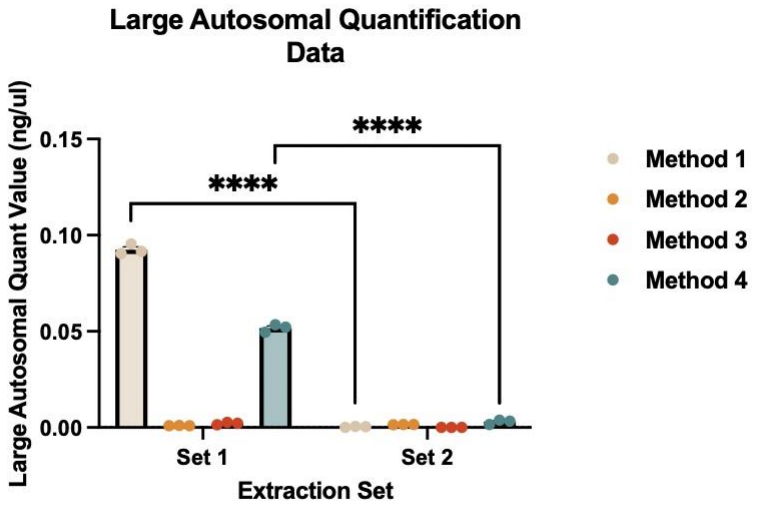

Figure 3. Sidak's Multiple Comparisons test performed on the means of the large autosomal quantification values obtained by the same methods in different extraction sets. The lines on the graph demonstrate significance.

Figure 4 displays the results of the Sidak's Multiple Comparisons test that was performed to compare the mean LA quantification values between methods within a single set. In the first set, there was a significant difference between the mean LA quantification values of Methods 1 and 4 between all other methods. Methods 2 and 3 were not found to be significantly different from each other. This result demonstrates that Methods 1 and 4 in set 1 displayed large autosomal quantification values that were significantly higher than other methods. **Figure 4** also

shows that there was no significant difference between the mean LA quantification values of the four methods in set 2. This indicates that the mean quantification values were all very similar to each other in set 2.

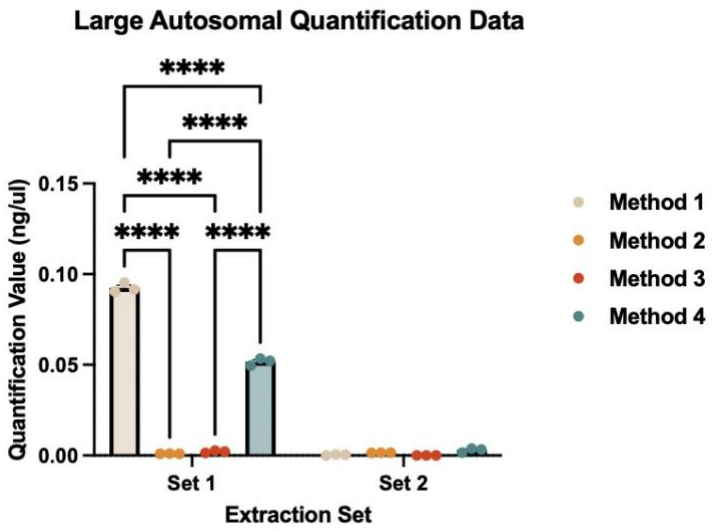

Figure 4. Sidak's Multiple Comparisons test performed on the means of the large autosomal quantification values obtained by different methods in the same extraction sets. The lines on the graph demonstrate significance.

The Sidak's Multiple Comparisons Test was performed on the means of the SA quantification values as well. **Figure 5** shows the results of the Sidak's Multiple Comparisons test that was done to compare the mean SA quantification values between individual methods in different sets. This shows that there is a significant difference between the mean SA quantification values of Methods 1, 3, and 4 in Sets 1 and 2. For all of these methods, the mean SA quantification values were significantly higher in Set 1 than in Set 2. **Table 5** shows the calculated P values for each comparison. The comparisons between Methods 1 and 4 in the two different extraction sets were very significant, each with P values of less than 0.0001. The comparison between Method 2 is less significant, with a P value of 0.0017. This supports the assertions made above that Methods 1 and 4 displayed the highest quantification values for both

LA and SA DNA in Set 1, but the results were not replicated in Set 2. There was no significant difference between Method 2 in Sets 1 and 2.

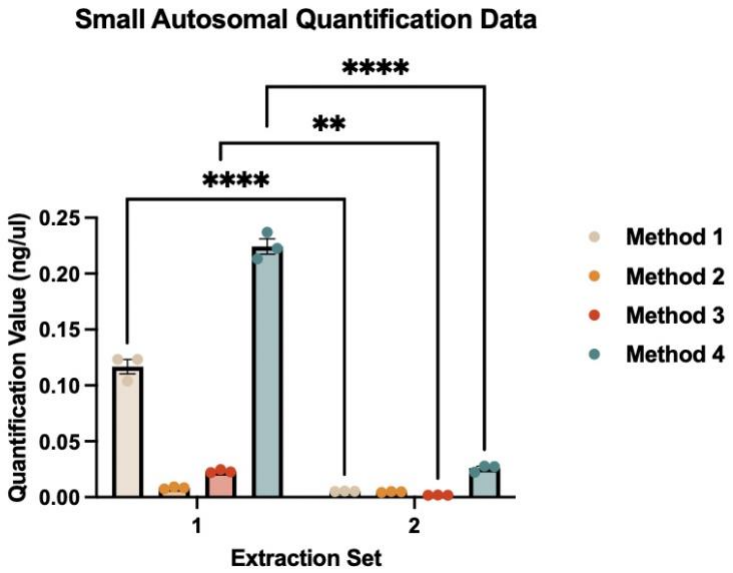

Figure 5. The Sidak's Multiple Comparisons test performed on the means of the small autosomal quantification values obtained by the same methods in different extraction sets. The lines on the graph show significance, and the number of asterisks demonstrates level of significance.

