Barrett, Lisa C. Effect of Collagenase Type 2 and Proteinase K Digestion on DNA Yield from Bone Samples Purified on the EZ1 Advanced XL. Master of Science (Biomedical Sciences, Forensic Genetics). July 2015. 39 pp., 16 Figures, 20 References.

Genetic results from bone samples often yield low quantities of DNA and poor quality of genetic data. Proteinase K is a proteinase that is commonly used in DNA extraction methods, however the target proteins of Proteinase K do not closely align with the makeup of bone. Collagenase Type 2 is a protease that is more specific to the breakdown of collagen, which bone is comprised of. This study looked at the potential effects of Collagenase Type 2 digestion on bone samples compared to the effects of Proteinase K on quantity and quality of genetic typing. This study also incorporates the EZ1 Advanced XL purification platform and the AmpFLSTR Globalfiler Amplification Kit.

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1. Introduction

DNA is significant in forensics as it aids in determining the source of a biological sample. DNA results are significant in the testing of bones in the forensic science community, because they are most likely related to a missing persons case. According to the NIJ there is an estimated 4,400 unidentified human remains cases every year and about 100,000 active missing persons cases on any given day [1]. Bone samples range in size from whole skeletons to tiny fragments. A likely scenario encountered when human remains are found are incomplete, degraded, or small fragments. In general, these types of bone samples tend to pose a problem for analysts because there is a limited quantity and quality of the DNA, as well as Polymerase Chain Reaction (PCR) inhibitors that can co-extract with the DNA and negatively affect the amplification process. With small fragments an analyst does not have a lot of chances to take a dissection for extraction, so it is important to have an extraction method that effectively breaks down potentially inhibiting proteins and maintains the integrity of the DNA.

There is a lack of studies that looks into the effect of different proteases on DNA yield and quality. Current extraction methods are performed using Proteinase K during the protein digestion step, however, the amino acid sites that Proteinase K targets are only minor constituents of collagen and is therefore possible that Proteinase K may not be the best protease to break down bone material and expose bone cells. The protease collagenase type 2 targets amino acid sites that are more prevalent in collagen and may perform better by breaking down collagen to access more DNA.

An optimal protease in conjunction with the use of an automated method like the EZ1 Advanced XL, DNA can be extracted with higher throughput and analyzed faster for the upload

of a DNA profile into a missing persons database. A method that yields higher DNA output will benefit in the analysis of small bone fragments or degraded bone samples.

Bone has a very complex hierarchal structure that provides it to have optimal mechanical performance [2]. Bone can be described as having a sandwich structure with a dense external shell known as the cortical bone and a spongy interior also known as the cancellous bone, though DNA testing is almost exclusively conducted on cortical bone samples.

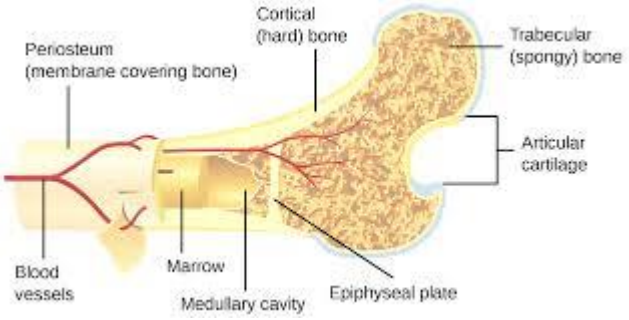

Fig. 1. Diagram of bone [3]

Cortical bone consists of collagen fibrils, known as lamellae that are reinforced by hydroxyapatite mineral platelets which also make up the matrix [4]. Majority of DNA in bones can be found in the osteocytes, which are bone cells that are located within the collagen-hydroxyapatite matrix. Cortical bone is arranged in units called osteons which are concentric rings of the collagen lamellae that run parallel to the long axis of the bone [4]. In the center of the osteon is a canal called the Haversian canal which surrounds a blood vessel [4]. Within the osteon are little spaces called lacuna, in which the osteocytes are found. Since osteocytes are embedded in the calcified matrix, the matrix provides a barrier to the osteocytes preventing the isolation of DNA [5].

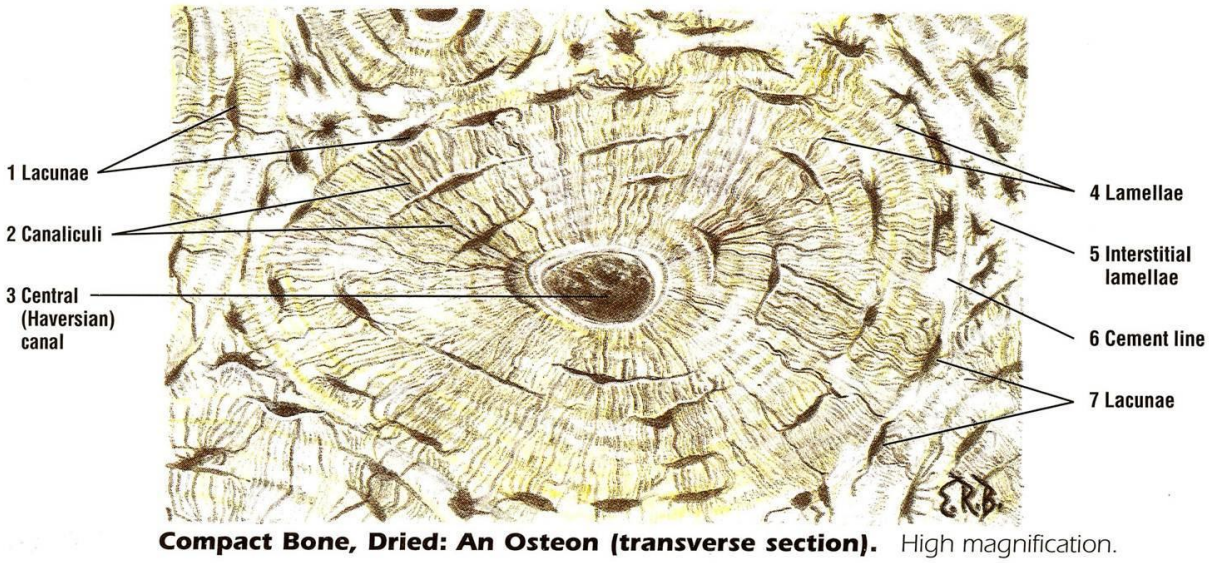

Fig. 2. Transverse section of an osteon in compact bone [6]

The collagen matrix has to be broken down in order to access the osteocytes which are embedded in the lacunae within the matrix. The bone has to be first ground up using liquid nitrogen in a freezer mill before being submerged in buffers that contain a protease for degradation of the collagen matrix so that osteocytes can be accessed by detergents for lysis.

The peptide bonds of the amino acids that Proteinase K cleaves are only minor components of collagen, which makes up bone material. Proteinase K has a very broad specificity, which makes it a good choice for digestion of proteins in biological samples. Proteinase K cleaves peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids. Though glycine, which is present in collagen, is aliphatic, the presence of aromatic amino acids are rare in collagen. For example, phenylalanine and tyrosine are present 1.4 and 0.3% respectively in type 1 collagen [7].

Collagen is the major fibrous component of animal extracellular connective tissue, constitutes about a quarter of the total protein content in most animals, and is a major component of bone. The collagen molecule consists of three polypeptide chains, called alpha chains, with the characteristic triplet repeat sequence Gly-Xaa-Yaa [8]. All three chains can be identical or a collagen molecule can contain two or three different chains with the difference being in the amino acids in the X and Y position. Any amino acid can be in the X and Y position but most often it is proline and hydroxyproline [9].

Collagenases are endopeptidases that digest native collagen in the triple helix region. The cleavage sites for clostridial collagenases are all at the Yaa-Gly bonds in the repeating Gly-X-Y collagen sequence [10]. Therefore, it is very specific for collagen. A distinguishing factor of collagenase is its ability to digest type I collagen which is the main component of bone.

The most critical step in the DNA analysis process to be able to accurately assess DNA quality and quantity is the extraction of the DNA from the biological evidence. There are various extraction techniques used for the purpose of isolating DNA from cellular components but organic extraction is a commonly used method that uses organic chemicals to isolate genomic DNA. Before the extraction process begins the bone is first ground to a fine powder, this powdered bone is incubated in an extraction buffer. The extraction buffer contains EDTA, detergents, and proteases which aid in the demineralization step. During demineralization, the detergents aid in lysing the cell, which exposes cellular components to proteases. EDTA is used to chelate calcium and inhibit it so that it does not interfere with the amplification process. The next step is purification to remove and separate the denatured proteins from the DNA. This step requires phenol chloroform isoamyl alcohol (PCIA). PCIA promotes the separation of non-polar and polar phases. The non-polar phase is the organic phase and contains non-DNA cellular

material and the polar aqueous phase contains the DNA. PCIA may be cost effective, but the process is laborious and time consuming. The process of digestion can take a day to complete alone. PCIA is hazardous to the analyst and the isolation step requires multiple tube transfers performed by the analyst and increases the chances of contamination and sample loss.

An alternative to organic PCIA purification is the use of an automated purification system. An automated system compared to manual organic purification is advantageous because they minimize contamination and sample loss, it is safer to used, and time spent on the process is shorter. An example of an automated purification system is the EZ1 Advanced XL. The EZ1 Advanced XL uses the EZ1 DNA Investigator Kit (Qiagen). The EZ1 DNA Investigator kit uses a magnetic-particle technology with silica coating. After protein digestion, the digested samples are added to the EZ1 tubes that contain the silica coated magnetic particles. DNA is isolated from the digest solution in a single step by binding to the silica surface of the particles in the presence of a chaotropic salt. The DNA binds to the silica coated particles via a salt bridge that forms between the negatively charged DNA and silica particles. Optimal conditions for the binding of the DNA occur when salt concentrations are high and the pH is low. Then the particles are separated from the digest solution by a magnet. The DNA is then washed with buffers that rinse away any degraded proteins and inhibitors without disrupting the salt bridge between the DNA and the silica coating, allowing the purified DNA to remain bound. The elution step can take place in either water or TE buffer, the EZ1 Advanced XL allows for an analyst to choose either one during instrument set up [11]. Studies involving the EZ1 automated method conducted by others such as Dukes et. al. at the North Louisiana Criminalistics Laboratory (NLCL) have shown that an automated method is capable of recovering DNA from

bones, however currently there is not an automated method that has consistently produced higher quality results from bone samples [12].

