OPTIMIZATION OF A METHOD FOR THE EXTRACTION OF DNA FROM HUMAN SKELETAL REMAINS FOR FORENSIC CASEWORK

A thesis presented to the faculty of the Graduate School of
Western Carolina University in partial fulfillment of the
requirements for the Degree of Master of Science in Biology

By

Sherri Marie Deaton

Director:
Dr. Mark R. Wilson
Associate Professor, Department of Chemistry and Physics
Director, Forensic Science Program

Committee Members: Dr. Jessica Moore, Department of Biology
Dr. John Williams, Department of Anthropology and Sociology
Dr. Scott Huffman, Department of Chemistry and Physics

June 2015

ACKNOWLEDGEMENTS

I would like to thank Dr. Mark Wilson for the opportunities I have been offered working in the forensic science department during my graduate career here at WCU. I also wish to thank the members of my committee for their insight and guidance, as well as Brittania Bintz for her guidance and help throughout my research. In addition, I'd like to extend my gratitude to Silvana Maas, an undergraduate student in our lab who performed the nuclear quantitation assays.

I'd also like to thank the National Institute of Justice, through which my work was funded.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

OPTIMIZATION OF A METHOD FOR THE EXTRACTION OF DNA FROM HUMAN SKELETAL REMAINS IN FORENSIC CASEWORK

Sherri Marie Deaton, B.S.

Western Carolina University (June 2015)

Director: Dr. Mark R. Wilson

Obtaining full DNA profiles from bone can be challenging due to the inherently low quantity and quality of DNA. An efficient extraction protocol is required to maximize DNA recovery from bone samples while minimizing the coextraction of the PCR inhibitors naturally present in bone. DNA extraction from bone involves three discrete steps: demineralization, lysis, and purification. Demineralization involves incubating bone that has been pulverized into a powder in a chelating agent in order to sequester the divalent metal cations that comprise the mineral matrix of bone tissue. This is followed by a lysis step, in which the demineralized bone material is incubated in a buffer that disrupts the cellular membrane, releasing the DNA and intracellular components. Purification is performed after lysis to separate the DNA from proteins and cellular debris, as well as to remove PCR inhibitors.

Several variables were examined in this study, including the use of three different lysis buffers, two different bone types, two different materials for the end caps and impactor bar used in the pulverization process, and the interactions between these variables. The effectiveness of each modification in the procedure was assessed using qPCR, which was used to measure mtDNA recovery in copies of mtDNA per gram of bone material, as well as by the assessment of inhibition using an internal positive control.

The actual effectiveness of each lysis buffer varied considerably with bone types examined. In addition, a novel modification to the pulverization step was found to significantly enhance mtDNA recovery by reducing inhibition detected in samples.

The findings of this work suggest that the current methodology is not fully optimized, and additional emphasis needs to be placed upon removing inhibitors but also investigating the effects of the bone lysate on the pH and ionic strength on the recovery of DNA from bone material. Preliminary work is presented from this study in developing a method for DNA extraction from human bone and elucidating the sources of inhibition that are introduced via the extraction method itself as well as those naturally present in bone tissue.

CHAPTER 1: INTRODUCTION

1.1. Methods of Forensic DNA Analysis

DNA analysis is a powerful tool for linking suspects to a crime scene and alternatively, exonerating the innocent; however, it is not uncommon in forensic casework to be presented with very challenging biological samples for analysis. In cases involving charred remains, missing persons, and mass burials, highly degraded bone fragments are often the only obtainable physical evidence for human identification. Numerous challenges exist with extracting DNA from bone; the structure and chemical composition of bone make extracting and amplifying DNA difficult, and the environmental conditions from which the bone is recovered can dramatically alter the preservation state of the bone material and consequently, the integrity and availability of the DNA (Ye et al. 2004, Kalmár et al. 2000). However, because of this unique structure, bone tissue is considerably more resistant to degradation than other tissues such as epithelial tissue, nervous tissues, etc. (Ye et al. 2004, Loreille et al. 2007).

Nancy Ritter, editor of the National Institute of Justice Journal, calls the issue of missing persons and unidentifiable human remains the "Nation's Silent Mass Disaster," noting that as of 2007, over 40,000 sets of human skeletal remains were unidentifiable using conventional means. Until more efficient DNA extraction methods are developed, many of these will remain in evidence rooms of medical examiners (2007). In addition, several government organizations such as the Joint POW/MIA Accounting Command-Central Identification Laboratory (JPAC) and the Armed Forces DNA Identification Laboratory (AFDIL) work diligently to recover and identify the remains of all missing military personnel. It is estimated that over 83,000 Americans are still unaccounted for from the previous conflicts of WWII, the Korean War, Cold War, and Vietnam War (Farrell et al. 2013, Edson et al. 2012).

As with all challenging bone samples, highly efficient DNA extraction methods are required to identify human remains. In situations involving very degraded bone material, it is often very difficult to obtain nuclear DNA for STR analysis (Holland and Parsons 1999). Nuclear DNA is typically analyzed in forensic casework by looking at short tandem repeats (STRs) present in non-coding regions of nuclear DNA. STRs are repeating stretches of short nucleotide sequences. The number of these repeated sequences is highly variable, so when multiple regions (loci) are examined, the probability of two of these STR profiles being identical is extremely unlikely if the individuals are unrelated, often with statistical probabilities of discrimination of one in many billions.

In forensic casework, evidence samples that may be considered robust under normal circumstances, such as bloodstains, may become too degraded for nuclear DNA analysis when subjected to harsh environmental conditions such as exposure to UV radiation, humidity, etc. Hair samples obtained as evidence often lack a root from which nuclear DNA can be extracted, and as a result, it is only possible to obtain mtDNA, due to it being significantly more numerous in cells (Melton et al. 2012). mtDNA can be extracted from casework samples in high quantities and because mtDNA is inherited maternally, it is also possible to obtain reference samples in missing persons cases from living maternal relatives (Holland and Parsons 1999).

1.2. Structure and Function of Deoxyribonucleic Acid

Deoxyribonucleic acid (DNA) is a biopolymer composed of two antiparallel strands of deoxyribose molecules connected by phosphate groups, referred to as the sugar phosphate backbone. Connecting each strand to one another are complementary, nitrogenous bases called nucleosides, which interact with one another via hydrogen bonding from opposing anti-parallel strands.

The nitrogenous bases that make up DNA include two purines, adenine (A) and guanine (G), which interact via hydrogen bonding with two pyrimidines, thymine (T) and cytosine (C), respectively (Fig. 1.1) (Watson and Crick 1953). In mammalian cells, DNA is found in the nucleus of a cell. It is supercoiled into linear structures called chromosomes (Banfalvi 1986). In addition to nuclear DNA, animal cells contain mitochondrial DNA (mtDNA), present in the mitochondria of a cell.

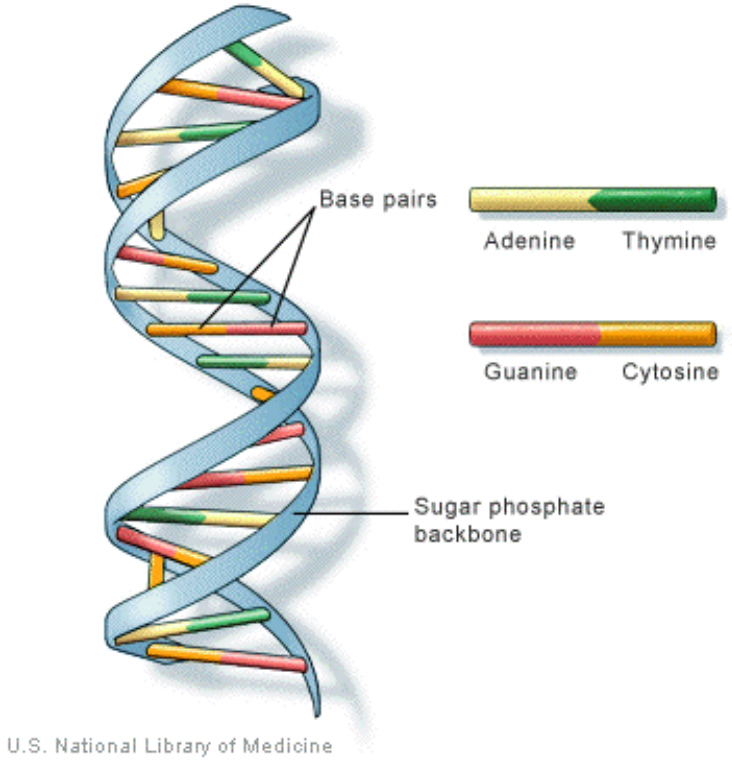

Figure 1.1: Structure and composition of DNA (NLM 2015).

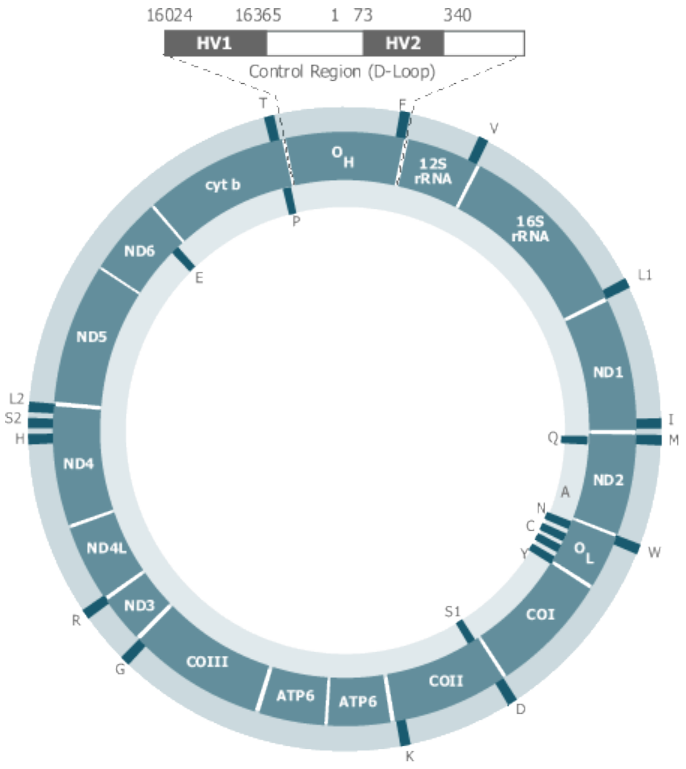

Figure 1.2: Mitochondrial genome showing the control region and hypervariable regions 1 and 2 (Tilstone 2007).

Mitochondria are double membrane organelles that are found in most eukaryotic cells that play an integral role in cellular respiration, signaling, and differentiation, and are an active part of the cell cycle, playing roles in growth, senescence, and apoptosis. mtDNA is a circular chromosome approximately 16,569 base pairs in length that contains 37 genes that code for proteins functioning in oxidative phosphorylation. mtDNA also contains genes which encode transfer RNA and ribosomal RNA (Anderson et al. 1981) (Fig. 1.2). Whereas a eukaryotic cell only contains two copies of nuclear DNA, mitochondria are abundant in many cell types, and as a result, mtDNA is significantly more numerous in most tissues than nuclear DNA with an

average of 500 copies of mtDNA per cell but up to as many as 2000 copies per cell in very metabolically active tissues (Satoh and Kuroiwa 1991).

1.3. Mitochondrial DNA Analysis in Forensic Casework

As previously mentioned, mtDNA is a circular chromosome found in mitochondria. A non-coding control region exists within the chromosome. This control region contains the origin of replication as well as the origins of transcription from each strand. Within the control region are two hypervariable regions, HVI and HVII (Anderson et al. 1981). Within these hypervariable regions, it is common to find length and sequence polymorphisms, which are analyzed to determine the mtDNA type of an individual (Holland and Parsons 1999). An mtDNA type is often expressed as a comparison to the revised Cambridge Reference Sequence (rCRS) in order to present a long sequence in shortened form. The rCRS has been used as a mitochondrial DNA reference sequence since 1999 (Andrews et al. 1999).

In forensic casework, the mtDNA type of the questioned sample found at the crime scene (Q) is often compared to known exemplar, or reference samples (K). In current practice, if both the Q and K samples contain identical bases at each position they are said to be in concordance with one another and cannot be excluded as originating from the same source. If a single nucleotide polymorphism (SNP) difference is found between any two sequences under comparison, then the comparison is deemed inconclusive. If more than one nucleotide difference is present, then the Q sample can be excluded as originating from the same source as the K sample (Budowle, Wilson, and DiZinno 1999). The power of forensic DNA analysis is a result of the ability to discriminate one person's genetic profile from another. Therefore, in order to increase the probability of discrimination of mitochondrial DNA, it is critical to examine a greater region of the mitochondrial genome (Coble et al. 2004).

1.4. Amplification of DNA Using Polymerase Chain Reaction

Due to the degradation arising from both the environment and intercellular mechanisms of necrosis, DNA extracted from forensically relevant sample types is often low in quantity and highly degraded. While not much can be done to address the degradation of DNA, amplification of the available DNA is integral in obtaining sufficient quantities for DNA analysis. One of the biggest advancements in molecular biology occurred in the early 1980's with the development of a method to amplify DNA in vitro. Polymerase chain reaction (PCR) utilizes an enzyme called DNA polymerase to synthesize new strands of DNA from an original template strand (Mullis et al. 1986). The result is amplification of the target strand across orders of magnitude. Key components of PCR include a DNA polymerase to catalyze the addition of nucleotides to the growing DNA strand, and DNA primers which are short oligonucleotides, typically about 20 bases in length, designed to be complementary to the target region of DNA. A key role of the primer is to provide a 3' hydroxyl group, which is necessary for the incorporation of nucleotides by DNA polymerase. Deoxynucleotide triphosphates (dNTPs) are included in the PCR reaction as the substrate of DNA polymerase to be added to the growing DNA strand. In addition to polymerase, primers, and dNTPs, a buffer is included to maintain proper pH and ionic strength, and divalent magnesium, which is a critical cofactor for polymerase activity.