Method 1	<0.0001
Method 2	0.9211
Method 3	0.0017
Method 4	<0.0001

Table 5. The calculated P values of each comparison of the same method in different extraction sets using Sidak's Multiple Comparisons on small autosomal data.

Figure 6 displays the results of the Sidak's Multiple Comparisons test that was performed on the means of the SA quantification values obtained by different methods within the same extraction sets. Similar to the results of this test with the LA quantification values, there are significant differences between Methods 1 and 4 and all other methods in Set 1. There is also a significant difference between Methods 2 and 3 in Extraction Set 1. **Table 6** shows the p-values

that were calculated for each comparison, and it can be seen that the significance of the comparison between Methods 2 and 3 was much lower, with a P value of 0.0444, than the significance of the comparisons that involve Methods 1 and 4. **Figure 6** also displays that there was found to be a significant difference between Method 4 and all other methods in extraction set 2. Method 4 was found to have a significantly higher mean SA quantification value, which could indicate the success of method 4 to recover higher amounts of DNA, even though the results of Set 1 were not replicated in Set 2.

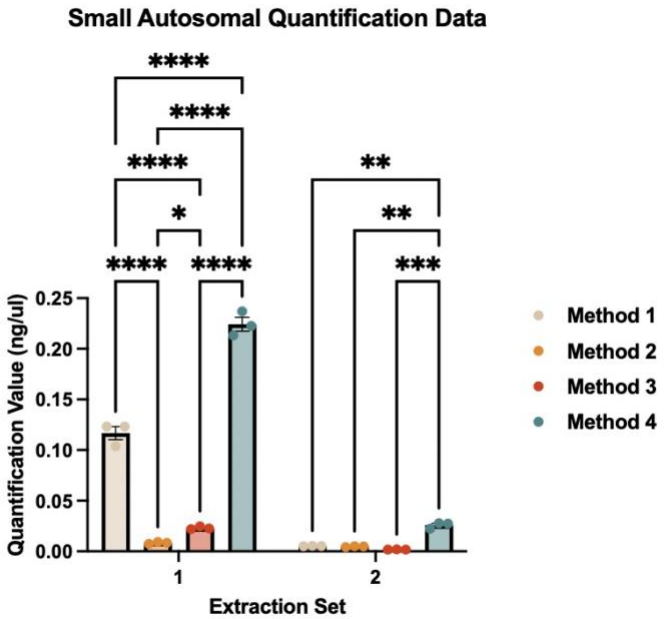

Figure 6. The Sidak's Multiple Comparisons test performed on the means of the small autosomal quantification values obtained by different methods in the same extraction set.

Extraction Set 1	**Method 1** vs. **Method 2**	<0.0001
	Method 1 vs. **Method 3**	<0.0001
	Method 1 vs. **Method 4**	<0.0001
	Method 2 vs. **Method 3**	0.0444
	Method 2 vs. **Method 4**	<0.0001
	Method 3 vs. **Method 4**	<0.0001
	Method 1 vs. **Method 2**	>0.9999

Extraction Set 2	**Method 1** vs. **Method 3**	0.9779
	Method 1 vs. **Method 4**	0.0036
	Method 2 vs. **Method 3**	0.9903
	Method 2 vs. **Method 4**	0.0029
	Method 3 vs. **Method 4**	0.0008

Table 6. The calculated P values of each comparison between methods using the Sidak's Multiple Comparisons test on small autosomal data. The lines on the graph show significance, and the number of asterisks demonstrates level of significance.

STR Genotyping

The positive and negative amplification controls and the reagent blanks gave the expected results. **Table 7** gives the total DNA input for the amplification step using the GlobalFiler PCR Amplification kit. Due to the known levels of degradation of these bones, their low quantification values, and their failure to generate profiles in preliminary studies, 15 µl of each sample was amplified (G. Williams, personal communication, October 10, 2023). For samples B4.2 and B1, this resulted in DNA inputs that were greater than 1ng. This did not appear to have an effect on the developed profiles for those two samples.

Total DNA Input (ng)	
Sample Name	**DNA input**
B1	1.7520
B1.2	0.0795
B2.1	0.3765
B2.2	0.2160
B3.1	0.3465
B3.2	0.0270
B4.1	3.3645
B4.2	0.3885

Table 7. The total DNA input for each sample.

Full or partial profiles were developed for all samples when compared to the known donor profile that was developed from a blood card. This known donor profile had a total of 40 allele calls. Sample B1 and Sample B4.1 generated the most allele calls, with 40 concordant

allele calls in the profile for Sample B1 and 37 concordant allele calls in the profile for Sample B4.1. Most importantly, Sample B1 developed a complete profile that was 100% concordant with the known donor. These samples (B1 and B4.1) had the highest quantification results and the highest total DNA input, which could explain why they generated the most complete profiles. Sample B3.2 generated the fewest allele calls, with only 12 concordant alleles. This sample had the lowest quantification values, which could explain the very partial profile. Sample B3.2 also had the lowest DNA input of only 0.0270ng, which is significantly lower than the manufacturers' recommended input of 0.5ng-1.0ng. The number of concordant alleles for each sample can be seen in **Table 8** below.

Sample	# Of Concordant Alleles	% Of Profile Complete
B1	40	100
B1.2	24	60
B2.1	31	77.5
B2.2	33	82.5
B3.1	31	77.5
B3.2	12	30
B4.1	37	92.5
B4.2	30	75

Table 8. The number of concordant alleles that were called in the profiles for each sample and the percent of the profile that was complete.