To assess quantity of samples this study uses Quantifiler Trio. Quantifiler Trio has two separate target specific human assays, one with a short PCR amplicon and one with a long PCR amplicon as well as a target specific human male DNA assay. The quantification process can also help determine if inhibitors are present in the samples. Quantifiler Trio is more sensitive and has a degradation index that allows an analyst to have an idea on the condition of the sample being analyzed/tested/examined. The small autosomal target has an amplicon size of 80 base pairs which aligns with sizes of "mini" STR loci and can better detect degraded samples. The large autosomal target has an amplicon size of 214 base pairs and is used as an indication of degradation. The kit also includes an internal PCR control (IPC) assay that contains synthetic DNA template that provides positive confirmation that all assay components are functioning properly [13]. The ratio of the quantification of the large autosomal targets is compared to the quantification results of the small autosomal targets. The degradation index indicates whether large DNA fragments will perform poorly in relation to small DNA fragments based on data observed when a sample displays a decrease in measured amount for large DNA fragments compared to small DNA fragments [13].

The next step which is amplification, makes copies of targeted regions of DNA that are highly discriminatory. For this study Globalfiler was used. The GlobalFiler kit is a 6-dye kit that amplifies 21 autosomal STR loci, sex determining marker Amelogenin, Y-STR DYS391, and one insertion/deletion marker on the Y chromosome (Y indel). Because GlobalFiler targets more than the standard 13 STR loci, the kit provides higher discriminatory power over other kits that contain less targets.

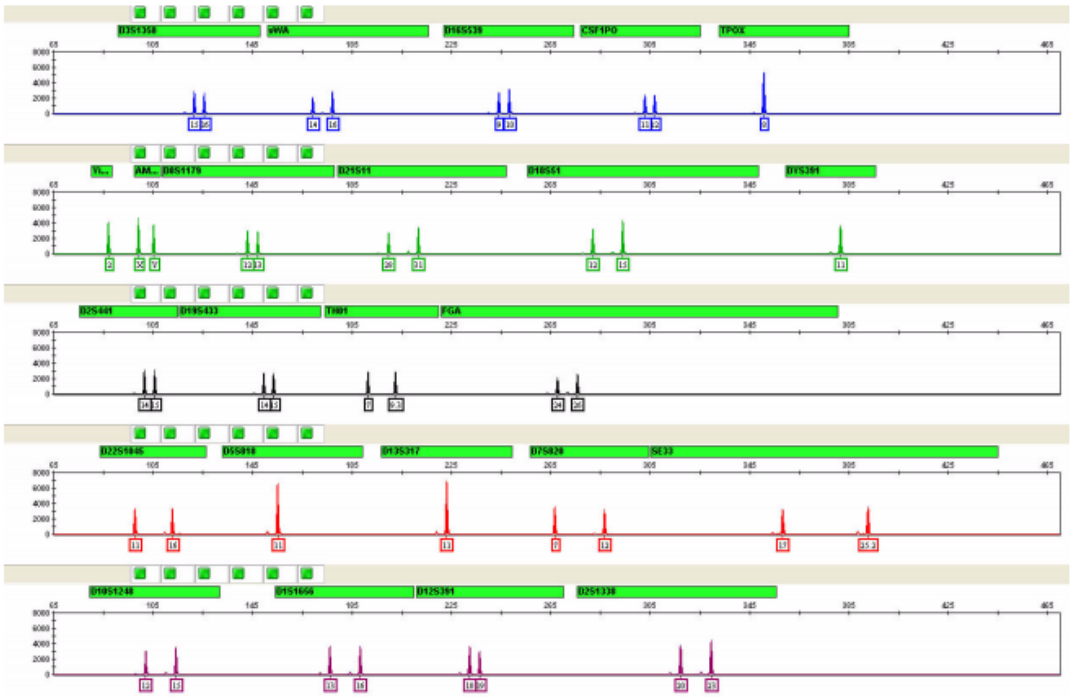

Fig. 3. Example of a GlobalFiler profile [14]

The isolation of DNA from bone samples recovered from human remains is very significant as it can potentially aid in the identification of a missing person. Bone samples pose a challenge as they usually produce low DNA yields because of degradation and low levels of intact cells. It is important to have an extraction method that is very efficient at isolating as much DNA as possible. Proteases are important in the extraction process because they break down the protein barrier that potentially prevents the isolation of bone DNA. Two proteases will be compared to each other to determine if they have differing effects in regards to the recovery of both DNA quantity and quality. DNA will be extracted from unembalmed human cadaver humerus, tibia, and femur samples using an automated method, the BioRobot EZ1 (QIAGEN, Germantown, MD). Samples will be allocated to one of three groups, Proteinase K (Pro K) digestion, collagenase type 2 (CLS2) digestion, or a combination of both Proteinase K and collagenase type 2 (PK+C2). The DNA quantification and profile analysis data from bone

samples extracted by the method using Proteinase K for protein digestion will be compared to bone samples extracted using collagenase type 2 for protein digestion and samples extracted in the presence of both proteases. The goal is to determine if utilizing collagenase type 2 for Proteinase K will have an effect on the DNA quantity and quality recovered from bones. Purification of the DNA will take place using the EZ1 automated method.

In general there is a lack of studies that examine the protein digestion step in regards to the effect of protease used during DNA extraction from bone samples. In most procedures Proteinase K is the proteinase of choice in DNA extraction methods, but it is unknown if Proteinase K is the most effective enzyme for digestion of bone samples. The amino acid sequences that Proteinase K targets are only minor constituents of collagen, which makes up a majority of bone structure [7]. Employing a proteinase that better matches the amino acid constituents of collagen will lead to a further degradation of the physical barrier surrounding the osteocytes and the DNA yield extracted from bone samples will be increased by utilizing collagenase type 2 for protein digestion.

2. Methods and Materials

2.1 Samples

The bones were harvested from an unembalmed human cadaver obtained from the Willed Body Program at the University of North Texas Health Science Center. The left and right humerus, left tibia, and right femur were obtained from the same cadaver for profile consistency, and each bone was frozen following harvesting and cleaning. Each bone was then documented and photographed. Measurements included proximal and distal diameter, maximum length, and anterior-posterior (AP) and medial-lateral (ML) diameter. Proximal and distal diameter refers to the interior diameter of the marrow cavity. This measurement was taken by inserting a sliding caliper inside the bone at each end. Maximum length was obtained by placing the bone next to a ruler and determining which side of the bone had the longest length. AP diameter was obtained by determining the maximum diameter of the bone by measuring with a sliding caliper from front to back. ML diameter was determined by measuring with a sliding caliper from side to side of the bone. AP and ML diameter are usually taken at the midpoint of the diaphysis; however, the midshaft of the right humerus and left tibia had been sampled previously, so an AP and ML measurement was taken from each end of the remaining diaphysis.

Table 1
Skeletal measurements by sample type in millimeters

Bone Measurements (mm)				
	Left Humerus	Right Humerus	Left Tibia	Right Femur
Max Length	244.0	216.0	257.0	225.0
AP Length I	16.08	17.31	38.53	29.25
ML Length I	15.09	16.99	23.81	27.52

AP Length II	N/A	15.02	27.00	N/A
ML Length II	N/A	17.17	17.58	N/A
Proximal Interior Diameter	16.32	12.38	31.75	16.21
Distal Interior Diameter	10.77	22.73	13.14	25.78

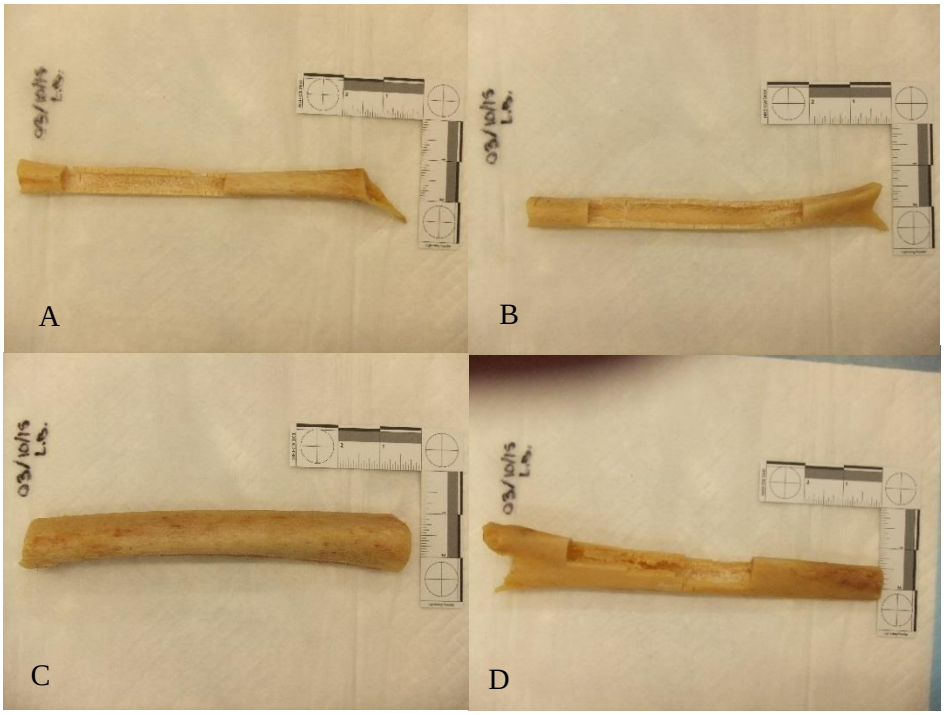

Fig. 4. The four skeletal elements: A. left humerus, B. right humerus, C. left tibia, D. right femur.