The PCR reaction is driven by thermal cycling, a repeating series of typically three temperature changes (Mullis et al. 1986, Saiki et al. 1988). The first step in PCR is called the denaturation step, in which relatively high temperatures weaken and disrupt the hydrogen bonds between the base pairs of double-stranded DNA, which results in the formation of single-stranded DNA.

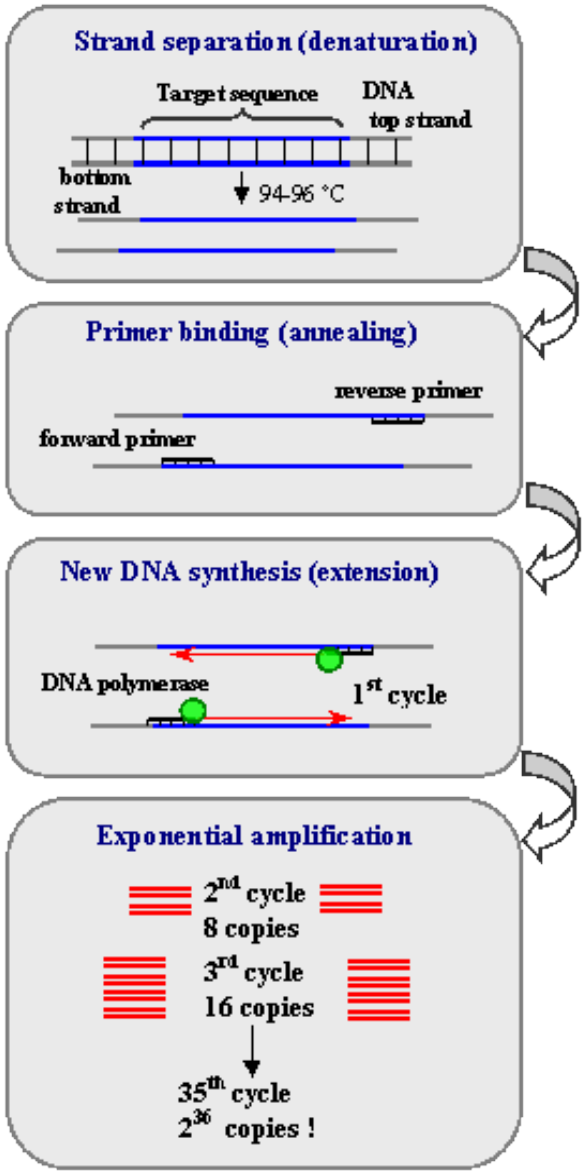

Figure 1.3: The steps involved in PCR (NCBI 2014).

The third step is extension, performed at the temperature for optimum polymerase activity and included to extend the newly synthesized strand past the region of the template that is complementary to the other primer in the reaction. This step is carried out for a time interval that is determined by the particular PCR product length (Fig. 1.3). The denaturation, annealing, and extension steps are then repeated between 20-40 cycles depending on the quantity of original template DNA. PCR concludes with a final extension step in order to ensure that all single stranded DNA strands have been extended. This is carried out at the temperature required for optimal polymerase activity as in the extension step (Mullis et al. 1986).

1.4.1. Inhibitors of PCR

PCR inhibitors are substances that prevent the amplification of DNA in a PCR reaction (Wilson 1997). These substances can inhibit the reaction in a variety of ways, such as by binding to or interacting directly with the DNA template; by affecting the availability of polymerase cofactors; by binding to and inactivating the DNA polymerase; or by interfering with polymerase activity such as during primer extension (Opel et al. 2010, Wilson 1997). In addition, the PCR process is affected by changes in pH, the presence of excessive salts, alcohols, and detergents (Wilson 1997, Weyant et al. 1990). Inhibitors can be removed through additional purification steps or dilutions, but these are not without the risk of additional DNA loss and are avoided in instances where the amount of extracted DNA is expected to be low (King et al. 2009). Certain PCR enhancers, such as bovine serum albumin (BSA) can also reduce the inhibitory effects of substances in the PCR reaction, although the precise mechanism is unknown (Kreader 1996). The DNA template to be amplified may itself be compromised from the interaction with several inhibitors, such as humic acid, which directly interacts with the DNA template (Opel et al. 2010).

The mechanisms of many inhibitors found naturally in bone have been studied. Calcium is present in bone material, and is believed to compete directly with divalent magnesium as a cofactor for DNA polymerase, therefore reducing the overall PCR efficiency. Another likely inhibitor that is found in bone tissue is the protein collagen. Collagen has been shown to decrease the processivity of the DNA polymerase and at high enough concentrations, it has been found to interact with the DNA template, particularly in later cycles of the PCR reaction (Opel et al. 2010). Collagen can be removed with the addition of collagenases rather than general proteinases, such as proteinase K, which is commonly used in DNA extraction methods (Scholz et al. 1998).

In addition, the PCR reaction can be enhanced with additives such as bovine serum albumin (BSA), betaine, dimethyl sulfoxide (DMSO), gelatin, and formamide. These enhance the reaction through different mechanisms. It is believed that BSA and gelatin stabilize the polymerase as well as providing an alternative substrate for many inhibitors (Farell and Alexandre 2012, Kreader 1996). Betaine, DMSO (Frackman et al. 1998 and Henke et al. 1997), and formamide reduce secondary structure formation, which occurs frequently in very GC rich DNA templates (Bessettii 2007).

1.4.2. Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR (qPCR) is widely used to quantify DNA extracted from forensic samples, which is critical for downstream procedures such as amplification and sequencing. This method uses traditional PCR in order to amplify the DNA; however, the DNA is detected in "real time" as it is being amplified, allowing the analyst to determine how much amplifiable DNA is present in a casework sample.

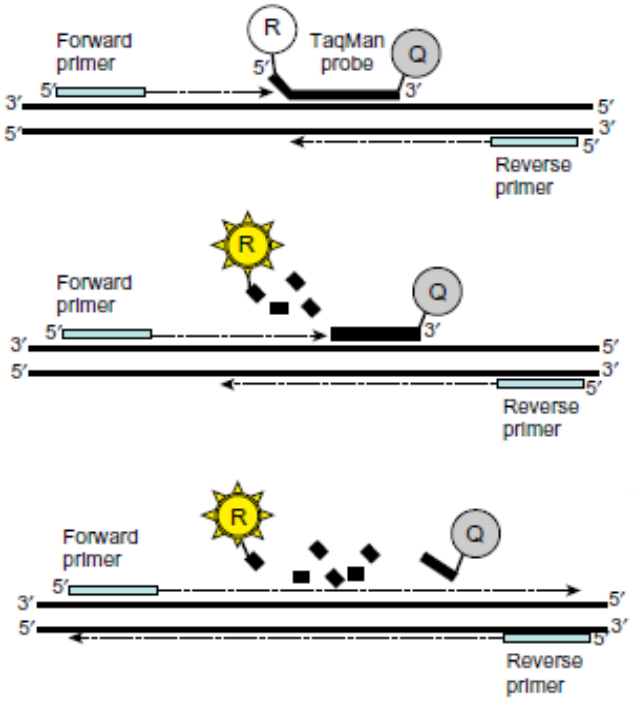

Figure 1.4: The mechanism of the 5' probe in qPCR (Butler 2011).

As mentioned previously, not all DNA in a sample will be amplifiable due to degradation. Knowing the quantity of amplifiable DNA present in a sample allows the analyst to decide whether there is sufficient DNA template to move forward to downstream procedures such as further amplification and sequencing (Heid et al. 1996). Non-specific intercalating fluorescent dyes and sequence-specific probes are used in many qPCR assays. The probes are comprised of single stranded oligonucleotides, which are labeled with a fluorescent reporter molecule on one end and a quencher molecule that absorbs the fluorescence on the opposite end. As DNA polymerase synthesizes the DNA strand, it degrades the probe, separating the reporter and the quencher, which prevents the absorbance of the fluorescence by the quencher (Fig. 1.4).

This allows a CCD camera to detect emitted fluorescence and therefore provides an accurate and effective means of quantifying the number of copies of DNA present in a volume of sample (Kavlick et al. 2011). DNA standards of known concentration are also run in order to quantify the DNA present in the unknown by reference to the knowns.

The cycle threshold (C_T) is the number of PCR cycles required for the detected fluorescence to exceed a pre-established threshold for detection of the signal above noise. The greater the concentration of DNA present, the fewer amplification cycles are required for the fluorescence to exceed the threshold (Fig. 1.5). Therefore, the C_T and the DNA quantity are inversely proportionate. In qPCR, known DNA quantities called standards are analyzed and a standard curve is generated as fluorescence as a function of the C_T values. Knowing the concentrations of each standard allow a curve to be generated and relative quantification of the DNA samples is possible (Heid et al. 1996). In addition, qPCR assays contain an internal positive control (IPC), which is a synthetic DNA template that is amplified with its own primers and probe in the PCR reaction.

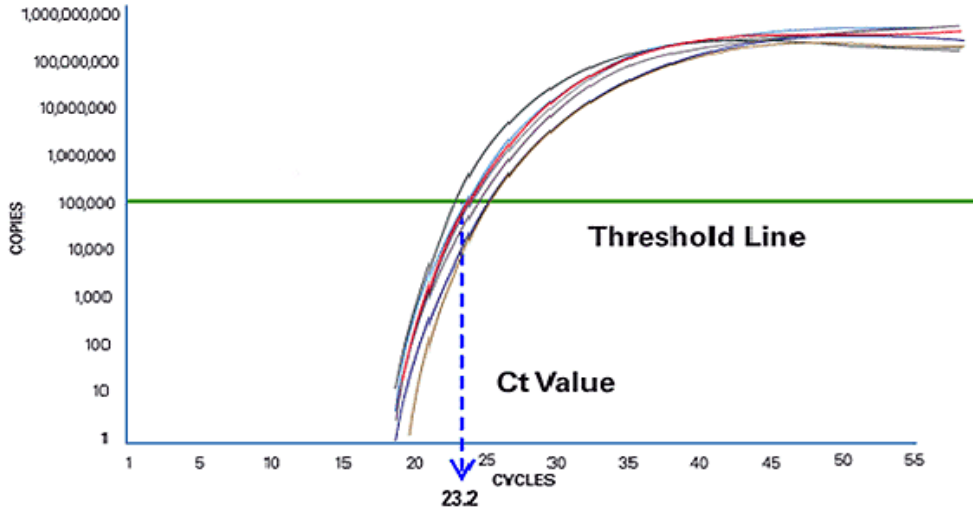

Figure 1.5: qPCR data showing the threshold line to determine the cycle threshold (C_T) value (Life Technologies™ 2015).

The purpose of the IPC is to detect inhibition that may be occurring in the PCR reaction. Amplification failure may occur when there is insufficient DNA template in the PCR reaction, or when there is adequate amount of DNA available but PCR inhibitors are present. In the case of the former, IPC would still amplify and be detected but the target DNA would not be, and in the latter, the IPC would fail to amplify as well as the target DNA. Varying levels of inhibition can be detected by comparing the IPC cycle threshold value (C_T) of the non-template control (NTC) samples to those observed in the samples. The non-template controls contain the PCR reaction but with molecular biology grade water (MBG) or Tris-EDTA (TE) buffer in place of the DNA template. An elevated IPC C_T as compared to this control would indicate that the entire quantity of IPC DNA is not being amplified and therefore inhibition is occurring (King et al. 2009).

In the quantification assay used in this study, samples showing an increase in the IPC C_T value of each sample compared to the IPC C_T of the NTCs and reagent blanks (RB) were

considered inhibited. A slight increase of less than one cycle indicates low levels of inhibition, and greater than two cycles indicates moderate levels of inhibition. If the IPC failed to amplify in the PCR reaction, the IPC C_T was said to be undetermined. When this occurred, very high levels of inhibition are present. In addition, a failure to quantify the DNA target from the samples in conjunction with an undetermined IPC C_T indicates very high levels of inhibitors (Kavlick et al. 2011). In this study, apparent inhibition caused by competition for reagents in the DNA standards containing greater than 10^5 copies/2 μL in the IPC was consistently observed. This was detected as a relative increase in the IPC C_T of greater than 1 cycle. This is not true inhibition of the PCR reaction, but simply a decrease in PCR efficiency from consumption of the reagents. Examining the IPC C_T of bone samples and comparing them to the observed IPC C_T values of the non-template controls and reagent blanks was a technique utilized in this work to assess inhibition.

1.5. Degradation of DNA Associated with Cell Death

DNA obtained from human remains is often highly degraded as it is subjected to not only environmental degradation processes but also the natural enzymatic and chemical processes that occur post mortem. The process of cell death is initiated approximately 4 minutes post mortem as tissues of the body become hypoxic. In these conditions, oxygen-dependent cellular respiration ceases, and in its place anaerobic processes which yield acidic byproducts will begin to synthesize ATP (Vass 2001). The process of DNA degradation begins as pH activated lysosomal proteases begin degrading the histone proteins, which contain the nuclear DNA. Upon being released from the histone proteins, DNA is then degraded by endogenous nucleases into oligonucleotide fragments of random length (Alaeddini et al. 2010). Endonuclease degradation of DNA has distinct characteristics and certain regions are more heavily targeted

than others, such as secondary structures. The degradation of these secondary structures often yields high molecular weight (longer) fragments of DNA. In addition, exogenous nucleases from the environment are also a source of DNA degradation. Other factors can affect the rate and size of fragmentation/degradation, such as environmental factors like pH and temperature, as well as the concentration of ions present, particularly divalent metals (Lindahl 1993).