Figures 7-10 show portions of the electropherograms (EPGs), specifically the 6-FAM dye channel. The four other dye channels had results that were similar to what is shown in these EPGs. These EPGs were developed from the known donor's reference card, Sample B1, Sample B4.1, and Sample B3.2. Samples B1 and B4.1 have shown themselves to be outliers, with the highest quantification values obtained. The y-axes of the EPGs give the peak height values in

relative fluorescent units (RFUs), and the range of values for the y-axes are different in the final EPGs, which should be taken into consideration when viewing the figures. The peak height values, or RFUs, give the strength of the fluorescent signal for each allele call. The profile that was developed for Sample B1 in **Figure 8** shows high allele peak heights that are consistent with or even higher than the allele peak heights shown in **Figure 7**. The profile developed for Sample B4.1 in **Figure 9** also shows consistently high allele peak heights, with the exception of the CSF1PO and TPOX loci. The lower peak heights for these two loci demonstrate the ski slope effect, which is a sign of degradation in the sample. Sample B4.1 had an average degradation index of 4.33, which could explain the observed ski slope effect. The profile in **Figure 10** that was developed for Sample B3.2, which had very low quantification values and low DNA input, shows significant allelic dropout, with only 2 allele calls in this dye channel. These allele calls show very low peak heights, and there is a significant amount of baseline noise.

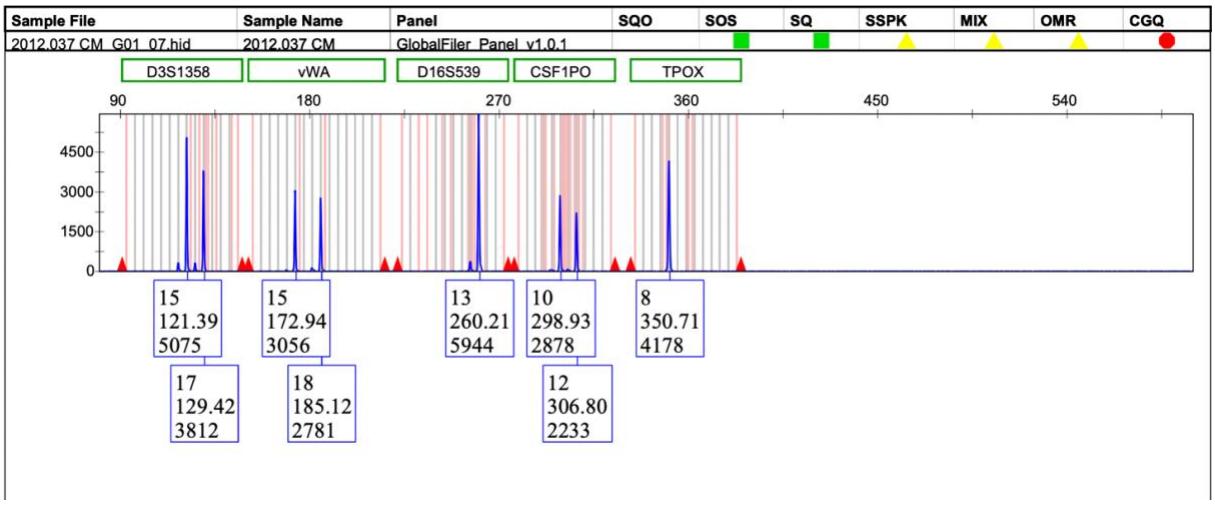

Figure 7. The 6-FAM dye channel from the profile developed from the known donor's reference blood card.

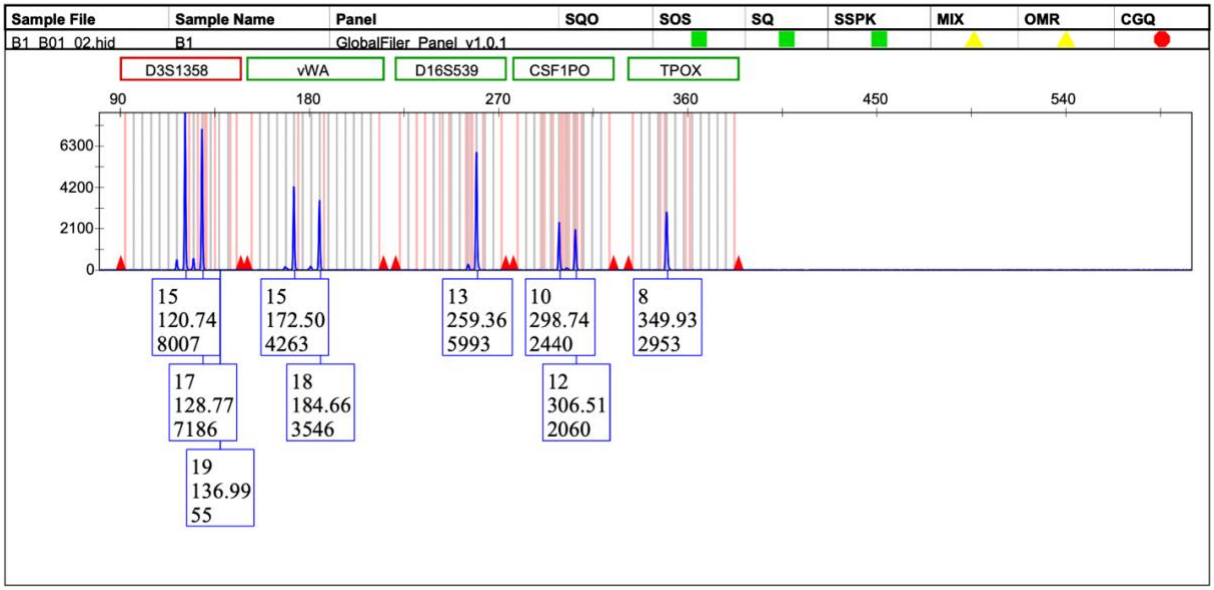

Figure 8. The 6-FAM dye channel from the profile developed from Sample B1.