2.2 Bone Sample Preparation

Bone samples were prepared according to the University of North Texas Center for Human Identification (UNTCHI) protocol, "Preparation of Skeletal Remains and Teeth for DNA Extraction." Required equipment was cleaned and UV irradiated for 30 minutes before and after each sample, and preparation was performed in a negative airflow sanding station to prevent sample loss or contamination. Each bone was sanded using a Dremel rotary tool (Dremel,

Racine, WI). An approximate area of 1 cm x 6 cm was sanded for the left and right humeri and the right femur. Due to the increased cortical thickness, a smaller 1 cm x 3 cm area was sanded on the left tibia. The sanded bone was wiped with 5% Tergazyme (Alconox, New York, NY) solution to remove dust. Thin sections of bone, approximately ¼ to ½ centimeter in width and about one centimeter in length, were cut with a Mopec Autopsy Saw 115V (Mopec, Oak Park, MI) and weighed.

Table 2
Weights of the cuttings of each skeletal sample in grams

Sample Weights (g)				
	Left Humerus	Right Humerus	Left Tibia	Right Femur
Cuttings	3.378	3.267	3.085	3.110
Pulverized	3.210	3.008	2.761	2.605

Approximately 3 g of bone was cut from each bone sample, the final sample weights are listed in Table X. The cuttings from each bone were placed in an individual 50 mL conical tube and covered with 2.8% sodium hypochlorite (NaClO) bleach solution, agitated, and soaked for 5 minutes. The NaClO solution was decanted, and followed by a water wash with deionized water in the same tube. The tube was agitated and the water decanted. The water wash was repeated five more times until the water was clean and there was no smell of bleach. A final wash was performed using 100% ethanol, and the waste decanted. The clean cut pieces of bone were placed on a weigh boat and allowed to air dry overnight in a hood.

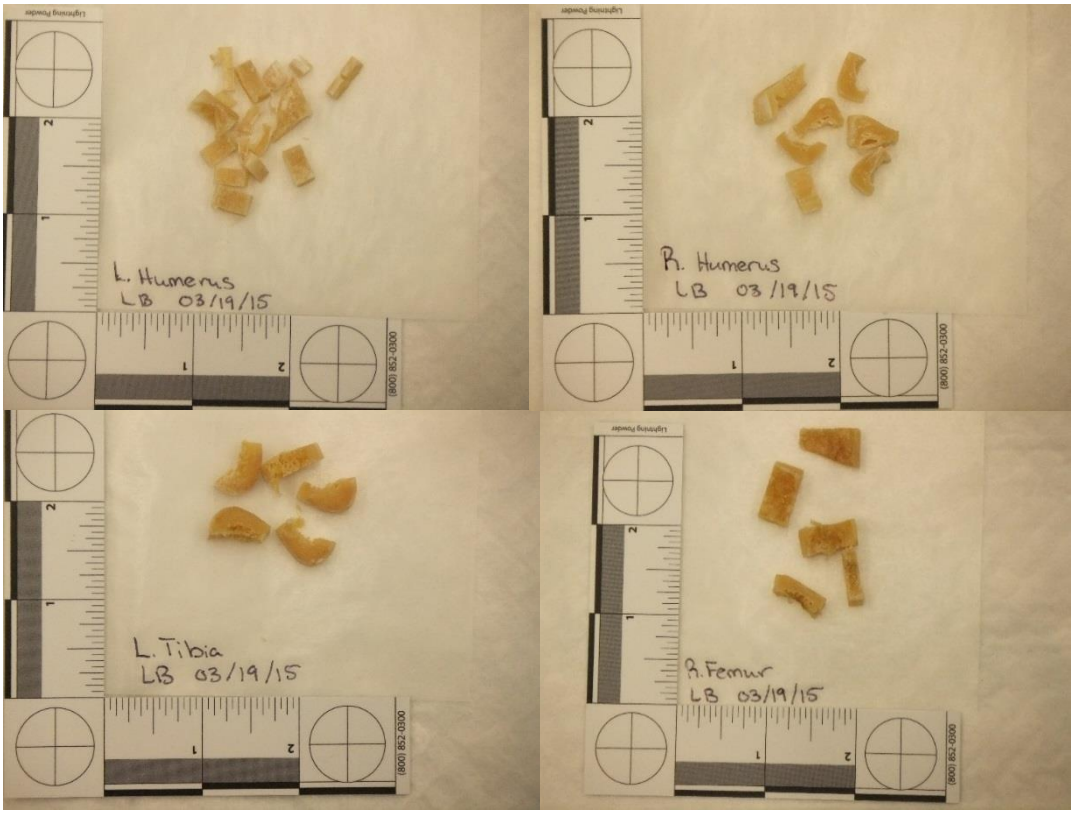

Fig. 5. The four skeletal bone samples after being cut and weighed

2.3 Cryogenic Grinding

The clean and dry bone cuttings for each sample were placed into a polycarbonate grinding cylinder tube. The cylinders were assembled by inserting the metal impactor with the cutting and sealing the cylinder with end caps. The reservoir of a 6770 SPEX Freezer Mill (Sample Prep, Metuchen, NJ) was filled with liquid nitrogen. After an initial chill period lasting 7 minutes, additional liquid nitrogen was added to the fill line in the reservoir before processing the samples. The cylinder containing the bone fragments was inserted into the freezer mill. Following a 4 minute pre-cool the samples were processed for 7 minutes and then visually

inspected to ensure thorough pulverization of the sample. After pulverization, the samples were allowed to return to room temperature. The room temperature bone powder of each bone sample was transferred into a new 50 mL conical tube and weighed. One 0.2 g aliquot of powder from each bone sample was prepared for each of the three extraction methods.

2.4 Preliminary Study

A buffer experiment was performed to determine whether Animal Tissue Lysis Buffer (Buffer ATL; QIAGEN, Germantown, MD) or Hanks Balanced Salt Solution (HBSS) would be more appropriate for use with CLS2 enzyme. Buffer ATL induces the lysis of cells to release and expose DNA. The function of a balanced salt solution is to maintain the pH balance of a solution during the extraction process. The CLS2 enzyme was prepared by first adding 250 mL of HBSS to a graduated cylinder, then 77 g of CLS2 was added an allowed to dissolve. The volume of HBSS was brought up to 1.5 mL, for a final concentration of 50 mg/mL. The trial was performed with previously pulverized bone samples from the same individual. Two gram aliquots of bone powder were placed into 0.2 mL tubes, one labeled HBSS and one labeled ATL. To each tube 75 µL of 50 mg/mL CLS2 was added, and 675 µL of HBSS or Buffer ATL was added to the respective tube. Each tube was vortexed for 10 seconds. The tubes were then placed in an orbital shaker at 37°C, 1100 rpm, and incubated for 5 hours. The tubes were visually inspected for any apparent differences prior to incubation and hourly thereafter.

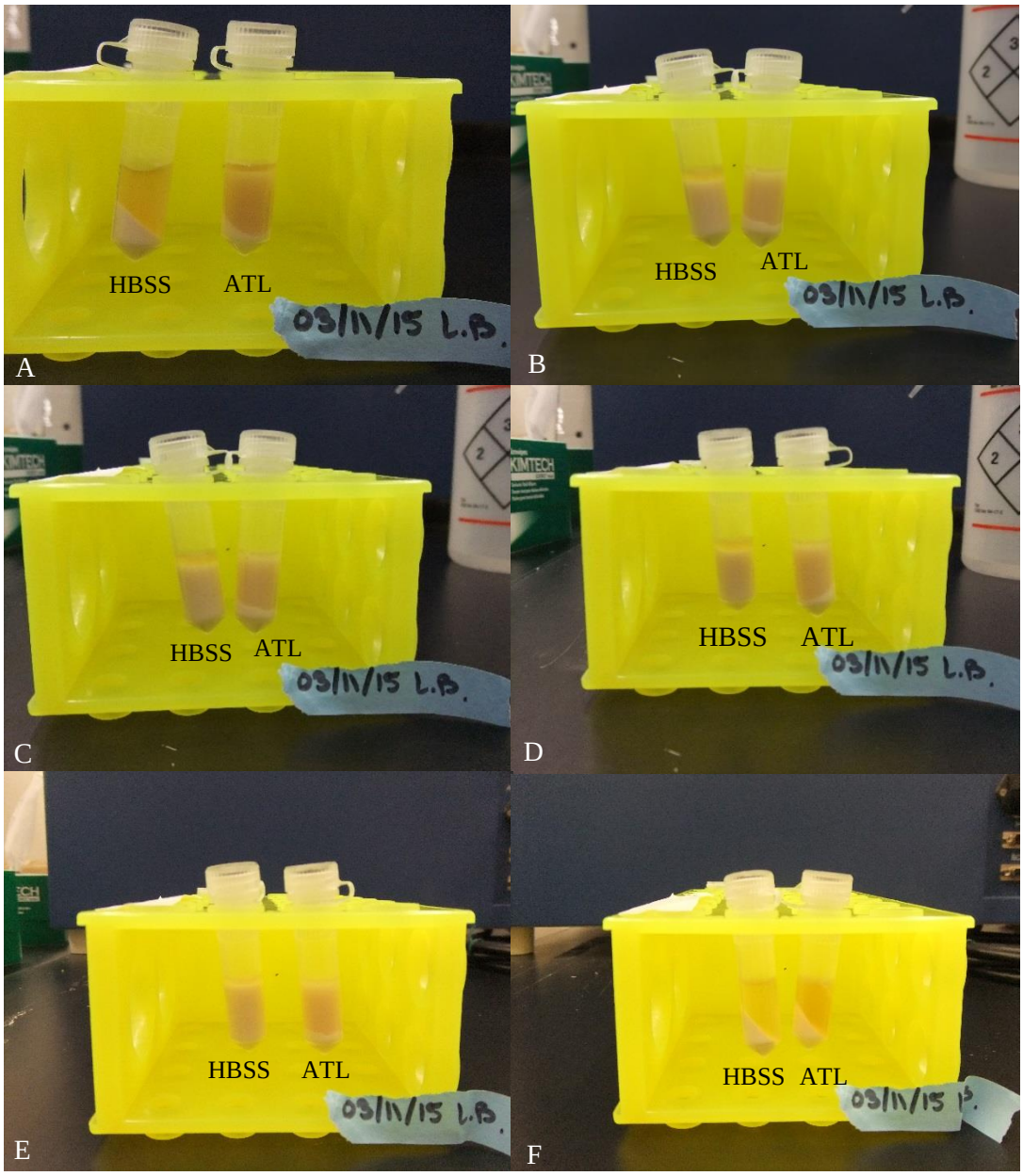

Fig. 6. Results of the buffer experiment from bone sample digested with HBSS versus ATL. Tubes were visually inspected for differences in digestion. A. prior to incubation, B. one hour, C. two hours, D. three hours, E. four hours, and F. five hours of incubation

After 5 hours the samples were removed from the incubator and centrifuged for 1.5 minutes at 10 xg. To both tubes, 750 µL of 0.5 EDTA and 60 µL of DTT were added and the samples were vortexed for 10-15 seconds. The samples were incubated for an additional 20 hours at 56°C, 1100 rpm. Following incubation the samples were centrifuged for 1.5 minutes at

10 xg. Two hundred and thirty microliters of supernatant from the samples were transferred into sample tubes and placed into the AutoMate Express™ Forensic DNA Extraction System (Applied Biosystems, Foster City, CA) for 30 minutes.