It is important to understand the mechanisms in which DNA is damaged, as these modifications will affect the integrity of the extracted DNA. The two most frequently encountered types of damage that occur post mortem in forensically relevant samples are double-stranded DNA breaks and oxidative damage. It is impossible to amplify extracted DNA when these modifications are present (Gilbert et al. 2003). Hydrolytic damage to the glycosidic base-sugar bond results in cleavage of the nitrogenous base, which crosslink formation. The formation of crosslinks prevents successful amplification of the DNA in downstream procedures for casework (Alaeddini et al. 2010). Deamination also occurs in the nitrogenous bases of cytosine, adenine, and guanine. This creates a problem in downstream amplification of the DNA template in that mis-incorporation of bases can occur (Pääbo 1989).

In low copy number samples additional PCR cycles or higher concentrations of reagents and polymerase may be added to the reaction. This will not be effective in cases where the DNA is present but highly degraded. In cases where DNA is highly degraded, MiniSTRs may be attempted, which consist of the set of forensic STRs that are present as smaller PCR amplicons (Butler 2003). This has been found to be effective as larger target regions of DNA will probabilistically contain more damage sites and smaller target regions are likely to contain less (Takahashi et al. 1997). In cases where the DNA is present in low copy number and is highly degraded, mitochondrial DNA analysis is the optimum choice. Because DNA obtained from

human remains is degraded from both environmental and internal processes, it is crucial to obtain adequate concentrations of DNA for downstream applications in a gentle method so as to not further degrade the extracted DNA.

1.6. Composition and Structure of Bone and Associated Challenges with DNA Extraction

Bone tissue is a type of connective tissue consisting of cellular and non-cellular components, the latter composed of metals and a collagen matrix (Clarke 2008). This structure renders the bone considerably more resistant to degradation than other bodily tissues and for this reason, bone may often be the only enduring physical evidence available for forensic analysis; however, the structure of bone presents unique challenges to the typing of DNA for identification purposes (Loreille et al. 2007). Bone tissue consists of two primary structures: cortical bone, a solid tissue which lines marrow cavities, and cancellous or trabecular bone, composed of trabeculae, which traverse the bone marrow cavities which contain vascular and neural tissue. In cortical bone, the Haversian system or osteon contains the vascular supply as well as neural tissue of the bone (Fig. 1.6). It is comprised of vertical, concentric rings known as lamellae. Osteocytes are located in lacunae, which are pockets located within the lamellae. These osteocytes are connected by small channels called canaliculi, which are further connected to the central canal, located in the middle of each osteon (Clarke 2008).

Bone types are characterized based upon their shapes: short, long, flat, irregular, and sesamoid. Each bone type contains different organization and content of cortical and cancellous bone, even within the individual bone.

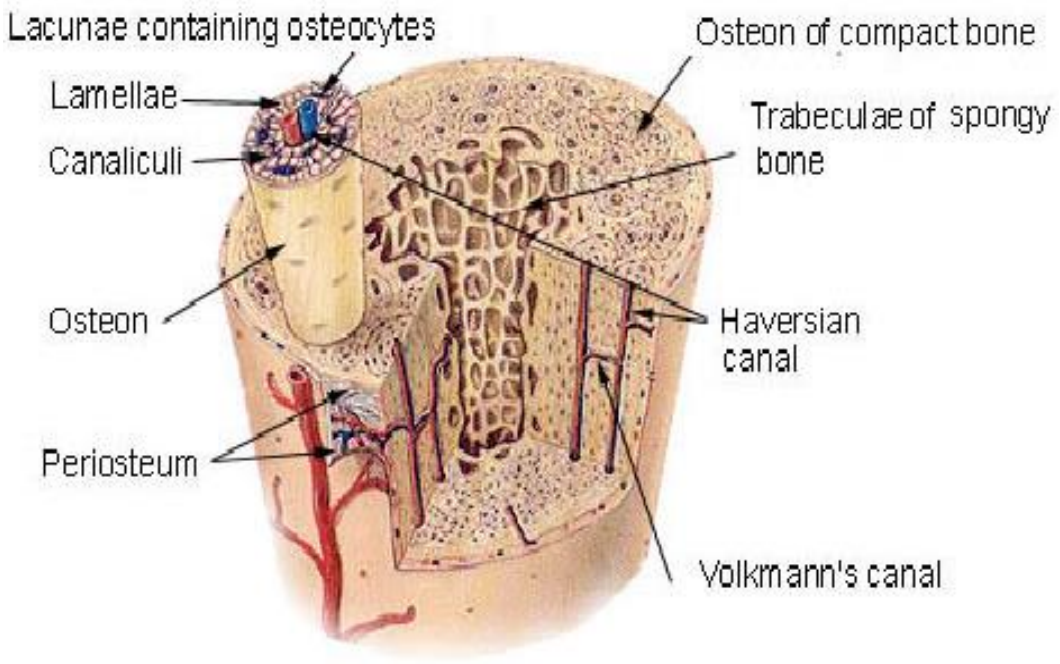

Figure 1.6: Structure of cortical bone showing cell types, location, and tissue organization (NCI [date unknown]).

Hydroxyapatite, $Ca_{10}(PO_4)_6OH_2$, comprises nearly 70% of the mineral content found in bone, with small amounts of carbonate, acid phosphate, and magnesium being present as well (Clarke 2008).

Type 1 collagen, a structural protein found in connective tissues rich in glycine and hydroxyproline, gives bone elasticity and makes up 90% of the organic content in bone (Filippini 2010). Collagen is another known inhibitor of PCR, which further illustrates the need of a highly efficient purification method in the extraction process (Opel et al. 2010).

The mineral content of bone along with its solid structure presents a physical barrier to efficient DNA extraction, primarily by blocking reagents from accessing the cellular material,

which in turn prevents liberation of the DNA. In addition, the divalent metal cations found in bone are inhibitors of PCR; therefore, their removal is critical for downstream forensic analysis (Loreille et al. 2007, Kalmár et al. 2000). The mineral to organic ratio in cortical or cancellous bone is consistent across species; however, cortical bone density and ash (hydroxyapatite) consistently exceeds that observed in cancellous bone, although the water content and ash: organic ratio is higher in cancellous bone (Gong et al. 1964). This may have implications in the amount and type of inhibitors present based on the type of bone analyzed.

1.7. Inductively Coupled Plasma Optical Emission Spectrometry

In addition to the metal inhibitors naturally occurring in bone tissue such as iron and calcium, it is critical to evaluate any potential introduction of inhibitors from the extraction method itself, particularly during the pulverization process. To further understand the PCR inhibition detected in bone samples, ICP-OES was used as a tool to detect and quantify calcium and other metals present in the DNA extract from bone.

ICP-OES is commonly used to detect and quantify trace metals. The instrument contains a torch, comprised of three concentric quartz or silica tubes contained in a copper radio frequency (RF) coil (Boss and Fredeen 2004). RF power is applied to the coil creating an oscillating current, resulting in an induced electromagnetic field. Argon gas flows through the torch and is sparked by a tesla coil, ionizing some of the argon gas molecules. The magnetic field accelerates the ionized argon molecules in a process called inductive coupling, resulting in collision with other molecules further ionizing the argon into plasma. The liquid sample is then delivered via a peristaltic pump into a nebulizer where it is converted into an aerosol and introduced into the plasma in argon gas (Fernández-Martínez et al. 2005, Boss and Fredeen 2004).

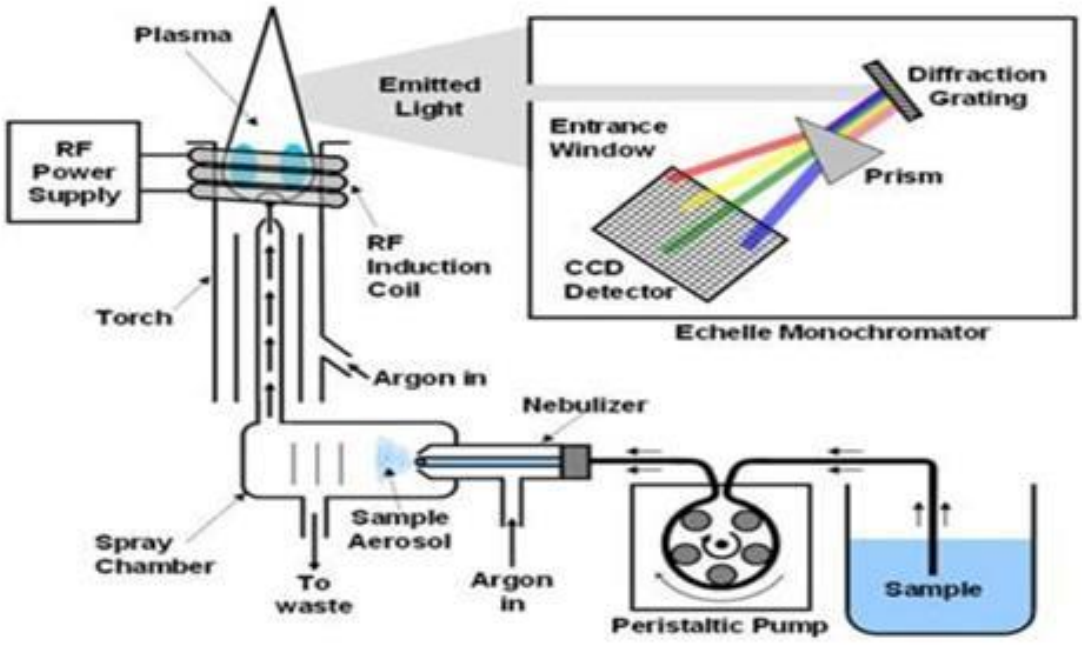

Figure 1.7: Schematic design of an ICP-OES instrument (Ramanaji 2014).

In the plasma, the sample solvent is removed from the droplets leaving a dry aerosol behind, and then the dry aerosol is broken down into gaseous molecules in a process called vaporization (Fernández-Martínez et al. 2005, Boss and Fredeen 2004).

The molecules are broken down further to their component atoms, which are then excited and emit radiation. This radiation is separated into its component wavelengths by a monochromator, which measures one wavelength at a time, or with a polychromator, which measures multiple wavelengths simultaneously. The separated wavelengths are detected using photosensitive detectors such as a charge coupled device (CCD) or photomultiplier tube (PMT). ICP-OES can be utilized in not only the detection of metals at low concentrations but also in

quantification. In order to do this, a calibration curve is created with known concentrations of analyte and the intensity measured at the appropriate wavelength (Fig. 1.7) (Boss and Fredeen 2004).

The ICP-OES is a sensitive instrument that can be utilized to characterize metals found in the purified DNA extracts, including calcium, which is a known inhibitor of PCR and present in bone tissue in high quantity. Elucidating which inhibitors are still present in bone extract following extraction is a critical step in optimizing extraction methods that are designed to remove them. In this study, DNA extracts were analyzed for the metals chromium, iron, and calcium. Because calcium is a known inhibitor of PCR and so prevalent in osseous tissues, it was quantified and compared to inhibition detected via the qPCR results.

1.8. Current Methodology in DNA Extraction from Osseous Tissues

In the extraction of DNA from bone, the surface of bone samples is typically first sanded with a Dremel rotary tool (Robert Bosch Tool Corporation, Mt. Prospect, IL) to remove dirt and exogenous DNA contamination and then wiped clean with ethanol. Fragments are cut into small sections in order to pulverize the bone fragments more completely. Prior to pulverization, the bone sections are cleaned with bleach (Lorielle et al. 2007) or Terg-A-Zyme® (FBI 2010) (Alconox Inc., White Plains, NY), an enzymatic detergent that removes proteinaceous soils, body fluids, etc., and finally they undergo a rinse with UV-irradiated molecular biology grade water. The bone material is then allowed to dry prior to pulverization.

In order to provide the maximum interaction between the demineralization and lysis agents and the bone material, the sample is then pulverized to a fine powder. This is frequently accomplished using either a cryogenic impact grinder such as the 6770 SPEX freezer mill (FBI 2010) (SPEX® SamplePrep Metuchen, NJ) or a Waring blender (Warring Torrington, CT). No

significant difference in DNA recovery has been observed when these two methods are compared (Lorielle et al 2012). For this study, the 6770 SPEX freezer mill was used set with the following parameters: 0 minutes of precool time, 5 minute run time, 2 minute cool time, and a rate of 15 cycles per second. The bone material was placed in a polycarbonate cylinder sample vial with a stainless steel impactor bar enclosed by two stainless steel end caps. The vial was then inserted into the freezer mill, which was filled to the appropriate level with approximately 5 L of liquid nitrogen (SPEX® SamplePrep 2008).

Following pulverization, the bone material undergoes a process known as demineralization in which the bone powder is incubated with a chelating agent such as Ethylenediaminetetraacetic acid (EDTA) for different lengths of time ranging from 24 hours (FBI 2010) up to three days, sometimes in conjunction with a detergent and other times as a separate incubation step at room temperature. This step is used to sequester divalent metal cations present in the mineral matrix of bone tissue (Lorielle et al. 2007).

Post demineralization, the EDTA can be removed by centrifugation and removal of the supernatant, or by concentrating the DNA with a commercially available centrifugal filter unit (Lorielle et al. 2007). In this study, the demineralization and lysis incubation steps were done separately and centrifugation was followed by subsequent wash steps to remove the chelated metal cations (FBI 2010).

In preliminary studies, a discoloration was sometimes observed when EDTA was added to bone powder, going from a white color to a gray color. In addition, some gray residue was present in the spin column after purification but was not observed in reagent blanks. Previous work that had been done suggested a relationship between discolored samples and higher levels

of inhibition. It is possible that the gray discoloration and debris observed are the result of stainless steel contamination as carryover from the pulverization process.