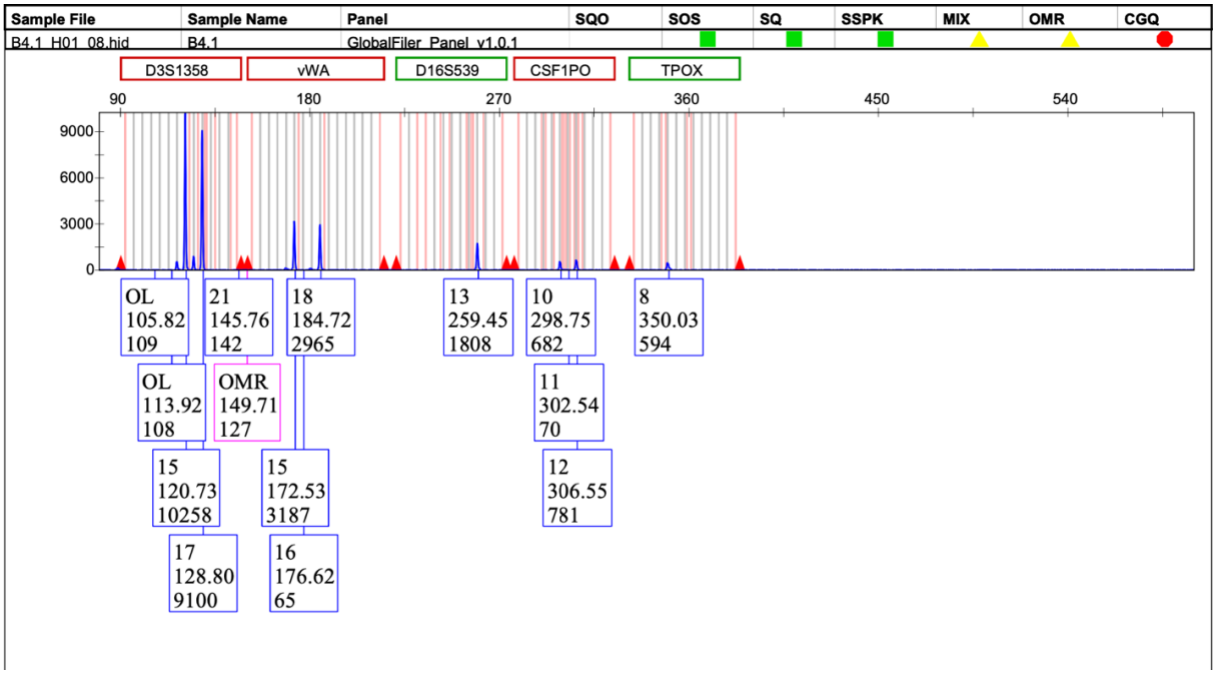

Figure 9. The 6-FAM dye channel from the profile developed from Sample B4.1.

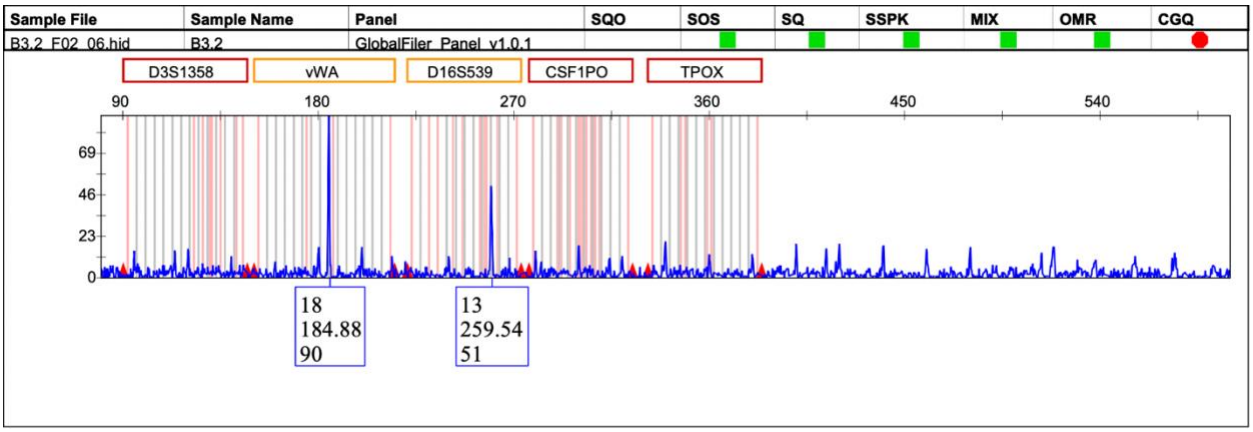

Figure 10. The 6-FAM dye channel from the profile developed from Sample B3.2.

To determine the quality of each profile, and the possible correlation between profile quality and DNA quantity, the signal strength metric was calculated for each sample based on the peak height values. The total signal strength metric for each profile was calculated by first finding the signal strength for each of the five dye channels in the generated EPGs. This was done by calculating the sum of the peak height values of the concordant allele calls in each dye channel. The sum of peak heights was divided by the expected number of alleles in that channel, which was determined by the number of loci in the channel. The signal strength from each dye channel was then summed to find the overall signal strength metric for each sample. The signal strength metrics, compared to the large autosomal and small autosomal quantification values, can be seen below in **Tables 9** and **10**. In Excel, the correlation coefficients were calculated between the quantification values and the signal strength metrics. These values can also be seen in **Tables 9** and **10** below.