2.5. Proteinase K Incubation

Bone powder aliquots were combined with 750 µL 0.5 M ethylenediaminetetraacetic acid (EDTA), 675 µL Buffer ATL (QIAGEN), 75 µL Pro K, and 60 µL of 1M DTT per sample. The samples were vortexed for 15 seconds on medium speed. Samples were placed on an orbital shaker at 56°C, 1100 rpm, for 24 hours. After incubation, the samples were centrifuged at 1.9 xg for 5 minutes. Five hundred microliters of supernatant from each sample was added to 3 separate 2mL EZ1 DNA Investigator Kit (QIAGEN) sample tubes.

2.5.1 Collagenase Type 2 Incubation

Bone powder aliquots were combined with 675 µL of Buffer ATL and 75 µL of 50 mg/mL CLS2. The samples were then vortexed for 15 seconds on medium speed. Samples were incubated at 37°C, 1100 rpm, for 5 hours. After incubation the samples were centrifuged. The samples were then combined 750 µL of 0.5M EDTA and 60 µL of 1.0M DTT. The samples were vortexed for 15 seconds at medium speed. The samples were incubated for an additional 19 hours at 56°C, 1100 rpm and then centrifuged at 1.9 xg for 5 minutes. Five hundred microliters of supernatant from each sample was added to 3 separate 2mL EZ1 DNA Investigator Kit sample tubes.

2.5.2 Combined Pro K and Collagenase Type 2 Incubation

Bone powder aliquots were combined with 675 µL of Buffer ATL and 75 µL of 50 mg/mL CLS2. The samples were vortexed for 15 seconds on medium speed. Samples were

incubated at 37°C, 1100 rpm, for 5 hours. After incubation the samples were centrifuged. The samples were then combined with 750 µL of 0.5M EDTA, 75 µL of Pro K, and 60 µL of 1.0M DTT. The samples were vortexed for 15 seconds at medium speed. The samples were placed on an orbital shaker set for an additional 19 hours at 56°C, 1100 rpm and then centrifuged at 1.9 xg for 5 minutes. Five hundred microliters of supernatant from each sample was added to 3 separate 2mL EZ1 DNA Investigator Kit sample tubes.

2.6 Automated Purification using EZ1 Advanced XL

DNA was purified from the lysate using the EZ1 Advanced XL. Purification reagents were added to each sample tube including: 400µL of Buffer Methylase (MTL), 30µL of sodium acetate (NaOAc), and 1µL of 1µg/µL of Carrier RNA. The samples were vortexed for 15 seconds on medium speed. Samples were loaded onto the EZ1 Advanced XL and purified according to the EZ1 Advanced XL DNA Investigator large volume protocol. Triplicate purification fractions were recombined using a single Microcon® 100 filters (Millipore, Billerica, MA) and eluted in 100 µL of TE^{-4}.

2.7 Quantification

The samples were quantified using the Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific, Carlsbad, CA) on the Applied Biosystems 7500 Real-Time PCR System for Human Identification (Thermo Fisher Scientific). For the preliminary samples, four total samples were quantified HBSS neat, HBSS 1:20, ATL neat, and ATL 1:20. The samples were quantified in duplicate along with a non-template control (NTC), according to manufacturer instruction. Experimental samples were quantified in triplicate along with a NTC in the same matter. Data collected from HID Real-Time PCR Analysis Software v1.2 (Life Technologies,

CA) includes cycle threshold values and quantification yields for each of the sample replicates, and mean and standard deviation of the mean for the replicates. Each sample was normalized to 1 ng/ 15 µL of DNA.

2.8 STR Amplification

Short tandem repeat (STR) amplification was performed in triplicate using the Applied Biosystems GlobalFiler® PCR Amplification Kit (Thermo Fisher Scientific). The amplification was performed according to the manufacturer protocols. Thermal cycling conditions for the GlobalFiler Kit were as follows: 1) 1 cycle at 95°C for 1 minute; 2) 29 cycles denaturation step at 94°C for 10 seconds, and anneal and extension at 59°C for 90 seconds; 3) final extension for 1 cycle at 60°C for 10 minutes; 4) 1 cycle hold at 4°C for up to 24 hours.

2.9 Capillary Electrophoresis and Data Analysis

Fragment analysis was performed in triplicate on the Applied Biosystems 3500xL Genetic Analyzer (Thermo Fisher Scientific). Parameters for the 3500xL were as follows: temperature set at 60°C, injection conditions set at 1.2 kV for 24 seconds, run conditions set at 13 kV for 1550 seconds, dye set J6. The data were analyzed using Applied Biosystems GeneMapper® ID-X (Thermo Fisher Scientific). The thresholds used for the analysis of the generated data were an analytical threshold of 120 relative fluorescence units (RFU) and a stochastic threshold of 250 RFU. Concordance was determined by comparing the STR profile obtained to one previously developed. The data collected were allele peak heights, percent of profile obtained, and concordance with the expected profile.

2.10 Statistical Analysis

Individual Cq values were summarized as the mean for each extraction method and are displayed as a scatter plot for the Internal PCR Control (IPC) and the large and small autosomal targets. The Cq values for the small and large autosomal targets were analyzed by an two-factor ANOVA with replication. Estimated quantity of amplifiable DNA, degradation indices, and percent of the expected profile are summarized in bar graphs comparing the different extraction methods between the different bone types. Peak heights are summarized as box and whisker plots, displaying median, quartiles, and the interquartile range. To determine if DNA quantity and quality differed among and within the different extraction methods the estimated quantity of amplifiable DNA, quantification cycle values, allele heights, and percent of the Globalfiler® STR profile obtained were compared.

3. Results

3.1 Preliminary Study

After the 20 hour incubation, a reduction in bone powder volume was observed in the sample incubated with HBSS and the supernatant was clearer than the sample incubated with Buffer ATL. The apparent reduction in bone powder volume was visually confirmed after centrifugation. Both sample extracts exhibited a faint brown coloration; however, the sample incubated with Buffer ATL was darker.

Quantification values and degradation index values were compared. The highest quantification values were determined from the Buffer ATL neat extracts. Neat sample extracts incubated with Buffer ATL had a mean quantification value of 1.33 ng/µL for the large autosomal target and a mean quantification value of 4.69 ng/µL for the small autosomal target. The 1:20 dilution of the sample extracts incubated with Buffer ATL had a mean quantification value of 0.07 ng/µL for the large autosomal target and a mean quantification value of 0.24 ng/µL for the small autosomal target. Neat sample extracts incubated with HBSS had a mean quantification value of 0.81 ng/µL for the large autosomal target and a mean quantification value of 3.07 ng/µL for the small autosomal target. The 1:20 dilution of the sample extracts incubated with from the HBSS had a mean quantification value of 0.03 ng/µL for the large autosomal target and a mean quantification value of 0.14 ng/µL for the small autosomal target. Lower degradation index values were obtained with samples incubated with Buffer ATL versus samples incubated with HBSS. Samples incubated with Buffer ATL neat and 1:20 dilution yielded a degradation index value of 3.52 and 3.32 respectively. Samples extracted with HBSS neat 1:20 dilution yielded a degradation index value of 3.79 and 4.08 respectively.

3.2 Comparison of Quantification Values between Pro K, CLS2, and PK+C2 Digested Bone Samples

The four bone samples incubated with Pro K, CLS2, and PK+C2 were compared to each other by determining the quantification cycle (Cq) values, quantity of amplifiable DNA, and degradation index yielded for each incubation method.

3.3 Quantification Cycle

For the internal PCR control (IPC) Cq, the right femur overall had higher values with an average Cq of 27.30, 27.43, 27.43 for Pro K, CLS2, and PK+C2 extractions respectively. The left tibia had the lowest values with an average Cq of 26.27, 26.22, 26.23 for Pro K, CLS2, and PK+C2 respectively. The left humerus had average Cq of 26.83, 26.75, and 26.73 and right humerus had average Cq of 26.54, 26.46, and 26.46.