The stainless steel components used in the SPEX 6770 Freezer Mill are made from magnetic 440C steel, which is comprised of iron as well as 0.95-1.2% carbon, 1% manganese, 1% silicon, 16-18% chromium, 0.75% nickel, 0.5% copper, 0.65% molybdenum, 0.04% phosphorus, and 0.3% sulfur. Iron containing compounds such as heme and chromium are known to be inhibitors of PCR (Opel et al. 2010, Thompson et al. 2000). The SPEX company also offers polycarbonate end caps and a polycarbonate-coated impactor bar for use in samples where metal contamination needs to be reduced. The use of polycarbonate end caps and impactor bar has not been currently studied for the pulverization of bone material in forensic casework. In this study, the performance of the SPEX polycarbonate end caps and polycarbonate coated impactor bar was evaluated.

1.8.1. Cell Lysis

An effective lysis buffer maintains pH, provides proper ionic strength, contains a detergent to disrupt the phospholipid bilayer of the cellular membrane, and a reducing agent to disrupt the disulfide bonds of cysteine amino acid residues found in proteins (Butler 2011). To prevent the degradation of the freed DNA by endonucleases, the broad-spectrum serine protease, proteinase K, is added to degrade nucleases. EDTA, a chelating agent, is also present to sequester divalent magnesium cations, a cofactor for nucleases, thus inhibiting their activity. Ethylene glycol tetraacetic acid (EGTA) is another chelating agent that is commonly used as it has a higher affinity for divalent calcium ions.

Previously in our lab, a commercially available buffer produced by the Qiagen company, lysis buffer ATL, was found to be more effective than SEB (10 mM TRIS, 100 mM NaCl, 39

mM dithiolthreitol, 10 mM EDTA, and 2% sodium dodecyl sulfate (SDS) in the digestion of hair shafts. The complete composition of ATL is proprietary; however, based on information provided in the MSDS, it can be inferred that buffer ATL does not contain EDTA or EGTA as a chelating agent, but does contain SDS at 2.5-10% as a detergent (Qiagen 2013). The lysis buffer BTA produced commercially by Life Technologies™ is specifically formulated for lysis of bone material; therefore, it may be effective as a lysis buffer in the development of this method. The chemical composition of BTA is also proprietary, but based on the information provided in the MSDS it has been determined that it contains EGTA at 10-30% (w/v) as a chelating agent at an unknown concentration and a detergent other than SDS (Life Technologies™ 2011). To maintain consistency between the lysis buffers, dithiothreitol (DTT) was added to each lysis buffer for a final concentration of 39 mM, and Proteinase K (Pro-K) was added for a final concentration of 1.2 U per lysis reaction, or the amount of enzyme required to turnover one micromole of substrate per minute.

1.8.2. DNA Extraction Methods

An efficient DNA extraction method is critical to isolate DNA and remove cellular debris and excess reagents that may interfere with downstream processes such as amplification. The steps of DNA extraction involve disrupting the cellular membrane to release the contents of the cell, separating the nucleic acids from the rest of the cell lysate, and then eluting the DNA into a stable solution to prevent degradation.

Past methods for extracting nucleic acids from tissues and biological samples typically involved lysing the cells and then precipitating out the nucleic acids in an alcohol, such as ethanol, under high salt conditions. In a commonly utilized organic extraction method, the solvents phenol, isoamyl alcohol, and chloroform are used to extract nucleic acids. As seen

with most methods, cells are lysed with a detergent, and then the lysate is introduced into the organic solvents at a certain pH and ionic strength. These conditions are critical to ensure that cellular debris remains in the organic phase and nucleic acids are solubilized into the aqueous phase. Alcohol precipitation is then used to extract the nucleic acids from the aqueous fraction. Possible limitations of this method include the potential for organic solvent and salt carry-over, as well as being labor intensive and time consuming (Hucklenbroich and Scherer 2013).

Solid phase methods for purification of extracted nucleic acids that use spin columns are based on ion exchange and hydrophobic interactions. Cells are lysed and buffers are then added to the lysate which contain a high concentration of chaotropic salts such as guanidinium thiocyanate or guanidinium chloride. Chaotropic agents act by disrupting hydrogen bonding in aqueous solution, which enables the negatively charged DNA to bind strongly to the positively charged silica substrate via the formation of a salt bridge at a pH of 7 or lower (Hucklenbroich and Scherer 2013, Esser et al. 2006). Cellular debris and inhibitors of PCR are removed by several wash and centrifugation steps, and then the DNA is eluted from the substrate with a change in the pH and ionic strength (Fig. 1.8) (QIAamp® DNA Mini Kit and QIAamp® DNA Blood Mini Kit Handbook 2003).

In magnetic bead-based purification systems such as Life Technologies™ PrepFiler®, DNA can be precipitated out of solution by non-specifically associating with silica-coated magnetic beads (Reeve 1997). In high salt conditions, alcohol can be added to render the DNA insoluble, although it does not fully precipitate from solution. Beads can be added to the cell lysate prior to or at the same time as a precipitating reagent such as alcohol.

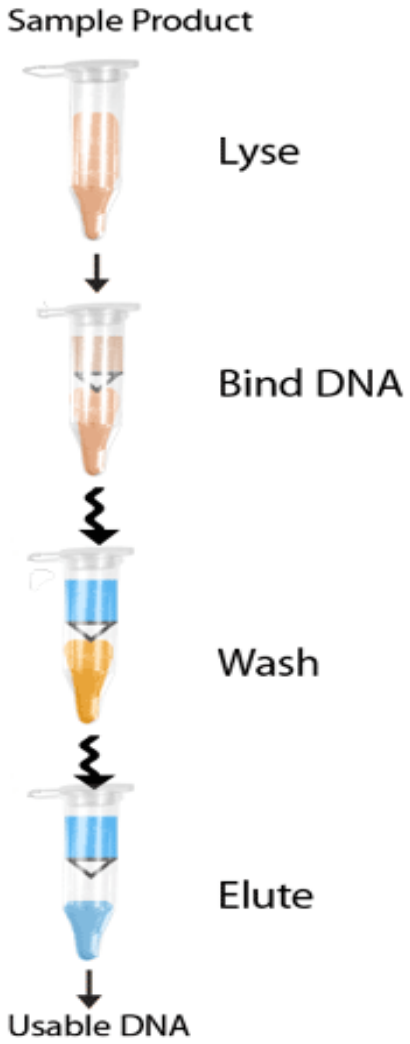

Figure 1.8: A schematic workflow for solid phase DNA extraction methods. (Tilstone 2007).

As the DNA becomes insoluble, it increases its association with the silica coated magnetic beads. When the sample is placed on a magnetic stand, the silica coated beads and associated DNA are drawn to the wall of the reaction tube nearest to the magnet. Ethanol or isopropanol is then used to wash the sample to remove cellular debris, inhibitors, and salts. This particular method is very rapid and does not rely upon centrifugation steps like other methods,

which utilize spin columns. This method also removes the need for multiple tube transfers, which are a potential source of DNA loss and contamination (Reeve 1997).

The use of high concentrations of EDTA in DNA extraction from bone, as well as the larger lysate volume, may interfere with optimum binding of DNA to the silica substrates via changes in pH. EDTA and DTT are added to bone material in the demineralization and lysis incubations, respectively, and will raise the pH of the cell lysate. This increase in pH can be corrected with the addition of sodium acetate after the lysis incubation, which has been shown to enhance DNA recovery (Dukes et al. 2012).

1.8.3. Quantification of DNA Using qPCR

A custom assay developed for human mtDNA was used for quantification of extracted DNA using an Applied Biosystems 7500 Real-Time PCR System with the 7500 Software V2.0.1. In this assay, a 105 bp region of the mitochondrial genome comprising part of the NADH dehydrogenase subunit 5 gene is targeted for amplification, corresponding to base positions 13,288 to 13,392 in the rCRS. This region does not contain many non-human homologues, making it ideal for avoiding the amplification of non-human DNA.

CHAPTER 2: METHODS AND RESULTS

2.1. Contamination Control for Processing Bone Samples

All sample processing was performed in laminar flow PCR hoods (AirClean® Systems, Creedmoor, NC) that were cleaned with 10% (v/v) sodium hypochlorite, 70% ethanol and UV-irradiated for 15 minutes. All microcentrifuge tube racks, microcentrifuge tubes, PCR plates, and materials for cutting and sanding bone were UV-irradiated for 15 minutes in a Spectrolinker® XL-1000 UV Crosslinker (Spectronics Corporation Westbury, NY) as were all reagents that did not contain nucleic acids or enzymes. The freezer mill pulverization vial and associated components (impactor bar and end caps) were cleaned with 10% bleach, 70% ethanol, and then UV-irradiated for 15 minutes (FBI 2010). To prevent contamination, all extractions were performed in a separate extraction laboratory and downstream procedures such quantitation of amplified DNA were performed in a designated post-amplification laboratory. Due to the inherent sensitivity of mtDNA to contamination, personal protective equipment including a lab coat, disposable sleeves, facial mask, and two pairs of nitrile gloves were used and changed frequently.

2.2. Overview of the Extraction Process

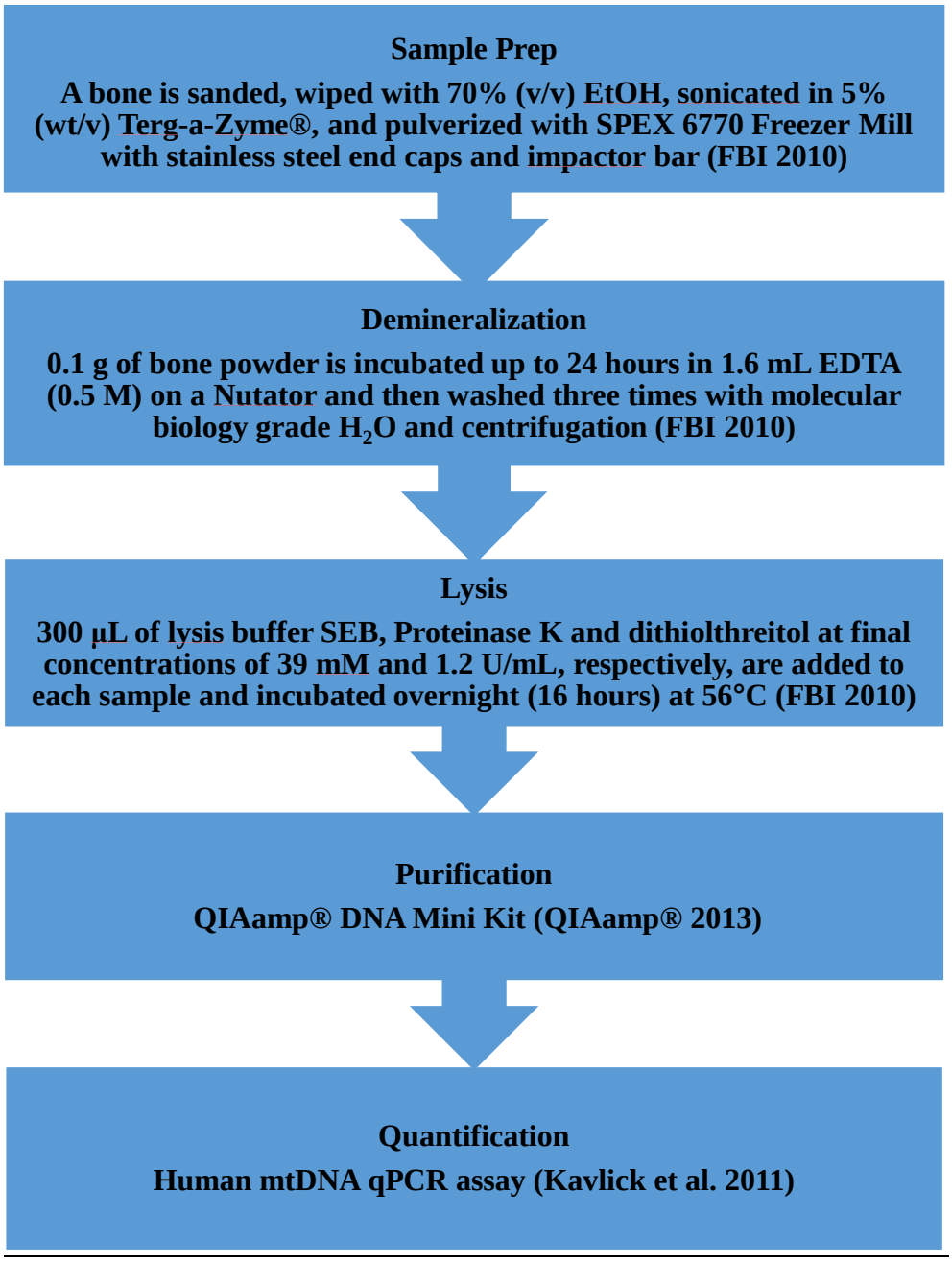

Figure 2.1: Extraction method overview for the recovery of mtDNA from human bone.

Commercially obtained human bones (Skulls Unlimited International, Inc. Oklahoma City, OK) were sanded thoroughly with a Dremel rotary tool (Robert Bosch Tool Corporation,

Mt. Prospect, IL) and then wiped down with 70% (v/v) ethanol to remove surface contaminants and exogenous DNA. The sanded bone was then cut into small fragments. Bone sanding and cutting was done in a ductless chemical fume hood (AirClean® Systems, Creedmoor, NC) that had been cleaned with 10% (v/v) sodium hypochlorite, 70% (v/v) ethanol, and UV-irradiated for 15 minutes. Bone fragments were sonicated for 15 minutes in UV-irradiated 5% (wt/v) Terg-a-Zyme®, an enzymatic detergent used to remove proteinaceous soils and contaminants, and then allowed to air dry (FBI 2010).

2.2.1. Pulverization of Bone Material

Pulverization of samples was performed using stainless steel end caps and impactor bar in a SPEX 6770 Freezer Mill. The freezer mill was set with the following parameters: 0 min of precool time, 5 min run time, 2 min cool time, and a rate of 15 cycles per second, or back and forth cycles of the impactor bar. A reagent blank was prepared by swabbing the empty polycarbonate cylinder, impactor bar and end caps, and then processed in the same manner as the bone samples (FBI 2010).