Sample Name	**Average LA Quantification Value (ng/µl)**	**Signal Strength Metric**
B1	0.0925	32573.2
B1.2	0.0004	1094.6
B2.1	0.0010	2095.7
B2.2	0.0015	1322.8
B3.1	0.0021	3158.5

B3.2	0	412.6
B4.1	0.0517	31066.2
B4.2	0.0029	4129.4
Correlation Coefficient	**0.956**	

Table 9. The average large autosomal concentration and the calculated signal strength metric for each sample.

Sample Name	**Average SA Quantification Value (ng/µl)**	**Signal Strength Metric**
B1	0.1168	32573.2
B1.2	0.0053	1094.6
B2.1	0.0084	2095.7
B2.2	0.0048	1322.8
B3.1	0.0231	3158.5
B3.2	0.0018	412.6
B4.1	0.2243	31066.2
B4.2	0.0259	4129.4
Correlation Coefficient	**0.921**	

Table 10. The average small autosomal concentration and the calculated signal strength metric for each sample.

There was a positive correlation between the signal strength metrics and the large autosomal quantification values for each method, with a calculated correlation coefficient of 0.956. There was also a positive correlation between the signal strength metrics and the small autosomal quantification values for each method, with a calculated correlation coefficient of 0.921. These positive correlation coefficients show that as the LA and SA quantification values increase for each sample, the signal strength metrics also increase. Samples B1 and B4.1 had the highest calculated signal strength metrics of 32,573.2 and 31,066.2, respectively. These two samples also displayed the highest quantification values. This data supports the calculated positive correlation coefficient, because the samples with the highest quantification values also had the highest quality STR profiles.

The graphs in **Figures 11** and **12** show the positive correlation between the signal strength metric and the quantification values, both LA and SA. As has been discussed throughout

this section, Samples B1 and B4.1 had the highest quantification values and the highest quality STR profiles. These samples can clearly be seen as outliers in the graphs below.

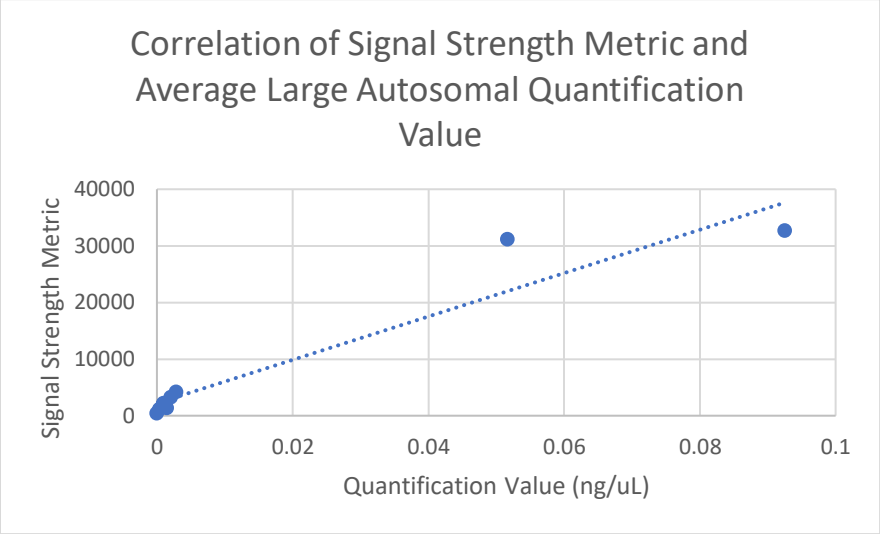

Figure 11. A graph showing the correlation between the large autosomal concentration and the signal strength metric.

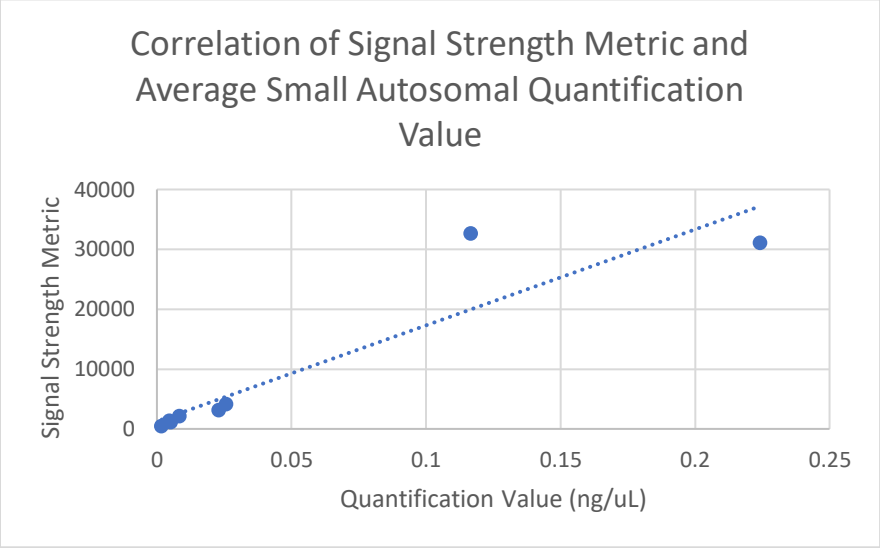

Figure 12. A graph showing the correlation between the small autosomal concentration and the signal strength metric.