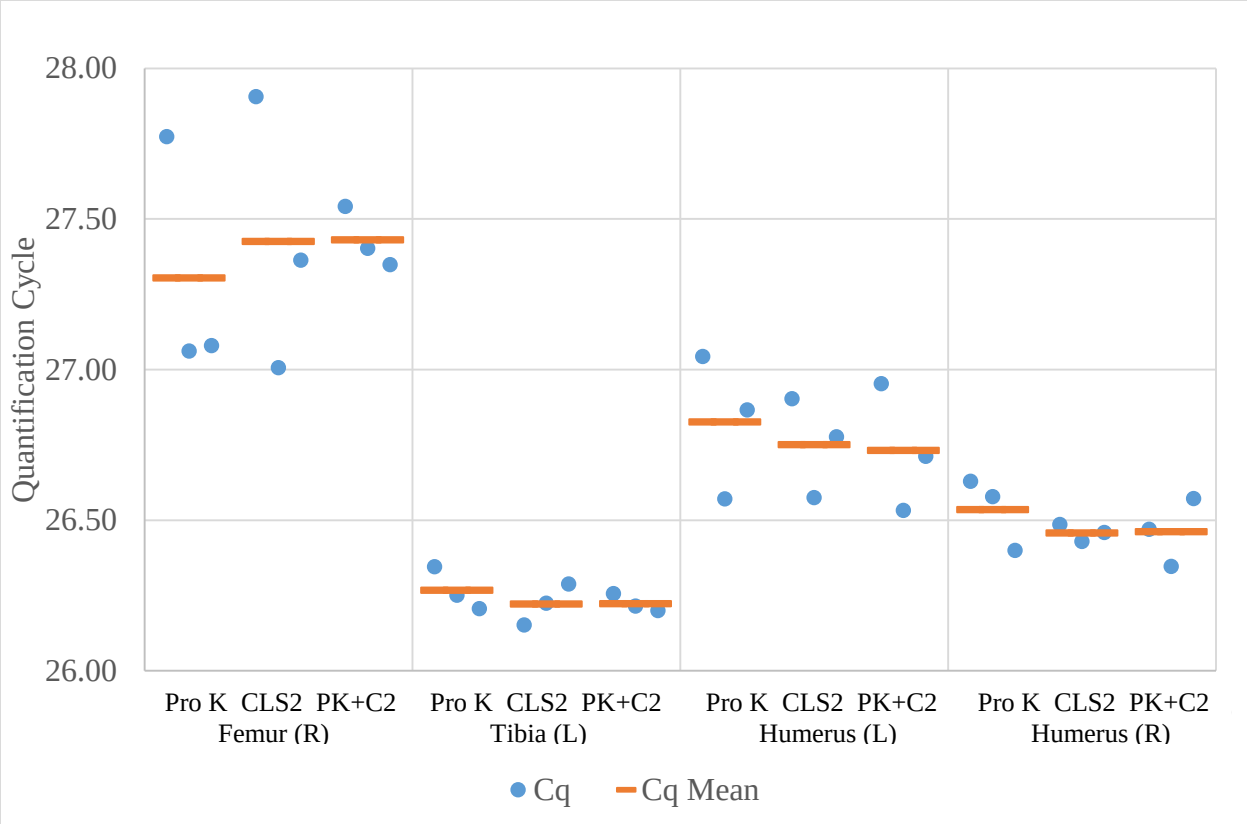

Fig. 7. Internal PCR control (IPC) Cq values. DNA was extracted from human cadaver right femur, left tibia, and left and right humerus samples using Proteinase K (Pro K), Collagenase Type 2 (CLS2), and combined Proteinase K and Collagenase Type 2 (PK+C2) for initial incubation. All samples were purified using an EZ1 Advanced XL and quantified in triplicate with Quantifiler Trio DNA Quantification Kit. Cq values were obtained from the IPC assay for each sample from each incubation method.The blue dots represent the Cq values for the IPC target for each individual sample and the orange lines represent the mean Cq value for each bone.

Each bone sample produced different Cq values from each other for both the small and large autosomal targets. The Cq values obtained for each bone were consistent within and between the replicates for each incubation method for both the small and large autosomal targets. The standard deviations of the small autosomal targets for the right femur ranged from 0.06 to 0.12, for the left tibia the range was 0.03 to 0.12, for the left humerus the range was 0.05 to 0.16, and for the right humerus the range was 0.10 to 0.20. The standard deviations of the large autosomal targets for the right femur ranged from 0.01 to 0.08, for the left tibia the range was

0.05 to 0.10, for the left humerus the range was 0.01 to 0.15, and for the right humerus the range was 0.02 to 0.12. The average small autosomal Cq values for the Pro K, CLS2, and PK+C2 respectively for the right femur are 22.92, 22.13, and 22.39. For the left tibia the values are 26.08, 26.07, and 25.45. For the left humerus the values are 24.26, 24.06, and 24.14. For the right humerus the values are 25.10, 24.96, and 24.91. The average large autosomal Cq values for the Pro K, CLS2, and PK+C2 respectively for the right femur are 21.07, 20.58, and 20.94. For the left tibia the values are 26.95, 27.07, and 26.67. For the left humerus the values are 22.58, 22.59, and 22.75. For the right humerus the values are 23.92, 24.01, and 24.00.

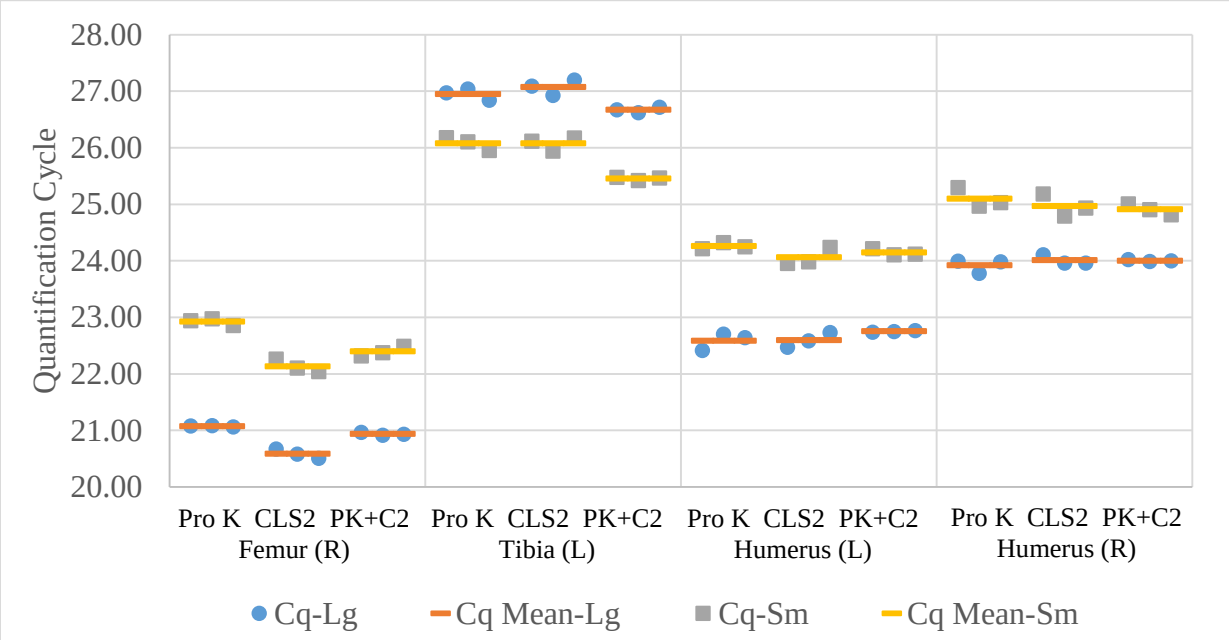

Fig. 8. Large autosomal and small autosomal Cq values. DNA was extracted from human cadaver right femur, left tibia, and left and right humerus samples using Proteinase K (Pro K), Collagenase Type 2 (CLS2), and combined Proteinase K and Collagenase Type 2 (PK+C2) for initial incubation. All samples were purified using an EZ1 Advanced XL and quantified in triplicate with Quantifiler Trio DNA Quantification Kit. Cq values were obtained for each of the samples from each incubation method for both the large and autosomal targets.The blue dots and grey squares represent the individual sample Cq values for large and small autosomal targets respectively. The orange and yellow lines represent the mean Cq values for the large and small autosomal targets for each bone.

The results of the ANOVA for the small autosomal Cq values show a significant difference at the 0.05 level for the type of skeletal element used for extraction. There is also a significant difference at the 0.05 level for the type of method being used for incubation. The results of the ANOVA for the large autosomal Cq values show a significant difference at the 0.05 level for the type of skeletal element used for extraction, but there is not a significant difference at the 0.05 level for the type of method used for incubation.

Table 3.
ANOVA for the small autosomal Cq values.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Skeletal Element	56.13	3	18.71	1378.33	5.48E-27	3.01
Incubation Method	0.87	2	0.44	32.19	1.61E-07	3.40
Interaction	0.99	6	0.16	12.12	2.94E-06	2.51
Within	0.33	24	0.01			
Total	58.32	35				

Table 4.
ANOVA for the large autosomal Cq values.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Skeletal Element	174.56	3	58.19	6843.23	2.59E-35	3.01
Incubation Method	0.03	2	0.01	1.56	0.23	3.40
Interaction	0.67	6	0.11	13.23	1.38E-06	2.51
Within	0.20	24	0.01			
Total	175.46	35				

3.4 Quantity

The bone samples that were incubated with Pro K had a mean quantification value of 12.55 ng/µL, samples incubated with CLS2 had a mean value of 7.31 ng/µL, and samples incubated with PK+C2 had a mean value of 5.82 ng/µL. The quantification values of bone samples incubated with each of the incubation methods were performed with both the large and

small autosomal quantification values. Bone samples digested with CLS2 or PK+C2 did not have a large difference in the quantity of amplifiable DNA within the same bone type. For the right femur the quantity of amplifiable DNA for the large autosomal target for CLS2 and PK+C2 respectively was 21.55 and 16.24 ng/µL, for the left tibia the quantities were 0.25 and 0.32 ng/µL, for the left humerus they were 5.42 and 4.70 ng/µL, and for the right humerus they were 2.05 and 2.00 ng/µL. For the right femur the quantity of amplifiable DNA for the small autosomal target for CLS2 and PK+C2 respectively was 36.05 and 28.37 ng/µL, for the left tibia the quantities were 2.25 and 3.21 ng/µL, for the left humerus they were 9.30 and 8.15 ng/µL, and for the right humerus they were 4.92 and 4.74 ng/µL. For the large and small autosomal target respectively the quantity of DNA reported for the right femur was 32.76 and 56.94 ng/µL, for the left tibia they are 0.64 and 6.11ng/µL, for the left humerus they are 11.94 and 22.08 ng/µL, and for the right humerus they are 4.88 and 12.27 ng/µL.