2.2.2. Demineralization and Lysis

Each bone sample consisted of approximately 0.1 g of bone powder, which was weighed out on an analytical balance (Mettler-Toledo, LLC Columbus, OH) and placed in UV-irradiated 2 mL microcentrifuge compatible Nalgene tubes. A volume of 1.6 mL of UV-irradiated 0.5 M EDTA was added to each sample, and the samples were incubated for 20-24 hours at room temperature on a Nutator. The reagent blank was also incubated in 1.6 mL EDTA and processed alongside all samples from the same extraction experiment. After the demineralization step, the microcentrifuge tubes were centrifuged at 10,900 rpm for 1 minute and the supernatant containing EDTA and chelated minerals were removed by micropipette. The samples were then

subjected to three wash steps with UV-irradiated molecular biology grade water (MBG) in an attempt to remove sequestered divalent metal cations. In these washes, 1 mL of UV-irradiated MBG H_2O was added to each sample, the samples were vortexed to mix and centrifuged for another minute, and the supernatant removed by micropipette (FBI 2010).

For cell lysis, 300 µL of the lysis buffer SEB was then added to each sample and reagent blank in conjunction with 1 M dithiothreitol (39 mM final concentration) and 2 µL of 600 U/mL Pro-K. All samples and reagent blanks were then incubated at 56°C on a Nutator overnight (16 hours) (FBI 2010).

2.2.3. QIAamp® Spin Column Extraction

Samples and reagent blanks were purified using the Qiagen QIAamp® DNA Mini kit, following the manufacturer's protocol for DNA purification from blood or body fluids with the following modifications: 300 µL of buffer AL, a buffer which provides proper pH and chaotropic salts for maximum binding of the DNA to the spin column, was added to each sample and the first ten minute incubation was performed at 70°C. The volume of absolute ethanol used post incubation with buffer AL was increased to 400 µL to accommodate the larger sample volume. Samples were centrifuged at 10,900 rpm rather than 8,000 rpm. DNA was eluted from the column using a volume of 60 µL of MBG H_2O (FBI 2010).

2.2.4. Quantification of Extracted mtDNA

The concentration of mtDNA present in copies per 2 µL of sample was assessed using a human mtDNA qPCR assay. Samples were often normalized in studies where samples were not diluted to a value of copies of mtDNA per gram of bone material. Inhibition in samples was assessed by a relative comparison of the IPC C_T values of the reagent blank (RB) IPC C_T and the sample IPC C_T values. In addition, reagent blank (RB) IPC C_T values were compared to the IPC

C_T values of the NTCs, which allowed assessment of inhibition that may be from reagent carryover.

Controls for this assay included three non-template controls containing MBG H_2O, Tris-EDTA buffer, and an Exogenous IPC Blocking Reagent, which blocks the amplification of the internal positive control (Applied Biosystems). The quantification reactions were set up in a MicroAmp® 96 well optical plate (Applied Biosystems) with master mix added to each well prior to the addition of non-template controls, samples, or standards. Non-template controls were run in duplicate to calculate the average quantity of mtDNA and IPC C_T values. Likewise, samples and reagent blanks collected from the extraction phase were run in triplicate in order to calculate the average quantity of mtDNA and IPC C_T values. These were capped and the standards were added on the unused outermost wells (Kavlick et al. 2011).

2.3. Examining the Effectiveness of Three Different Lysis Buffers and Interactions with Different Bone Types

To observe the effects of using different lysis buffers in the DNA extraction process, samples taken from a human femur and two human ribs were processed following the method outlined in section 2.2. Each sample was treated with 300 µL of one of three lysis buffers, buffer ATL, BTA, or SEB, for the overnight lysis incubation.

2.3.1. Lysis Buffer Performance with Different Bone Types

Regardless of the lysis buffer utilized, bone samples taken from the femur used in this study routinely yielded greater quantities of mtDNA per gram of bone powder than samples from human ribs, $t(50) = -4.40$, $p < 001$ (Fig. 2.2). The overall effectiveness of the lysis buffers ATL, BTA, and SEB as determined by apparent mtDNA recovery varied with the type of bone sampled (Fig. 2.2).

Treatment with lysis buffer ATL yielded the highest mean quantity of recovered mtDNA in samples taken from femur cross sections, although this was not found to be significantly higher than SEB or BTA at the 95% confidence level, F(2,25) = 3.39 p ,= 0.24 (Fig. 2.2).

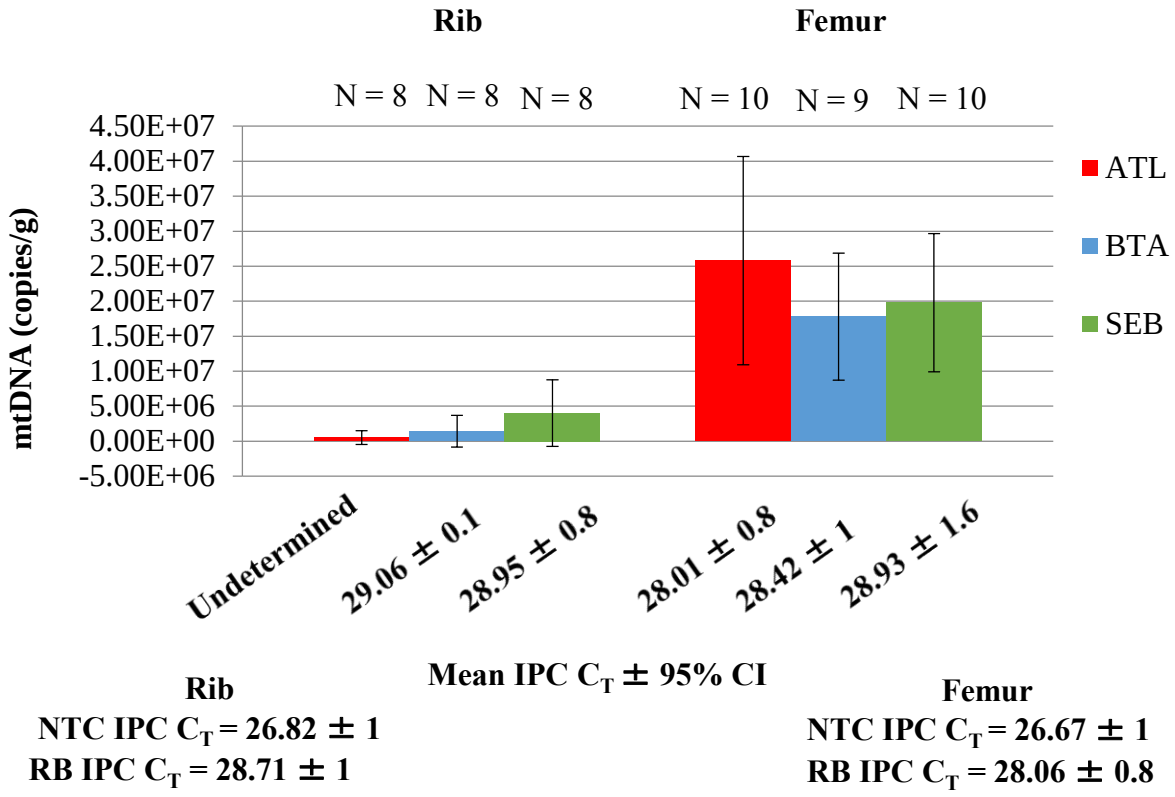

Figure 2.2: The effects of different lysis buffers on mtDNA recovery from two human ribs, F(2,22) = 3.47, p = 0.41) and human femur, F(2.25) = 3.39, p = 0.24. When all variables are combined, the femur used in this study routinely yielded greater quantities of mtDNA than the ribs, t(50) = -4.40, p < 001. Error bars represent 95% CI.

The IPC C_T values of the samples processed from human femur cross sections were elevated by 1 cycle number relative to the IPC C_T of the NTCs; however, they were not elevated relative to the RB IPC C_T. This indicates that low levels of inhibition are occurring in both the samples and reagent blanks (Kavlick et al. 2011) (Fig. 2.2). The IPC C_T of the reagent blanks were elevated relative to those of the NTCs by an average of 0.93 cycles; this was observed in all qPCR assays regardless of which lysis buffer was used, $F(2,6) = 1.40$, $p = 0.32$ (Fig. 2.3).

For human ribs, the difference in the performance of the three lysis buffers was also found to be insignificant at the 95% confidence level, $F(2,22) = 3.47$, $p = 0.41$ (Fig. 2.2). Samples treated with lysis buffer ATL consistently yielded the lowest quantities of mtDNA per gram of bone powder obtained from rib material. The IPC C_T was undetermined in 50% of samples treated with lysis buffer BTA or SEB, and 100% of samples treated with ATL indicating very high levels of inhibition. Overall, undetermined IPC C_T values were observed in 67% of rib samples compared to 46% of femur samples. As seen in femur samples, the reagent blank IPC C_T values in the rib samples were elevated an average of 1.89 cycles higher than those observed in the NTC IPC C_T (Fig. 2.2).

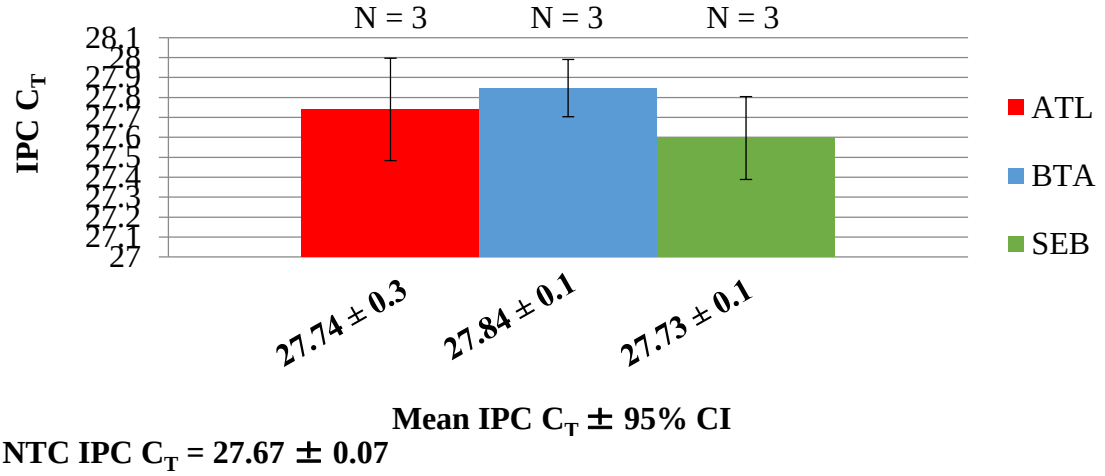

Figure 2.3: A comparison of the reagent blank IPC CT values from human femur samples that were treated with either lysis buffer SEB, BTA, or ATL. No difference in cycle threshold values was observed at the 95% confidence level, $F(2,6) = 1.40$, $p = 0.32$. The IPC C_T values for the reagent blanks showed an increase of approximately 1 cycle relative to the IPC C_T of the NTC. Error bars represent 95% CI.

2.4. Comparison of Stainless Steel and Polycarbonate Pulverization Components

It was difficult to determine the effectiveness of each lysis buffer as tremendous variation in performance was observed within samples processed from a femur and those processed from ribs. Further variation was observed between identical samples processed side by side in samples exhibiting high levels of inhibition. In addition, in some instances a color change was observed when EDTA was added to powdered bone samples in the demineralization step. Upon quantification, these samples showed very high levels of inhibition of both the internal positive control and the target region of mtDNA. Dark gray debris was also observed in the spin column following purification in these highly inhibited samples (Fig. 2.4).

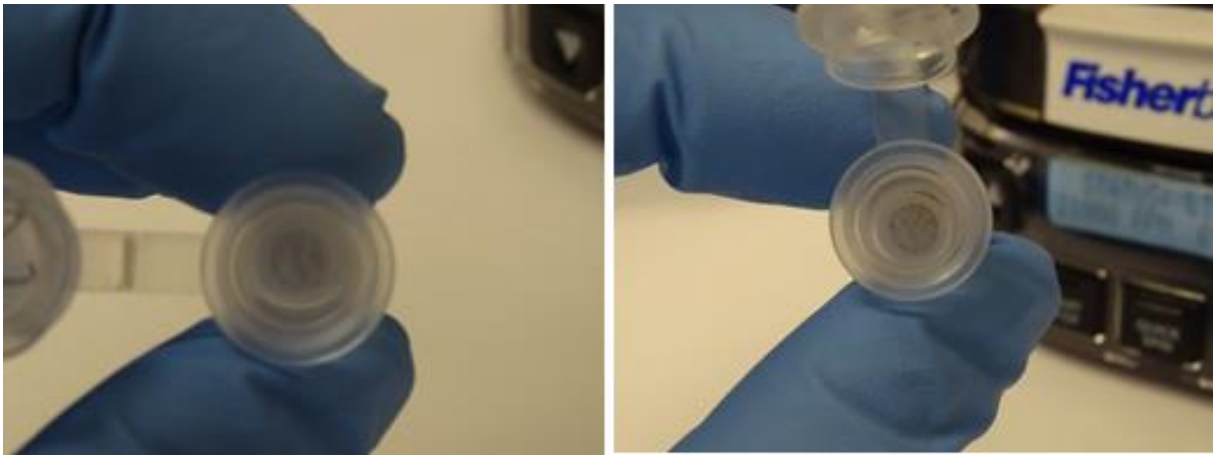

Figure 2.4: QIAamp® spin columns after extraction. The sample on the left was pulverized with polycarbonate, while the sample on the right used stainless steel. Debris was present in the sample processed with stainless steel.

Upon examination of the SPEX pulverization end caps and impactor bar, it was observed that even after one use, the metal end caps exhibited chipping. For the data presented in this study, only new stainless steel pulverization components were utilized and discarded after a single use.