Research Results and Discussion

The results of this study showed that Method 1 and Method 4 produced DNA extracts with the highest quantities of DNA, specifically Sample B1 and Sample B4.1. In future studies that are done with Theralin, the results from Samples B1 and B4.1 will be what researchers hope

to see. These two samples, included in set 1, were identified as outliers and shown to have significance in the statistical analysis that was performed. Methods 1 and 4 were found to have LA quantification values that were significantly different from all other methods in extraction set 1. These results were not replicated in Extraction Set 2, as demonstrated by Method 1 and Method 4 in Set 1 being significantly higher than Method 1 and Method 4 in Set 2. Methods 1 and 4 were also found to have significantly different SA quantification values from all other methods in Set 1, and Method 4 was shown to have significantly different SA quantification values from all other methods in Set 2. The high SA quantification results were again not repeated in Set 2, as there was a significant difference between the SA quantification values for Methods 1 and 4 in Set 1 and Methods 1 and 4 in Set 2. The reasons for why these results were not replicated in Set 2 will be explored throughout this section.

Samples B1 and B4.1 showed not only high quantities of SA, LA, and male DNA, but also high-quality STR DNA profiles. Sample B1 generated a profile that was 100% complete, and Sample B4.1 generated a profile that was 92.5% complete. These two samples were not only outliers because of their quantification values; they were also outliers in the dataset that included signal strength metric calculations. Their status as outliers is clearly seen in **Figures 11** and **12**, where Samples B1 and B4.1 are plotted much further away from the rest of the data points, which are all skewed towards the left side of the graph. The high signal strength metric calculations of these two samples were indications of the high-quality STR profiles that were developed from both of these samples. Sample B1 was extracted using the Qiagen QIAamp DNA Investigator kit protocol only, while Sample B4.1 was extracted using the PrepFiler BTA lysis buffers with the Qiagen MinElute columns. The purification step in both of these methods was the same; the use of the Qiagen MinElute column. The Qiagen MinElute column captures

the DNA, which is then purified with three individual washes; two with wash buffers provided by Qiagen, and one with ethanol (9). When used with Samples B1 and B4.1, the column was shown to be extremely effective in capturing DNA from the bone material and purifying it for PCR amplification and STR genotyping. However, as previously mentioned, this result was not replicated for Sample B1.2, which also underwent extraction using only the Qiagen kit and the MinElute columns. Samples 2.1, 2.2, 3.1, and 3.2, which were purified using the PrepFiler Magnetic Beads, did not display comparatively high LA or SA quantification values. In fact, Sample 3.2 displayed the lowest LA quantification values, with two replicates having 0ng/µl of DNA detected. These samples also generated low signal strength metrics. This data indicates that the PrepFiler Magnetic beads, which were used in the extraction protocols for each of these samples, were not as effective in recovering high quantities of quality DNA.

The indication that the PrepFiler Magnetic Beads were not as successful in purifying the DNA comes in contrast to what has been reported in previous literature. As was discussed in the literature review, Parson, et. al used the Qiagen lysis buffers, Buffers ATL and AL, in combination with the PrepFiler Magnetic Beads to extract mtDNA from hair shafts and was successful (18). Another previous study published by Burnside, et. al, displayed this same success when extracting mtDNA from hair shafts using the aforementioned combination method (24). This combination method was used as Method 3 in this research project and did not show the same success with Theralin-demineralized bone samples. The results of the current study indicate that this method, while efficient in the extraction of mtDNA from hairs, was not efficient in the extraction of nuclear DNA from bones. The data from the current study can inform future studies that are done on the extraction of DNA from bones, as it's important to know that silica-based columns, as opposed to silica-coated beads, appear to produce higher

quantities of quality DNA from bone samples when they are used in conjunction with the Theralin demineralization process.

The reason for the difference in quantification values for both LA and SA DNA and profile quality between samples that were extracted using the same method could be due to the length of time of the Theralin demineralization process. In this experiment, the bone material that was used for the first set of extractions (Sample B1, B2.1, B3.1, and B4.1) was only exposed to washes with Theralin for one overnight period. The samples that were used for the second set of extractions (Samples B1.2, B2.2, B3.2, and B4.2) were exposed to Theralin for a much longer period of time: 19 days. The reason for the inconsistent time period is that the first set of samples demineralized more quickly in Theralin. After one overnight period of Theralin washes, this set had been converted into a gum-like state and the bone material was ready to be cut. The second set of samples did not demineralize this quickly, and more Theralin was required for the demineralization to be completed. Once the Theralin had been obtained, the samples could finish the demineralization process. This difference in length of time that each sample spent in Theralin seems to have impacted the quantity and quality of the DNA extracts. For example, Sample B1.2 had extremely low large autosomal quantification values, but Sample B1 had the highest large autosomal quantification values. This impact is seen in the statistical tests. Sample B1 was found to be significantly different from Sample B1.2, even though the same method was used. Additionally, Sample B1.2 demonstrated significantly higher DI values than Sample B1. This discrepancy could mean that when a sample spends a long period of time in Theralin, larger fragments of DNA in the sample become more degraded, and the breakdown of these fragments is evident in subsequent analyses. This would explain why the large autosomal quantification values decreased for most methods from one sample set to the next, because there were fewer

intact large fragments of DNA. There was also a significant difference in the small autosomal quantification values for Methods 1, 3, and 4 between extraction sets. The samples in set 1 gave significantly higher amounts of small autosomal DNA. These results indicate that overall, Theralin negatively effects the recovery of all DNA fragment sizes if the samples are left in it for too long. This project has shown that 19 days is too long for the samples to be exposed to Theralin.