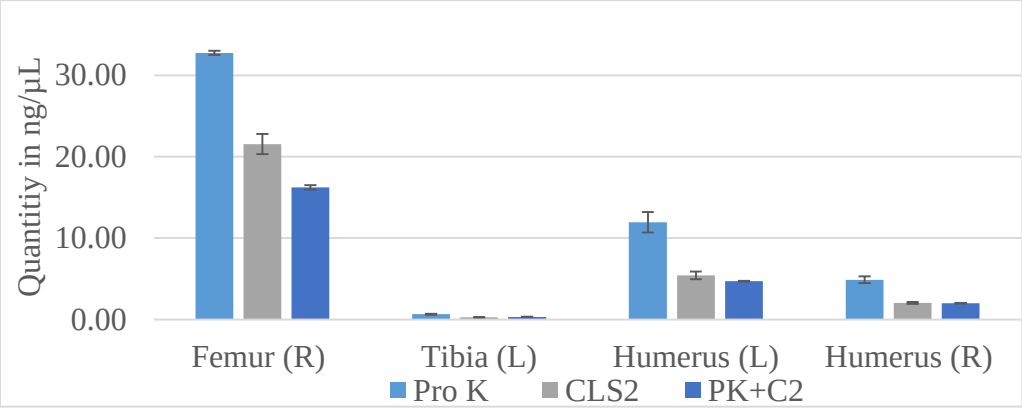

Fig. 9. Quantity of amplifiable DNA. DNA was extracted from human cadaver right femur, left tibia, and left and right humerus samples using Proteinase K (Pro K), Collagenase Type 2 (CLS2), and combined Proteinase K and Collagenase Type 2 (PK+C2) for initial incubation. All samples were purified using an EZ1 Advanced XL and quantified in triplicate with Quantifiler Trio DNA Quantification Kit. Quantities of amplifiable DNA were obtained for each bone sample from each incubation method. Quantities are estimated by the large autosomal target and are reported in ng/µL.

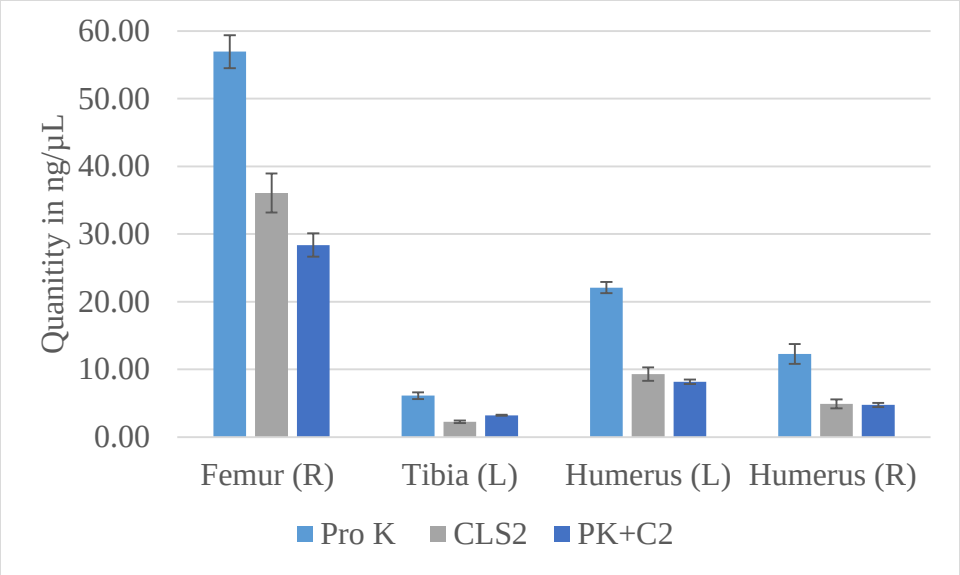

Fig. 10. Quantity of amplifiable DNA. DNA was extracted from human cadaver right femur, left tibia, and left and right humerus samples using Proteinase K (Pro K), Collagenase Type 2 (CLS2), and combined Proteinase K and Collagenase Type 2 (PK+C2) for initial incubation. All samples were purified using an EZ1 Advanced XL and quantified in triplicate with Quantifiler Trio DNA Quantification Kit. Each bone sample was quantified in triplicate. Quantities are estimated by the small autosomal target and are reported in ng/µL.

3.5 Degradation Index

The range of degradation index values for all samples was from 1.67 to 9.99. Respective of Pro K, CLS2, and PK+C2, the degradation indices for the right femur were 1.74, 1.67, and 1.75; for the left tibia were 9.55, 9.0, and 9.99; for the left humerus were 1.86, 1.72, and 1.74; and for the right humerus were 2.51, 2.39, and 2.37. When comparing the degradation index values between samples from different bones, the left tibia overall had the highest degradation index values while the right femur, and left and right humerus had lower values.

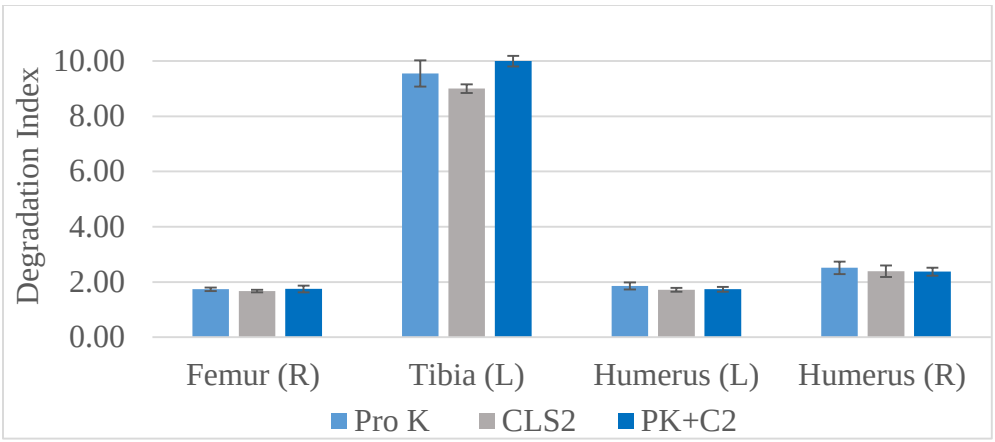

Fig. 11. Degradation index values. DNA was extracted from human cadaver right femur, left tibia, and left and right humerus samples using Proteinase K (Pro K), Collagenase Type 2 (CLS2), and combined Proteinase K and Collagenase Type 2 (PK+C2) for initial incubation. All samples were purified using an EZ1 Advanced XL and quantified in triplicate with Quantifiler Trio DNA Quantification Kit. The mean degradation index value is reported for each bone sample from each incubation method.

3.6 Percent of Expected Profile Obtained

The goal of this study was to determine the percentage of the expected profile obtained from each bone sample extracted with each incubation method and amplified with the GlobalFiler ™ Amplification Kit. This kit targets 24 markers in total. To determine percent profile obtained from the samples only 22 of the 24 markers were used. The two markers that were not used, Yindel and DYS391 were excluded from analysis because they are targets of the Y chromosome and the bones were known to originate from a female individual. When comparing the percent profiles of the Pro K small and large autosomal targets, respectively, the right femur had a recovery of 93% and 100%, the left tibia had a recovery of 46% and 97%, the left humerus had a recovery of 100% and 41%, and the right humerus had a recovery of 90% and 97%. All bone samples yielded a full profile when incubated with the CLS2 method, as well as

the right femur, left tibia, and right humerus samples incubated with the combination PK+C2 method. The DNA profile obtained from the left humerus was 98% of the expected.

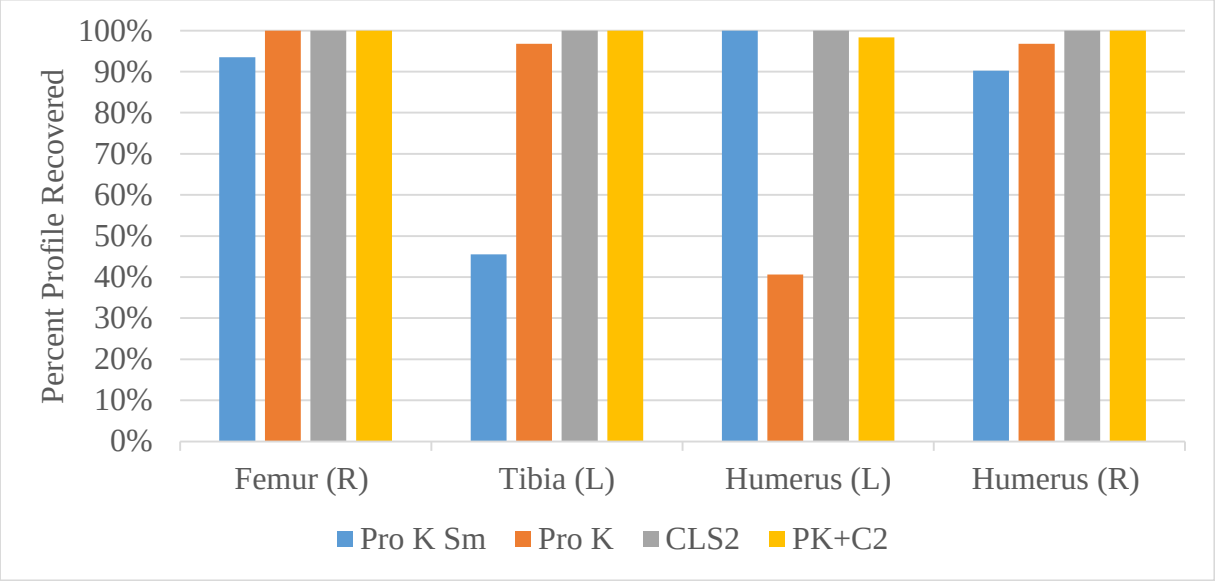

Fig. 12. Percentage of the expected GlobalFiler® STR profile. DNA was extracted from human cadaver right femur, left tibia, and left and right humerus samples using Proteinase K (Pro K), Collagenase Type 2 (CLS2), and combined Proteinase K and Collagenase Type 2 (PK+C2) for initial incubation. All samples were amplified in triplicate with GlobalFiler® PCR Amplification Kit and analyzed with GeneMapper ID-X. Profile obtained was compared to a reference profile to determine how much of the profile was recovered. The profile obtained for each replicate was compared to a reference profile to determine how much of the profile was expected twenty-two forensic markers were used to determine full profile status.