Because the composition and structure of bone varies with different types of bone, the performance of each lysis buffer was analyzed with respect to these different bone types, in this case, a femur and rib. In addition, the combined effects of bone type, pulverization component, and lysis buffer were examined. Two adjacent cross sections were cut from a human femur. These cross sections were again cut in half, and then divided equally to ensure homogeneity of sampling, and processed with either polycarbonate or stainless steel end caps and impactor bar.

The samples were processed following the method outlined in section 2.2, with one third of the samples processed with stainless steel treated with lysis buffer ATL, one third treated with

BTA, and one third treated with SEB. The same treatment was performed on samples pulverized with polycarbonate pulverization components.

2.4.1. Optimizing the Bone Pulverization Step

The apparent mtDNA recovery with polycarbonate end caps and impactor bar was significantly greater at the 95% confidence level than that observed when stainless steel end caps and impactor bar were used, $t(45) = 2.68$, $p < 001$, regardless of the lysis buffer used or bone type analyzed (Fig. 2.5). Moderate levels of inhibition were seen to be occurring in samples processed with stainless steel as well as with polycarbonate (Fig. 2.5). The lysis buffer used did not make a difference in the reagent blank IPC C_T for samples processed with polycarbonate $F(2,3) = 2.14$, $p = 0.27$ (Fig. 2.5).

For samples processed from a human femur, samples pulverized with polycarbonate end caps and impactor bar yielded significantly high recovery of mtDNA, $t(26) = 6.48$, $p < 0.001$. The performance of the lysis buffers was not affected by the use of stainless steel end caps and impactor bar, $F(2,14) = 0.20$ $p = 0.82$, or polycarbonate end caps and impactor bar, $F(2,9) = 0.15$, $p = 0.86$, at the 95% confidence level.

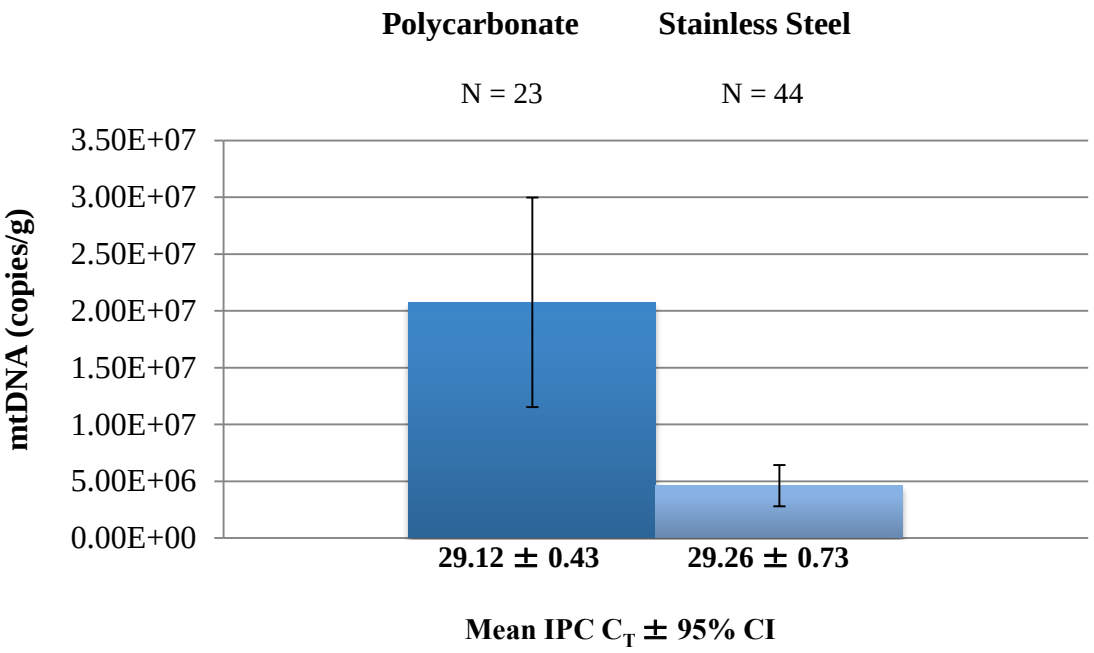

Figure 2.5: A compilation of data from different bone types and lysis buffers from samples processed with polycarbonate or stainless steel pulverization components. Regardless of the lysis buffer used or bone type, samples pulverized with polycarbonate end caps and impactor bar yielded significantly greater mtDNA recovery at the 95% confidence level, $t(45) = 2.68$, $p < 001$. Undetermined IPC C_T values were observed in 62% of samples processed with polycarbonate and 60% of samples processed with stainless steel, which is indicative of very high levels of inhibition. Error bars represent 95% CI.

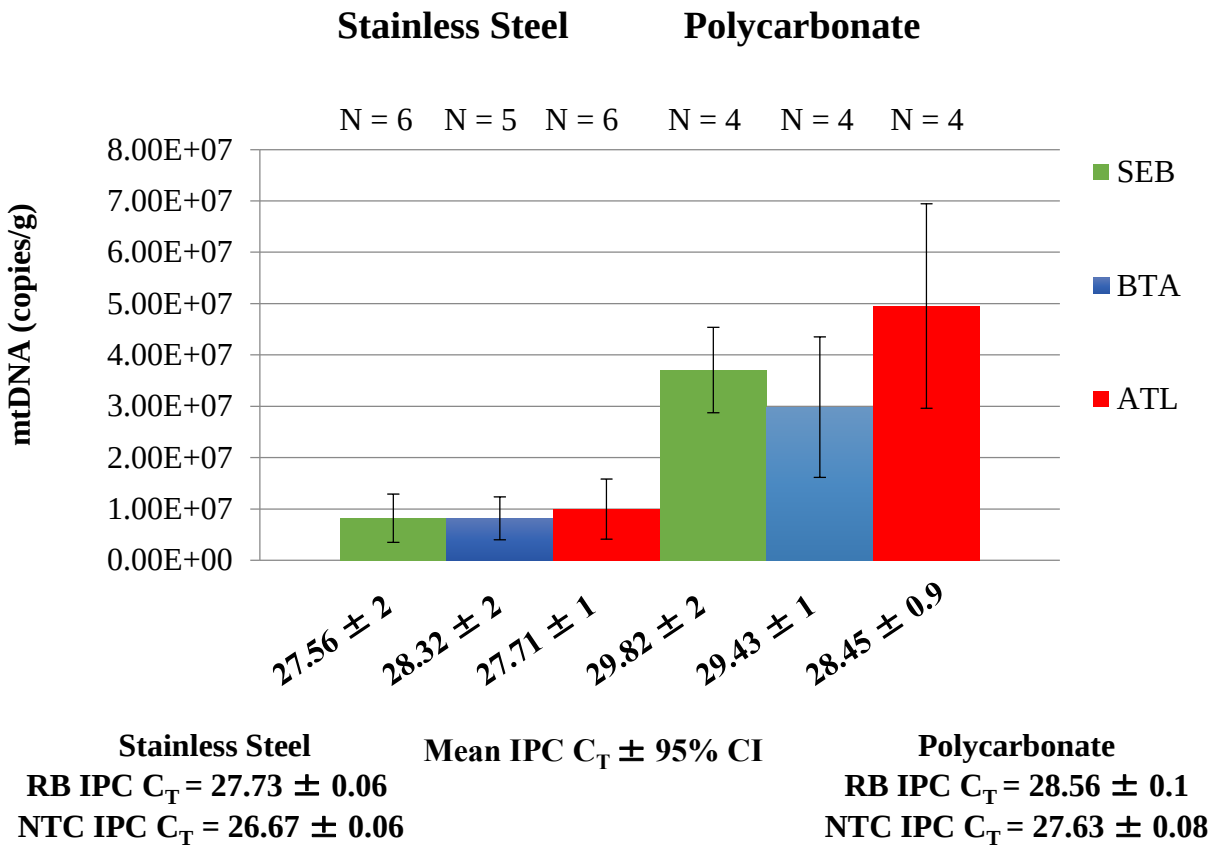

Figure 2.6: A comparison of quantities of mtDNA recovered from human femur using three different lysis buffers and either stainless steel or polycarbonate freezer mill end caps and impactor bar. The samples pulverized with polycarbonate end caps and impactor bar yielded significantly high recovery of mtDNA at the 95% confidence level, $t(26) = 6.48$, $p < 0.001$. The performance of the lysis buffers was not affected by the use of stainless steel end caps and impactor bar, $F(2,14) = 0.20$ $p = 0.82$, or polycarbonate end caps and impactor bar, $F(2,9)$ $f = 0.15$, $p = 0.86$, at the 95% confidence level. Error bars represent 95% CI.

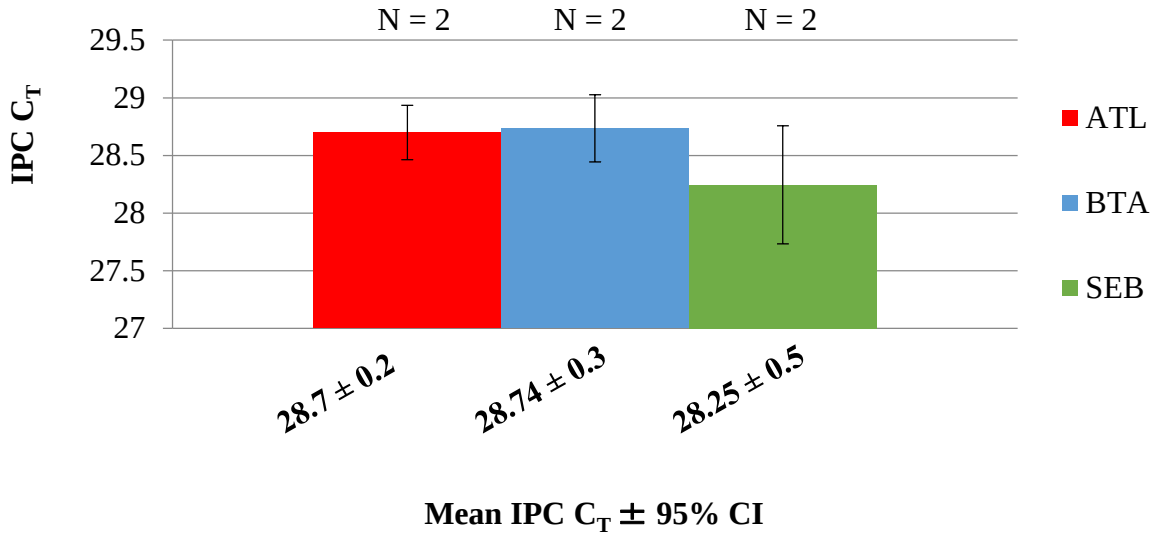

Figure 2.7: A comparison of the reagent blank IPC C_T values from human femur samples processed with either lysis buffer SEB, BTA, or ATL and polycarbonate pulverization components. No difference in cycle threshold values was observed at the 95% confidence level, $F(2,3) = 2.136$, $p = 0.265$. The IPC C_T values for the reagent blanks are increased by approximately 1 cycle relative to the IPC C_T of the NTC. Error bars represent 95% CI.

Samples processed with polycarbonate, regardless of the lysis buffer used, had reagent blank IPC C_T values an average of 1.06 cycles higher than those observed with the NTC IPC C_T (Fig. 2.7). Likewise, samples processed with stainless steel regardless of lysis buffer used had reagent blank IPC C_T an average of 0.93 cycles higher than the NTC IPC C_T (Fig. 2.3). This difference of approximately one cycle is equivalent to an order of magnitude and represents low levels of inhibition.

2.4.2. Direct Comparison of Pulverization Components with a Human Rib.

To confirm that samples pulverized with stainless steel did exhibit higher levels of inhibition and a lower apparent mtDNA yield, an experiment was designed to do a direct comparison of rib samples processed with either polycarbonate or stainless steel. A human rib was cut, cleaned, and the fragments equally divided prior to pulverization with either stainless steel or polycarbonate end caps and impactor bar. The samples were lysed with buffer SEB and processed according to the method outlined in section 2.2. In addition, a second quantification was performed of the purified mtDNA at a 1:10 dilution in order to observe whether or not any difference in mtDNA recovery could be attributed to inhibition. By diluting out potential inhibitors, it was possible to observe if the difference in recovered mtDNA was caused by the addition of inhibitors from the use of stainless steel pulverization components. If there was no difference in the quantities of recovered mtDNA at a 1:10 dilution, this suggests that the difference is caused by the presence of inhibitors in the undiluted samples.

All samples showed inhibition and low recovery of mtDNA; however, the samples pulverized using stainless steel components exhibited complete inhibition of samples and IPCs. However, when samples from a human rib were pulverized side by side with polycarbonate or stainless steel, diluted ten-fold and then quantified, the difference in mean quantity of mtDNA recovered using either polycarbonate or stainless steel end caps and impactor bar was found to be insignificant at the 95% confidence level, $t(4) = 0.53$ $p = 0.62$.