In addition to the difference in amount of time spent in Theralin, there was a visual difference between the first set of samples and the second set of samples following the incubation periods to digest the bones. For example, Sample B1, which can be seen throughout the incubation periods in **Figure 13**, retained its solid state even after the third Proteinase K spike and 1-hour incubation at 56ºC. Sample B1.2, which can be seen in **Figure 14**, did not retain its solid state following the incubation periods. The solid bone material completely dissolved following the overnight incubation period. To keep things consistent between sample sets, the amount of Proteinase K spikes and additional incubations was not changed, and this could have been detrimental to the second set of samples, which were exposed to Theralin for longer and therefore more demineralized than the first set of samples. It's possible that the bone material in the second set of extractions was *too* digested, and the subsequent spikes of Proteinase K and 1-hour incubations at 56ºC began to cause degradation of the bone material, which led to lower quantification values being obtained. Further studies that directly compare the length of time spent in Theralin and the number of additional 1-hour incubations are needed. Additional studies can also be performed to determine a method of automating this process with Theralin, using robotic instruments to switch out the Theralin washes for a specific amount of time.

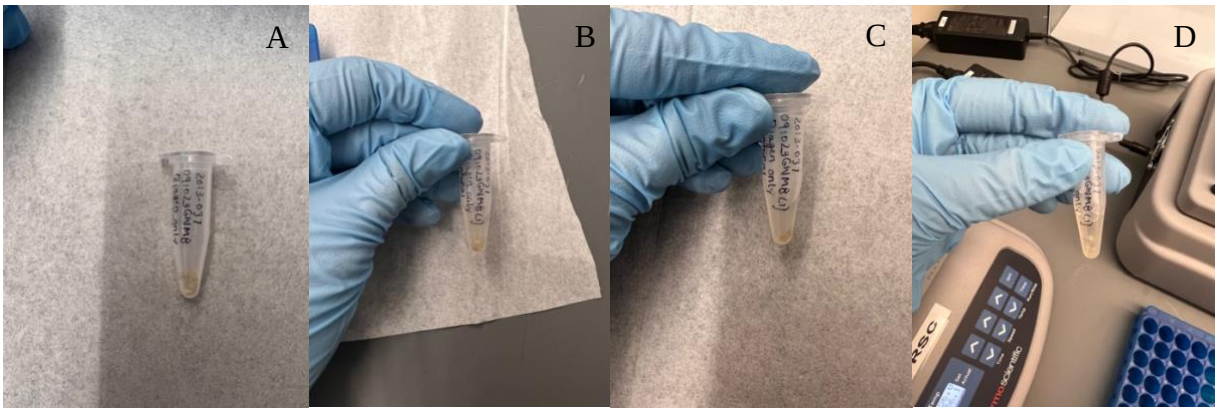

Figure 13. This figure shows Sample B1 before the lysis protocol was initiated (A), the bone sample following the overnight incubation with the Qiagen lysis buffers (B), the bone sample following the first 20uL spike of Proteinase K and a 1-hour incubation period (C), and the bone sample following the third 20uL spike of Proteinase K and a 1-hour incubation period (D).

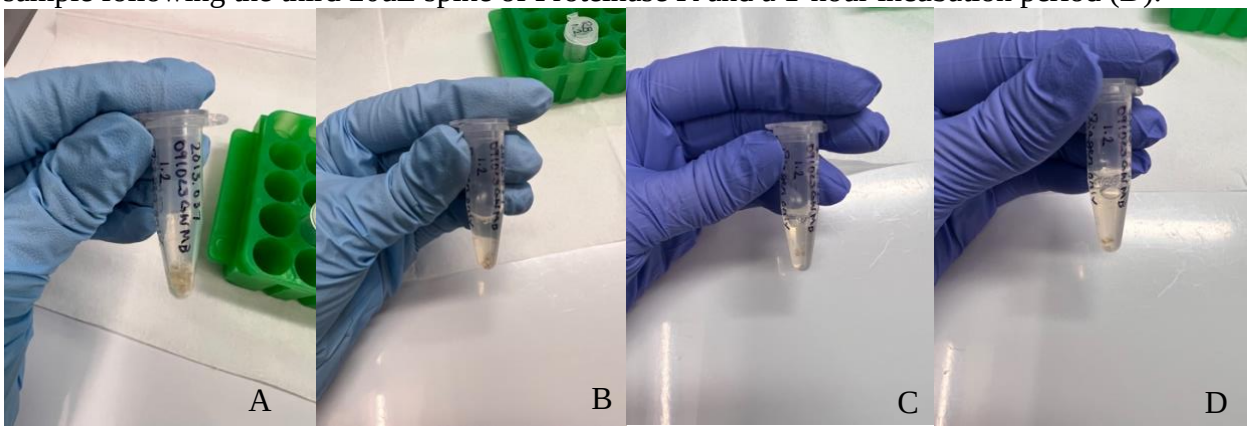

Figure 14. This figure shows Sample B1.2 before the lysis protocol was initiated (A), the bone sample following the overnight incubation with the Qiagen lysis buffers (B), the bone sample following the first 20uL spike of Proteinase K and a 1-hour incubation period (C), and the bone sample following the third 20uL spike of Proteinase K and a 1-hour incubation period (D).

In addition to the differences between quantification values, there were many differences between calculated DI values. While these differences within individual methods were too variable to warrant statistical analysis that would have led to meaningful results, they are notable enough for further discussion. All DI values increased from the first set of extraction to the second set of extraction except for Method 2, which had DI values that decreased in the second set of extractions. As was discussed previously, this change can most likely be attributed to the amount of time the bones in the second set of extraction spent in Theralin. Due to the formula

that is used to calculate the DI values, this variability between samples is likely due to the different large autosomal quantification values that were obtained. Samples that contain more intact large DNA fragments will have lower DI values. Sample B1.2 exhibited the highest DI value, and it also had one of the lowest large autosomal quantification values. In contrast to Sample B1.2, Sample B1 had the highest large autosomal quantification values, and the lowest DI values.