3.7 Peak Heights

The purpose of this analysis was to determine the difference in the quality of the GlobalFiler® profile obtained from samples incubated with Pro K, CLS2, and PK+C2 combination. For Pro K, the relative fluorescence units (RFU) of the peak heights did not exceed 6,133 RFU for extracts normalized using the Quantifiler® Trio small autosomal target. When the Quantifiler® large autosomal target was used for normalization, Pro K samples showed a maximum peak height of 26,267 RFU; however, the majority of the profile peak heights did not

exceed 5,000 RFU. The maximum peak height reported from the samples extracted with CLS2 was 32,158 RFU, and the maximum peak height reported from the samples incubated with the PK+C2 combination was 32,466 RFU.

For the Pro K, RFU had a minimum value of 438 RFU for extracts normalized using the Quantifiler® Trio small autosomal target. When the Quantifiler® large autosomal target was used for normalization, Pro K samples showed a minimum peak height of 84 RFU. The minimum peak height reported from the samples extracted with CLS2 was 1,835 RFU, and the minimum peak height reported from the samples incubated with the PK+C2 combination was 1,249 RFU.

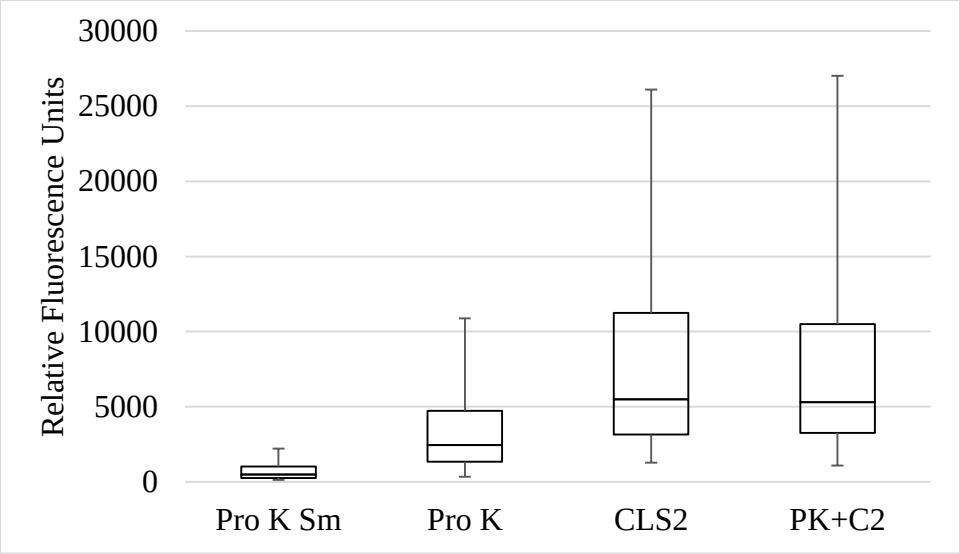

Fig. 13. The range and quartiles of allele peak heights, expressed as relative fluorescence units, from right femur samples obtained using the three incubation methods. DNA was extracted from human cadaver right femur, left tibia, and left and right humerus samples using Proteinase K (Pro K), Collagenase Type 2 (CLS2), and combined Proteinase K and Collagenase Type 2 (PK+C2) for initial incubation. All samples were amplified in triplicate with GlobalFiler® PCR Amplification Kit and analyzed with GeneMapper ID-X. The Relative Fluorescence Units (RFU) were obtained for each incubation method and compared between the incubation methods. Samples incubated with Pro K were normalized using the quantification estimates obtained for the Quantifiler® Trio small (Pro K sm) and the large (Pro K) autosomal targets, whereas other samples were only normalized using the quantification estimates obtained from the large target.

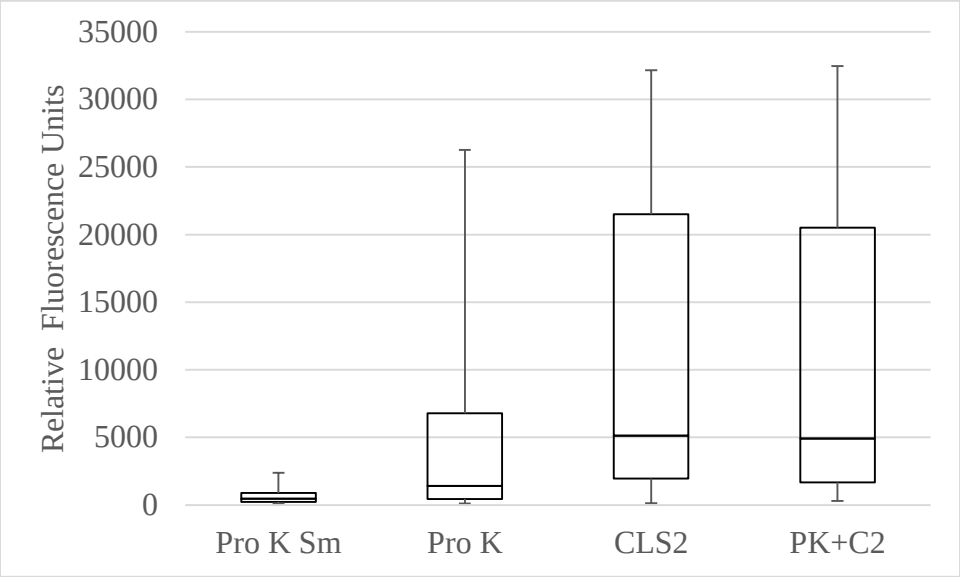

Fig. 14. The range and quartiles of allele peak heights, expressed as relative fluorescence units, from left tibia samples obtained using the three incubation methods. DNA was extracted from human cadaver right femur, left tibia, and left and right humerus samples using Proteinase K (Pro K), Collagenase Type 2 (CLS2), and combined Proteinase K and Collagenase Type 2 (PK+C2) for initial incubation. All samples were amplified in triplicate with GlobalFiler® PCR Amplification Kit and analyzed with GeneMapper ID-X. The Relative Fluorescence Units (RFU) were obtained for each incubation method and compared between the incubation methods. Samples incubated with Pro K were normalized using the quantification estimates obtained for the Quantifiler® Trio small (Pro K sm) and the large (Pro K) autosomal targets, whereas other samples were only normalized using the quantification estimates obtained from the large target.

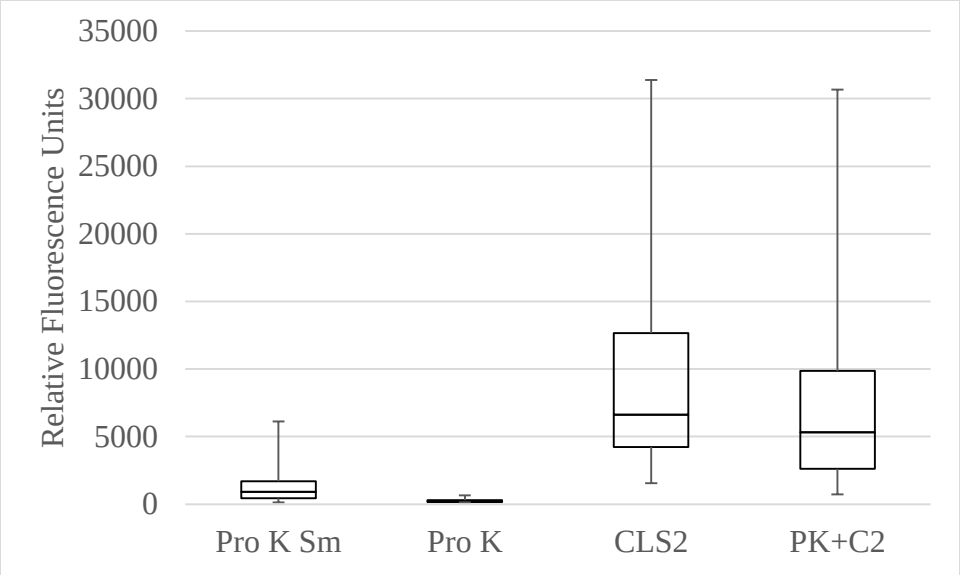

Fig. 15. The range and quartiles of allele peak heights, expressed as relative fluorescence units, from left humerus samples obtained using the three incubation methods. DNA was extracted from human cadaver right femur, left tibia, and left and right humerus samples using Proteinase K (Pro K), Collagenase Type 2 (CLS2), and combined Proteinase K and Collagenase Type 2 (PK+C2) for initial incubation. All samples were amplified in triplicate with GlobalFiler® PCR Amplification Kit and analyzed with GeneMapper ID-X. The Relative Fluorescence Units (RFU) were obtained for each incubation method and compared between the incubation methods. Samples incubated with Pro K were normalized using the quantification estimates obtained for the Quantifiler® Trio small (Pro K sm) and the large (Pro K) autosomal targets, whereas other samples were only normalized using the quantification estimates obtained from the large target.