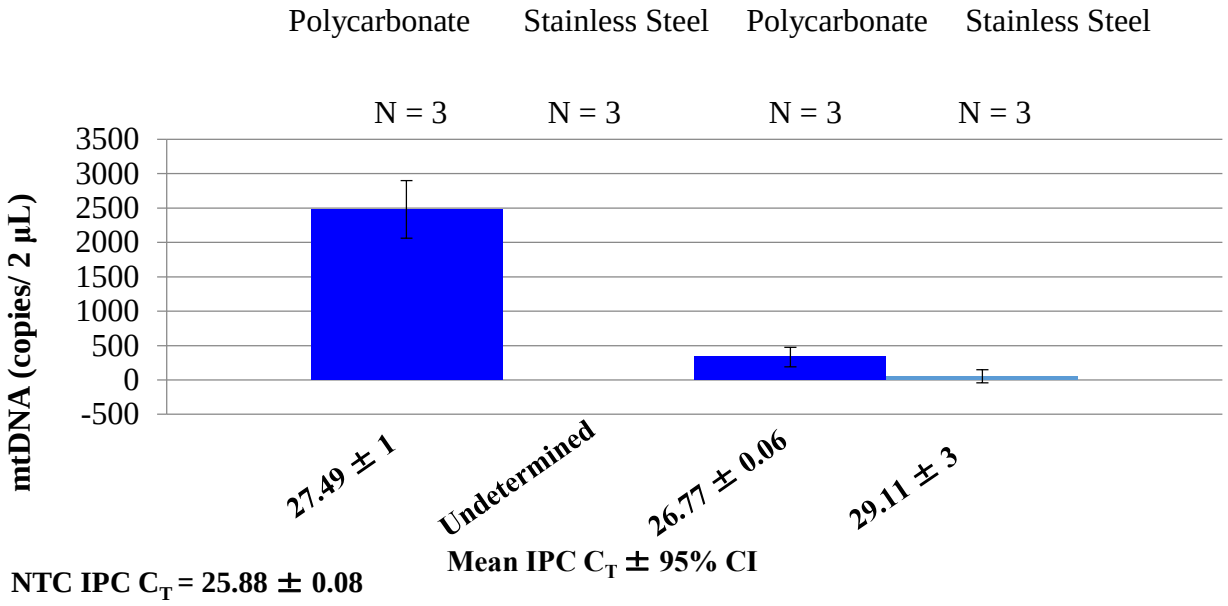

Figure 2.8: The mean quantity of mtDNA recovered from human rib when either polycarbonate or stainless steel pulverization components were used. The difference in mtDNA recovery when samples were diluted ten-fold was not found to be significant at the 95% confidence level, $t(4) = 0.53$ $p = 0.62$. Error bars represent 95% CI.

Even at the 1:10 dilution, a relative increase of the IPC C_T was observed in samples processed with stainless steel compared to the NTC IPC C_T. This was not observed in samples processed with polycarbonate.

2.5. Bone Sample Mass Effects on mtDNA Recovery from Human Femur

Inhibition was observed in mtDNA extracted from bone even when samples were pulverized using polycarbonate end caps and impactor bar. In an attempt to reduce this observed inhibition, the mass of bone powder used was reduced to 0.05 g and compared to 0.1 g samples quantified from pulverized bone from the same femur cross section.

Samples were processed with either lysis buffer ATL, SEB, or BTA, following the method outlined previously in section 2.2. If divalent calcium was a source of the observed inhibition, then increasing the ratio between the chelating agent EDTA and the quantity of bone powder used may reduce the levels of inhibition observed. However, the quantity of recovered mtDNA from bone samples of 0.1 g compared to 0.05 g was not found to be significant at the 95% confidence level, $t(16) = 0.35$, $p = 0.73$ (Fig. 2.9).

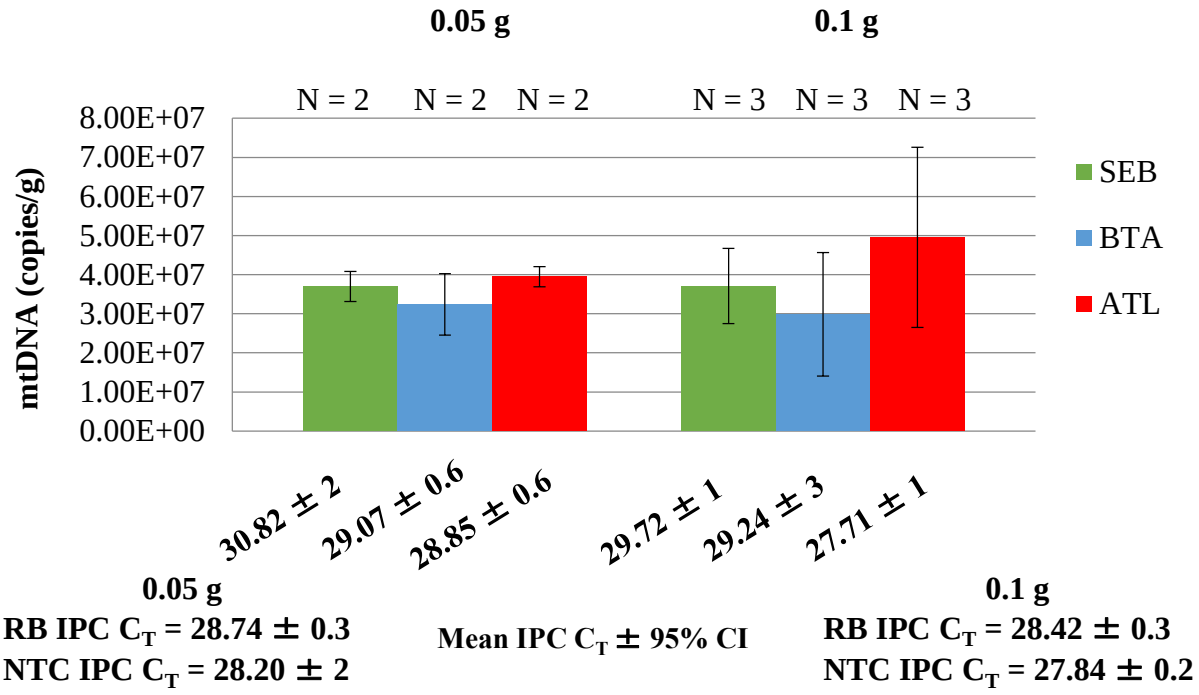

Figure 2.9: Mean quantities of recovered mtDNA from small rib using three different lysis buffers and either 0.1 g or 0.05 g bone powder. The difference was not found to be significant at the 95% confidence level, $t(16) = 0.35$, $p = 0.73$. No difference in the effectiveness of each lysis buffer was observed for samples processed from 0.05 g of bone powder at the 95% confidence level, $F(2,3) = 0.18$, $= 0.98$) or with samples processed from 0.01 g, $F(2,6) = 0.11$, $p = 0.89$). Error bars represent 95% CI.

2.6. Dilution of Samples to Observe the Behavior of Lysis Buffers with Inhibitors

To confirm that the difference in apparent mtDNA recovery in samples processed with polycarbonate was due to inhibition, samples from two different ribs and a femur were processed according to the method outlined in section 2.2 with samples treated with either lysis buffer SEB, BTA, or ATL for the lysis incubation. Following the initial mtDNA quantification of neat extracts, dilution series were made of each sample and quantified. The quantity of recovered mtDNA was analyzed, as well as the overall qualitative performance of each lysis buffer in each dilution.

2.6.1. Lysis Buffer Behavior and Dilution Effects with Femur Samples

Samples from a femur cross section were processed using polycarbonate pulverization components, treated with different lysis buffers and then processed according to the method described in section 2.2. If inhibitors were present in samples, dilution can be used to an extent to reduce their effect. If samples are diluted to a point in which inhibition is no longer detectable and there is no difference in mtDNA recovery with different lysis buffers, this strongly suggests that the apparent difference in mtDNA recovery in undiluted samples is due to inhibition. A dilution series of the mtDNA extracts from bone were quantified with qPCR. The quantities of mtDNA recovered from the 1:10 was multiplied by ten and five, to estimate the actual quantity of the neat and 1:2 diluted samples in the absence of PCR inhibitors.

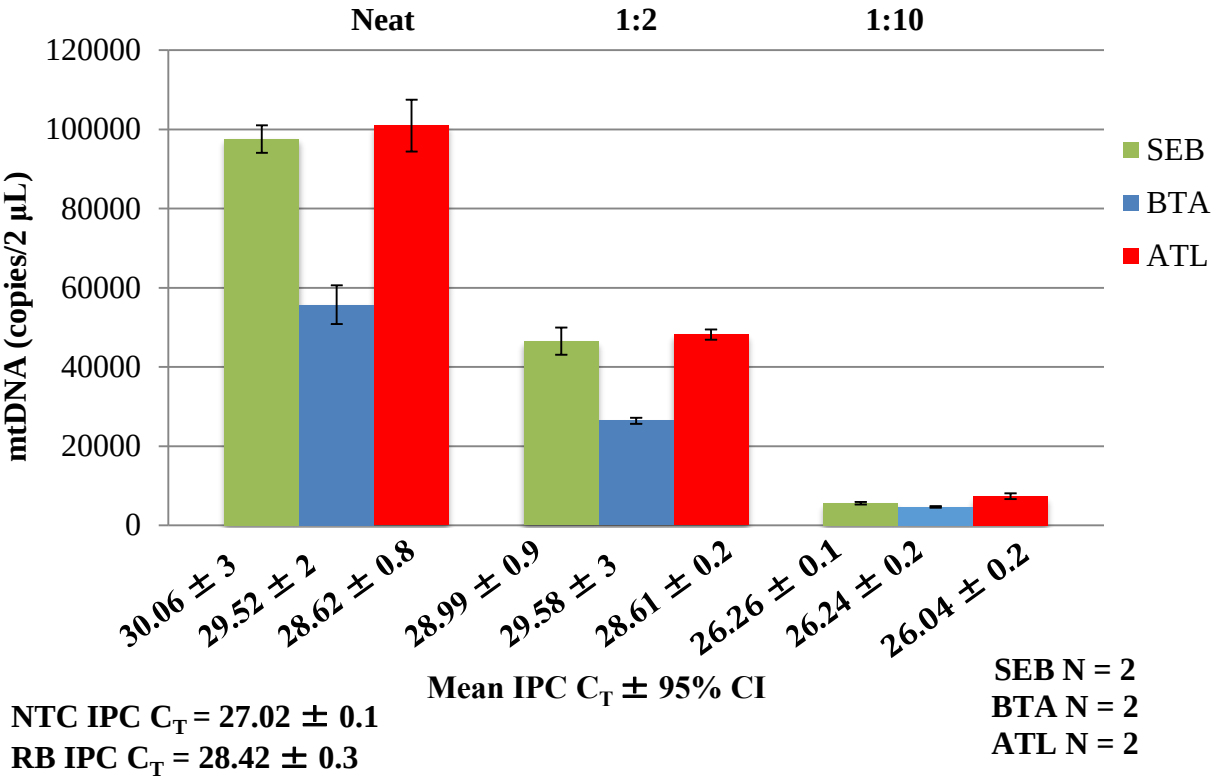

Figure 2.10: Mean quantities of recovered mtDNA from a femur cross-section using three different lysis buffers and polycarbonate SPEX components. Samples were prepared in a dilution series and quantified. Error bars represent 95% CI.

It was observed that the performance of each lysis buffer remained consistent throughout the dilution series based on the mtDNA recovery relative to each lysis buffer. At the 1:10 dilution, there was no significant difference in lysis buffer performance at the 95% confidence level, $F(2,3) = 2.87$, $p = 0.20$ (Fig 2.10). The elevated IPC C_T values indicate low levels of inhibition in the neat samples and those at a 1:2 dilution (Fig. 2.10). In the 1:10 dilutions, the IPC C_T values fell within one cycle of the NTC IPC C_T, which indicates that any inhibitors were diluted. Based on the calculated expected values, samples treated with BTA, SEB, or ATL exhibited 2%, 4%, and 3% more mtDNA recovery than expected, respectively.

A similar difference was observed with the 1:2 diluted samples. The actual recovered quantities of mtDNA for samples BTA, SEB, and ATL showed 1%, 7%, and 3% more mtDNA than expected, respectively. This indicates that very little inhibition is occurring in these samples processed from a femur and any that inhibition that is occurring is not affecting the performance of the lysis buffers.

2.6.2. Lysis Buffer Behavior and Dilution Effects with Large Rib Samples

When the same study was performed using a large human rib, samples treated with buffer ATL appeared significantly more inhibited than those treated with BTA or SEB in neat samples. Complete inhibition of not only the IPC but also the target mtDNA in the sample is observed in neat and 1:2 diluted samples. However, when the samples processed from a large rib were diluted ten-fold, no significant difference in recovered mtDNA was observed from either lysis buffer ATL, BTA, or SEB, $F(2,3) = 0.06$, $p = 0.94$ (Fig. 2.11). Therefore, the difference of mtDNA quantities observed in the neat samples and 1:2 diluted samples is likely due to the presence of inhibitors.

The IPC C_T values for SEB and BTA samples at the 1:10 and 1:2 dilutions were not elevated from the NTC IPC C_T value indicating that no inhibition is occurring. In neat samples, samples processed with SEB or ATL exhibited 10% and 100% less mtDNA recovery than

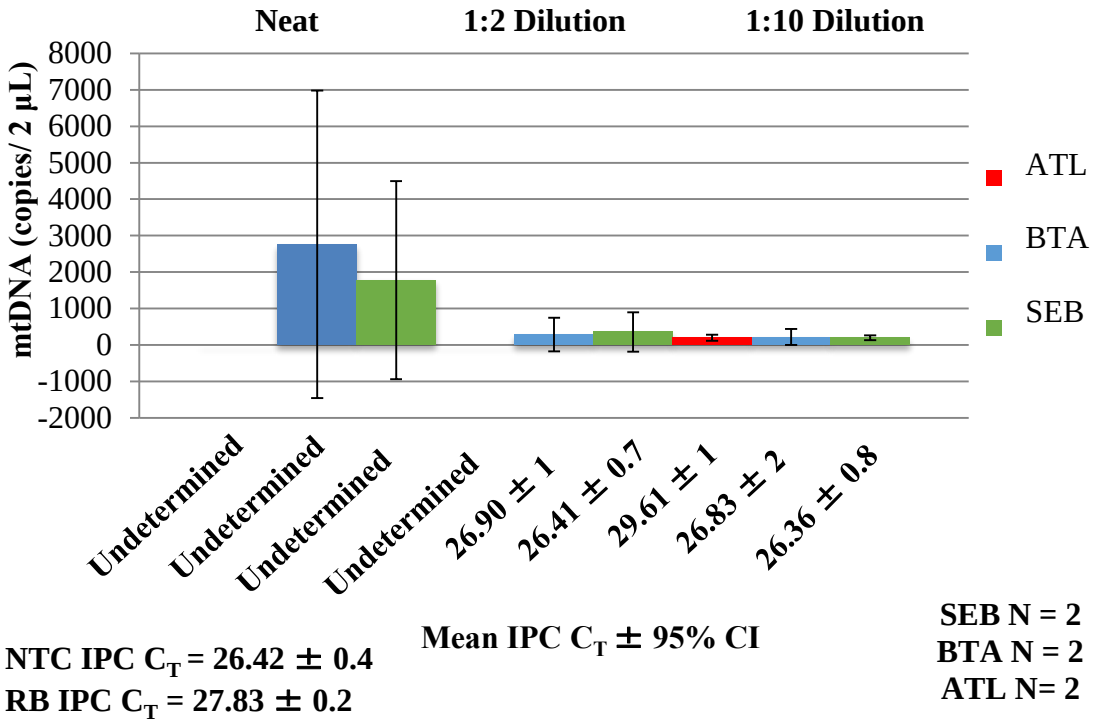

Figure 2.11: Mean quantities of recovered mtDNA from large rib using three different lysis buffers and polycarbonate SPEX components. Samples were quantified neat, diluted two fold and ten-fold. Error bars represent ± 95% CI.

expected, but samples processed with BTA exhibited 25% greater mtDNA recovery than expected. A larger difference between the actual and expected neat mtDNA recovery was observed in the 1:2 diluted samples. Samples processed with BTA or SEB showed 74% and 67% greater mtDNA recovery than expected, respectively. However, for samples processed with ATL, there was 100% less mtDNA recovered than predicted. It is possible that large quantities of DNA were also diluted out as well as inhibitors. This illustrates the importance of inhibitor removal with samples that contain low copy number DNA.