It is also possible that the degradation indices can be used to show how successful the extraction method was in recovering quality DNA from samples. All of the samples used in this study came from the same bone. Because of this, it's plausible that the DI values being so variable is due to the different extraction methods being more or less efficient in recovering different fragment sizes within the sample. In future studies, a degradation parameter that is developed by TrueAllele® could be used to help determine the efficiency of each extraction method.

Overall trends in this data show that Methods 1 and 4, which both used the Qiagen MinElute columns, produced extracts that were outliers in the data and had the highest quantity and quality DNA. Specifically, the extracts that led to these outliers in the data came from Samples B1 and B4.1. The status of these extracts as outliers in the data is most likely due to the amount of time spent in Theralin (one overnight period), the state of the bone following multiple incubation periods, and the success of the Qiagen MinElute columns in purifying the DNA. Method 4 was shown to be significantly different from all other methods when analyzing small autosomal and large autosomal DNA concentrations. This could indicate that the combination of PrepFiler lysis buffers with Qiagen MinElute columns is the most effective method to extract DNA from Theralin-demineralized bone samples. Samples B1 and B4.1 exhibited the highest

signal strength metrics, meaning they produced allele calls that had consistently high peak heights. These allele calls were also concordant with the known genotype, indicating that these profiles are useful for forensic identification purposes. The results that were obtained from these two outliers are the desired results from all bone samples that are demineralized using Theralin. The goal of future studies will be to determine how the methods of extraction can be optimized to recover these quantities of high-quality DNA from all, or most, bone samples, since the outliers have shown that it is possible.

Conclusion

The results of this study show that it is possible to obtain DNA extracts of sufficient quantity and quality for downstream applications from bone samples that have been demineralized using Theralin, as was displayed most prominently with Samples B1 and B4.1. Samples B1 and B4.1 demonstrated results that are sought every time DNA is extracted from bone samples. The results of this study indicate that the amount of time a sample spends in Theralin is an important part of this process. If the sample is in Theralin for too long, it appears to degrade the DNA further and cause less recovery of DNA fragments of all sizes. This study tested four different methods of extraction: the Qiagen QIAamp DNA Investigator Kit, the Qiagen lysis buffers with PrepFiler Magnetic Beads, the PrepFiler BTA Forensic DNA Extraction Kit, and the PrepFiler BTA Lysis Buffers with Qiagen MinElute columns. While DNA was obtained from every method of extraction, the use of PrepFiler BTA Lysis Buffers with Qiagen MinElute columns obtained quantities of DNA that were found to be significantly different from the other methods. Quality STR profiles that displayed a high number of concordant allele calls were generated from the Qiagen QIAamp DNA Investigator Kit method and the PrepFiler BTA Lysis Buffers with Qiagen MinElute columns method. Partial profiles

that displayed concordant allele calls were generated from all methods of extraction that were tested.

There were some limitations to this experiment. First, due to a limited amount of sample, each extraction method could only be tested twice. Future studies will need to be performed to determine if the results from this paper can be replicated using more samples. These future studies can also include a diverse set of bone samples that are different ages and have been exposed to different environmental conditions. Another limitation is the lack of consistency in the amount of time each sample spent exposed to Theralin. There was a 19-day difference between Set 1 and Set 2 of the samples, which led to inconsistent results, though this did offer important insight into the length of time that a sample should spend in Theralin. One final limitation of this study is that the only bone that was used in this project was one that had been exposed to harsh environmental conditions. While it is essential to the study of DNA extraction from bone that challenging samples are used, a control bone sample that was in pristine condition could not be used in this study for comparison purposes.

This study has an important impact on forensic science. Theralin demineralization has not previously been tested in a study of this nature to determine if it can yield useful results. Based on this study, Theralin is a viable option for the demineralization of bone samples that are used by forensic scientists for identification purposes. A full DNA profile that was concordant with a known donor was developed from a ten-year-old bone sample that was exposed to the environment to mimic real casework samples. This speaks to the success that can be found when Theralin is used. As was explained in the literature review, Theralin is different from other methods in that it uses whole bone chips and includes a digestion step that only involves commercially available DNA extraction reagents. From these simple demineralization and

digestion steps, Theralin-prepared bone can then be used for extraction purposes to generate STR profiles. The implications of this study are immense when it comes to the amount of unidentified human remains that exist in the United States. With the addition of future studies that can and will be done using Theralin, this method of demineralization could be used on bone samples that will help to identify these unknown victims.

There are many future directions for this research. It will be necessary to perform a study that directly compares the results of this study using Theralin to the demineralization method that uses EDTA and powderization of bone. This future study could show the benefits of Theralin compared to the current most commonly used demineralization method. In addition to this, it is crucial to perform studies in the future that determine the ideal amount of time for a bone sample to be exposed to Theralin during the demineralization process. This study showed that an overnight exposure to Theralin gave successful results, but that 19 days is too long. The future studies would also need to include how many subsequent incubations need to occur to digest the bone sample, and how long those incubations should be. Future studies can be done to optimize the extraction methods that were found to be the most successful in this study, which were the methods that used Qiagen MinElute columns. These optimizations could include changing the pH of the column to improve the binding of the DNA or changing the length of time of the incubation with the lysis buffers and Proteinase K. Included in this future research could be a step that involves concentration of the DNA extract, which would allow for more DNA to be used in amplification and STR genotyping. Other future studies could be done that include MPS and Single Nucleotide Polymorphism (SNP) genotyping. The use of MPS is becoming increasingly significant in forensic science, especially with the rise of Investigative Genetic Genealogy (IGG). SNP genotyping can produce data that, when uploaded to a genealogy

database, generates a family tree that can connect an individual to a relative who is also in the database. The implications of IGG for identifying currently unidentified victims are very important, and there can be future studies done to test the success of using Theralin-demineralized bone samples with library preparation methods and MPS to generate data for IGG.

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