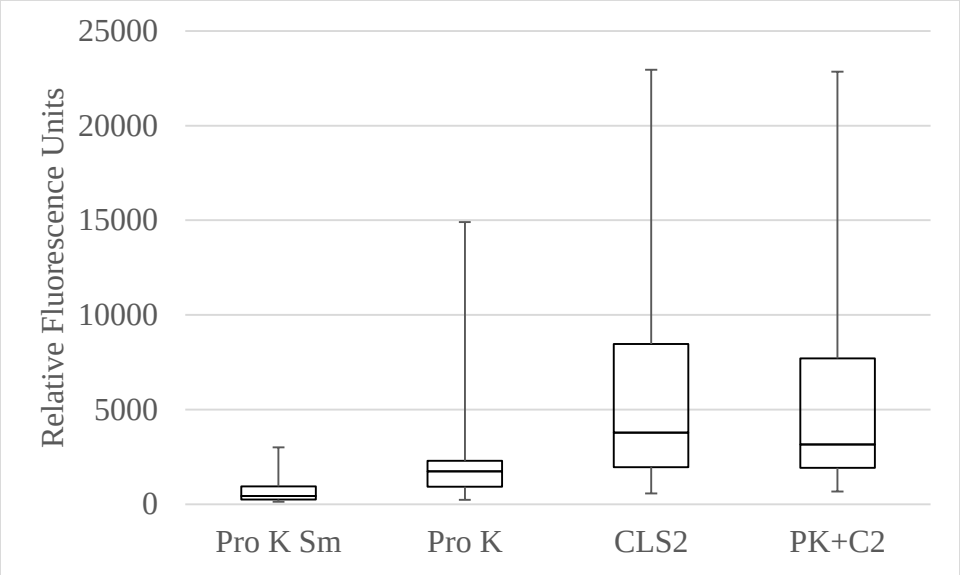

Fig. 16. The range and quartiles of allele peak heights, expressed as relative fluorescence units, from right humerus samples obtained using the three incubation methods. DNA was extracted from human cadaver right femur, left tibia, and left and right humerus samples using Proteinase K (Pro K), Collagenase Type 2 (CLS2), and combined Proteinase K and Collagenase Type 2 (PK+C2) for initial incubation. All samples were amplified in triplicate with GlobalFiler® PCR Amplification Kit and analyzed with GeneMapper ID-X. The Relative Fluorescence Units (RFU) were obtained for each incubation method and compared between the incubation methods. Samples incubated with Pro K were normalized using the quantification estimates obtained for the Quantifiler® Trio small (Pro K sm) and the large (Pro K) autosomal targets, whereas other samples were only normalized using the quantification estimates obtained from the large target.

4. Discussion

The goal of the preliminary study was to determine if Buffer ATL was going to have an inhibitory effect on CLS2 enzyme activity, due to the concentration of sodium dodecyl sulphate (SDS) detergent [15]. Buffer ATL is a proprietary cell lysis buffer from QIAGEN that breaks open the cell and releases the cellular components. Buffer ATL induces the lysis of cells to release and expose DNA and was the recommended buffer for extraction of DNA from bone according to the Dukes et al. (2012) protocol for use the EZ1 Advanced XL purification system that also included incubation with Pro K enzyme [12]. The recommended protocol for bone digestion using the CLS2 enzyme includes the use of Hank's balanced salt solution (HBSS), and the function of this solution is to maintain the pH balance of a solution during the extraction process [16]. The results of this experiment indicated that Buffer ATL does not have an inhibitory effect on the enzymatic activity of CLS2, and the resulting extract had a higher estimates of amplifiable DNA quantity, as well as higher peak heights and greater percent of the expected GlobalFiler® STR profile.

The difference in Cq values for each bone was less than 1 Cq between the three incubation methods. The minimum IPC Cq for the right femur was 27.01 and the maximum was 27.91, for the left tibia the minimum was 26.15 and the maximum was 26.53, for the left humerus the minimum was 26.53 and the maximum was 27.04, and for the right humerus the minimum was 26.35 and the maximum was 26.63. In order to determine if samples may be affected by high sample concentration or inhibition, the user manual suggests comparing the IPC values of your samples to the mean IPC value of the standards. If sample IPC values are higher relative to the standard means then it is an indication of high sample concentration or inhibition [13]. A 95% confidence interval was used to determine if a sample IPC Cq is high when compared to the

mean IPC of the standards. The mean IPC Cq of the standards is 26.74, with a lower 95% confidence interval of 26.43 and an upper 95% confidence interval of 27.05. All of the Cq values for the right femur fall above the upper CI and therefore meet the manufacturer's interpretation of high sample concentration or inhibition. Based on the high quantification values obtained for the femur, it is the likely cause for the high IPC Cq values when compared to the IPC Cq values of the standards.

The cycle threshold defines the level of detectable fluorescence and the threshold value indicates the number of cycles it took for fluorescence to be detected. Cq values will increase with decreasing amounts of template, meaning samples with more DNA will have a lower Ct value [13]. The femur had the lowest small and large autosomal Cq values for all three incubation methods, an average of 20.86, and the tibia had the highest large autosomal Cq values, an average of 26.9. There is an inverse relationship between the Cq value and the starting template copy number [13]. The higher the Cq value the lower the starting templet copy number and the lower the Cq value the higher the starting templet copy number; therefore, it was expected that the femur would have a higher quantity of amplifiable DNA and that the tibia would have a lower quantity of amplifiable DNA. The Cq values obtained for the large autosomal target for each sample had lower values, with the exception of the left tibia. This could be an indication of degradation in the DNA extracted from the tibia because the Cq value for the large autosomal target has a higher value than the small autosomal target, and the small autosomal target has a length of 80 bases while the large autosomal target has a length of 214 bases [13]. Based on the results of ANOVA displayed in Table 3 and 4, the selection of the type of skeletal element used for extraction was a more significant source of variation than the type of enzyme used for incubation.

The quantities estimated by the small autosomal target had higher values than the large autosomal target. The small autosomal target has a smaller amplicon size and therefore there is preferential amplification of it compared to the large autosomal target. The quantification values indicate that the skeletal element selected effects the amount of template extracted. The Pro K incubation method yielded the highest amounts of amplifiable DNA from all bone types for both small and large autosomal targets. The right femur had the highest quantification values for both small and large autosomal targets and the tibia had the lowest quantification values for both small and large autosomal targets regardless of incubation method. The two humeri had values between the right femur and left tibia. A study reporting DNA quantification results from 200 to 500 year old skeletal remain samples ranked the skeletal elements from highest quantity to lowest quantity, which was the femur, followed by the humerus, and the tibia [17]. A study examining DNA yield rates for different skeletonized elements from recently skeletonized individuals reported higher DNA quantities from skeletal elements found in the lower extremities versus skeletal elements from the upper extremities [18]. Based on quantification values the femur is the bone type that yielded the highest amount of amplifiable DNA, which is consistent with the literature, being that the femur is a skeletal element of the lower extremity. The tibia, which is also a skeletal element of the lower extremity, had the lowest quantification values. This finding is consistent with the results from the old skeletal remains, but not the recently skeletonized study.

The degradation index provides an indication of how the large fragments of DNA are performing compared to the small fragments of DNA. This is calculated by the software by dividing the estimated DNA concentration from the small autosomal target by the large autosomal target [13]. A degradation index less than 1 is an indication that there is no

degradation or inhibition of the sample. A degradation index from 1 to 10 is an indication that there is degradation or inhibition of the sample, with the degradation ranging from slight to moderate as the index value approaches 10. [13]. The degradation index value will give an indication to the performance and quality of the STR profile. Degradation index results of 9.0, 9.55, and 9.99 indicate for the left tibia an expectation that allelic drop out, low peak heights, and peak height imbalance will be observed in the STR profile [14]. The femur did not have a degradation index exceed 1.75, the left humerus did not have a degradation index exceed 1.74, and the right humerus did not have a degradation index exceed 2.51. For the femur, and left and right humerus, it is expected that the STR profiles will have minimal to no allelic drop out, high peak heights, and peak height balance.

No drop out was observed in any of the samples incubated with CLS2 alone. A study that tested the successfulness of generating STR profiles from different skeletal elements found that skeletal elements originating from the lower extremity had higher success rates in generating profiles than skeletal elements originating from the upper extremities [19]. Similarly, a study that examined the preservation of DNA in skeletal elements from World Trade Center victims found that skeletal elements that were weight-bearing from lower limbs had higher success rates of generating STR profiles than upper limbs [20]. The femur overall had the largest percent of profile recovered for all incubation methods, which was consistent with the study that tested successfulness of STR profile generation and the World Trade Center study. The percentage of the profile recovered from the tibia was also consistent with both studies, except for the samples incubated with Pro K and normalized based on the small autosomal target quantification values. Both the left and right humeri had 90% or greater recovery of the profile, which was not consistent with either study, except for the left humerus samples incubated with Pro K. Those

samples, diluted based on the large autosomal target quantification values, had a recovery of 41%. The success study showed an average success rate of 46% for the humerus and the World Trade Center study showed an average rate of 60% for the humerus.

Based on the results of the Cq, quantification, and degradation index values, the left tibia was expected to have small peak heights. Despite this, large peak heights were obtained for the left tibia; the medians for CLS2 and PK+C2 were approximately 5,000 RFU and the highest peak was 32,466 from the PK+C2 incubation method. All skeletal elements generated profiles with high peak heights when incubated with CLS2 and PK+C2. The smallest peak heights were observed in samples incubated with Pro K regardless of bone type. Similar peak heights and medians were seen from CLS2 and PK+C2 an indication that Pro K in the PK+C2 combination is not contributing much to the breakdown of the collagen matrix bone barrier. Based on the quality of the STR profiles, the left tibia, followed by the right femur, and then the left and right humerus, was the best bone type for generating STR profiles and the CLS2 was the best enzyme to use for incubation.

5. Conclusion

Many cases involving skeletal remains are associated with a missing persons case. Events such as mass disasters or abductions can lead to a crime scene where only skeletal remains exist [REF]. These remains can vary in size and damage depending on the environmental conditions; therefore, it is important to have an efficient extraction procedure with an enzyme that can effectively break down the collagen matrix and cellular proteins to release the maximum amount of DNA possible. It is also advantageous for an analyst to understand which skeletal elements are most likely to yield the best STR profiles. Based on the results of this study, it is recommended to perform DNA extractions on skeletal elements that are from the lower extremities when available. It is also recommended to use CLS2 versus Pro K during the incubation step. Using CLS2 for incubation of samples obtained from skeletal elements will provide STR results with less allelic drop-out, higher peak heights, and improved peak height balance. In a missing persons case, a greater number of alleles included in an STR profile will provide higher confidence in results when comparing the profile of the remains to a familial profile or a profile in a database.

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