2.6.3. Lysis Buffer Behavior and Dilution Effects of Small Rib Samples

When the study was repeated with samples processed from a small rib, the same decrease in the performance of buffer ATL was observed. Samples processed with ATL showed complete inhibition of the sample and IPC in neat samples (Fig. 2.12). When samples processed from a small rib were diluted ten-fold no significant difference in recovered mtDNA was observed between samples treated with different lysis buffers in that the 95% confidence intervals overlapped one another. Therefore, the difference of means observed in the neat samples and 1:2 diluted samples is likely due to the presence of inhibitors.

In neat samples, samples processed with SEB or BTA exhibited 95% and 72 % greater mtDNA recovery than expected, but samples processed with ATL exhibited 100% less mtDNA recovery than expected. For the 1:2 diluted samples, samples processed with BTA, SEB or ATL showed 26%, 9%, and 67% less mtDNA recovery than expected, respectively. This suggests that a large portion of mtDNA was also diluted with the inhibitors and demonstrates a concern for simply diluting samples that are low copy number.

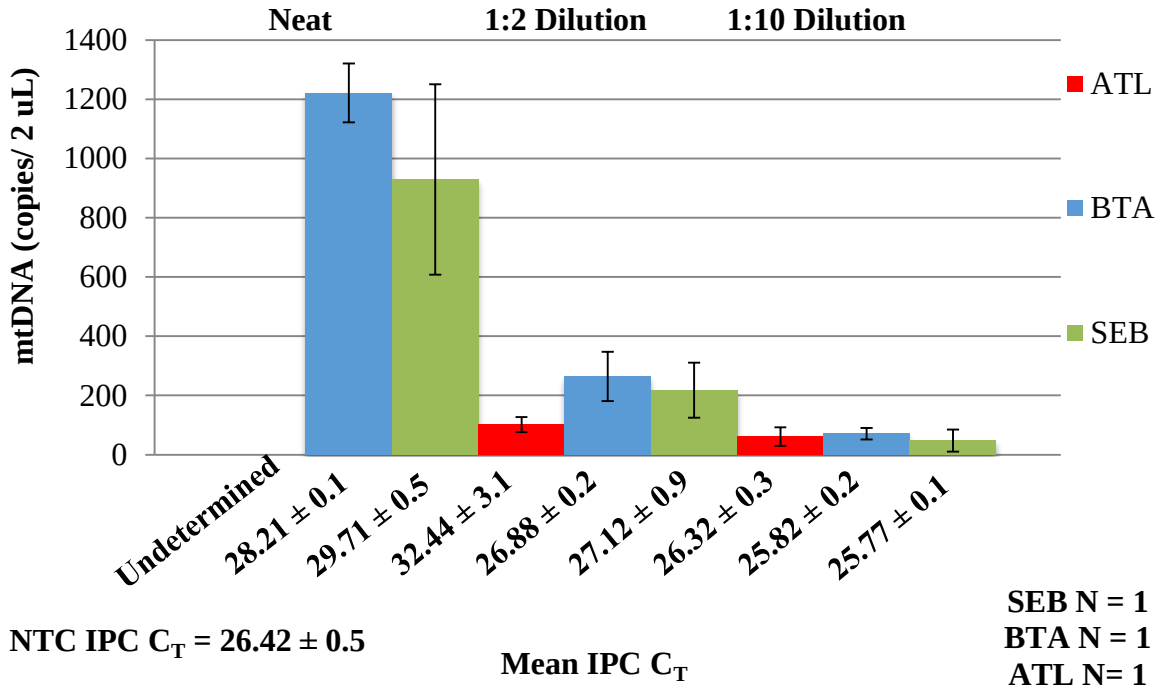

Figure 2.12: Mean quantities of recovered mtDNA from small rib using three different lysis buffers and polycarbonate SPEX components. Samples were quantified neat, diluted two fold and ten-fold. Error bars represent ± 95% CI.

2.7. Addition of a Chelating Agent to Buffer ATL

Lysis buffer ATL performed very well in femur samples, but overall poorly in ribs. A possible explanation is that lysis buffer ATL does not contain a chelating agent. Dilution studies of heavily inhibited rib samples showed that when samples were diluted ten-fold, there was not a significant difference in the effectiveness of each lysis buffer based on mtDNA recovery. The decrease in the performance of lysis buffer ATL in these samples may be explained by the fact it does not contain a chelating agent. Because lysis buffer ATL performed well with femur samples, a chelating agent, EDTA, was added to produce a final concentration of 10 mM to

buffer ATL. A quarter of samples from a large rib were treated with the ATL + 10 mM EDTA, one quarter with ATL, one quarter with BTA, and one quarter with SEB. The samples were processed following the protocol outlined previously in section 2.2.

2.7.1. Enhancing Lysis Buffer ATL with a Chelating Agent

In neat samples, ATL containing 10 mM of EDTA was found to significantly reduce mtDNA recovery. However, when diluted 1:10, the difference in mean mtDNA recovery using each lysis buffer was not significant at the 95% confidence level, $F(3,4) = 0.55$, $p = 0.68$ (Fig. 2.13)

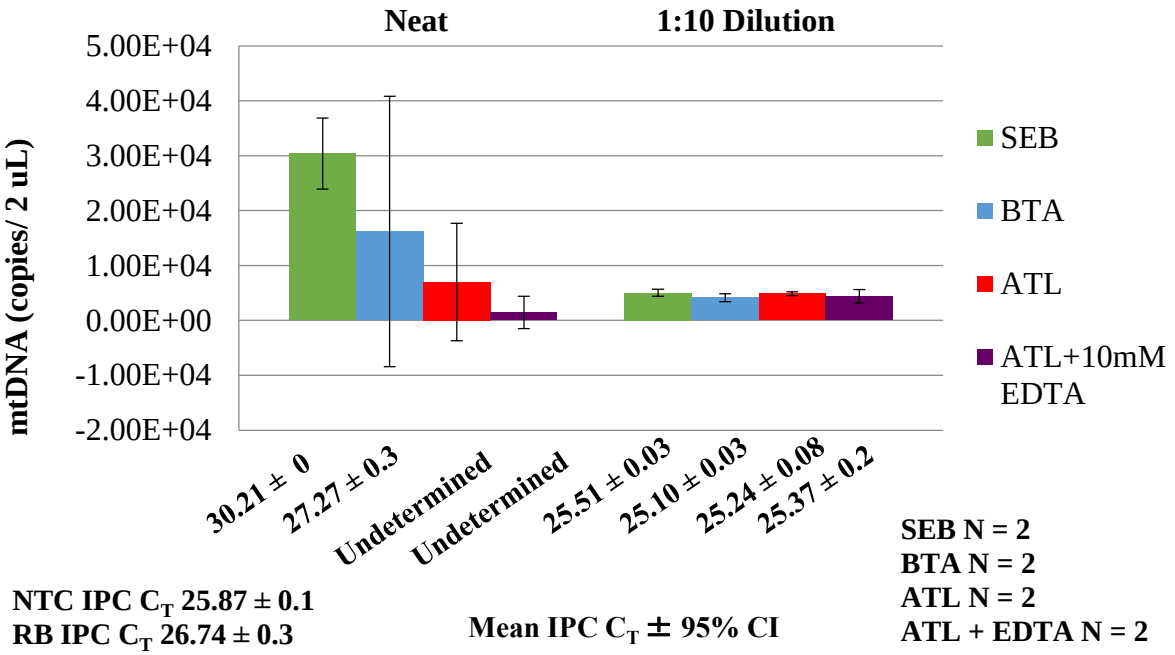

Figure 2.13: A comparison of mean mtDNA yields from rib samples using the lysis buffers SEB, ATL, and BTA, and ATL spiked with 10 mM EDTA. High levels of PCR inhibition were observed. Error bars represent 95% CI.

2.8. Preliminary Work on Comparison of QIAamp® and Prepfiler®

Two adjacent cross sections were cut from a human femur and pulverized with stainless steel end caps and impactor bar in a SPEX 6770 Freezer Mill. The samples were processed following the protocol outlined in section 2.2 with the following manipulations: all samples were treated with lysis buffer SEB, and were purified using either the Qiagen QIAamp® kit or Life Technologies™ PrepFiler® magnetic bead-based purification system. The following modifications were made to the manufacturer protocol as these were previously found in our lab to be effective for the recovery of mtDNA from hair shafts: for the PrepFiler® purification, 300 μL of isopropanol was added to each sample. Qiagen buffer AL was added to samples post lysis and samples were incubated at 70°C for ten minutes prior to the addition of absolute ethanol and loading onto the spin column to maintain consistency within the protocol (FBI 2010).

2.8.1. Preliminary Data on Purification Method

Samples purified using the QIAamp® solid phase extraction kit yielded an overall higher mean recovery of mtDNA than those processed with PrepFiler®; however, this was not found to be significant at the 95% confidence level, $t(4) = 1.82$, $p = 0.14$ (Fig. 2.14). Interestingly, the IPC C_T of samples processed with PrepFiler® were an average of 0.80 cycles lower than those in samples processed with QIAamp®, suggesting that Prepfiler® may be more efficient in the removal of PCR inhibitors.

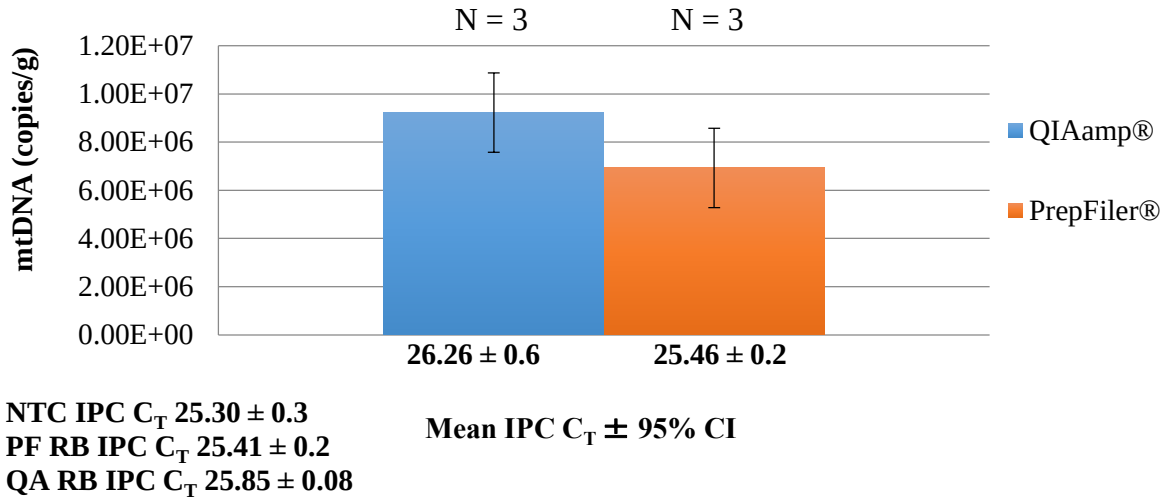

Figure 2.14: mtDNA recovery from human femur cross-sections using either PrepFiler® or QIAamp® in conjunction with using stainless steel end caps and impactor bar and lysis buffer SEB. The difference observed in overall mtDNA recovery was insignificant at the 95% confidence level, t(4) = 1.82, p = 0.14. Error bars represent 95% CI.

2.9. Utilization of Spectroscopy to Identify Metal Carryover in mtDNA Extracts

In samples processed previously, it was observed that inhibition was still occurring in samples that were processed with polycarbonate SPEX components, particularly in rib samples. Bone naturally contains many inhibitors of PCR, including large quantities of divalent calcium. It had been previously observed that lysis buffer performance appeared to be dependent upon the presence of a chelating agent, suggesting that calcium or another metal may be a source of the observed inhibition.

Inductively coupled plasma optical emission spectrometry (ICP-OES) was utilized in order to quantify the amount of calcium present in purified mtDNA extracts from bone. The Instrument used in this study was the Optima 4100DV (Perkin Elmer, Inc., Waltham, MA).

Quantifying the amount of calcium present in samples processed with stainless steel or polycarbonate may make it possible to determine if calcium is present in concentrations high enough to result in inhibition of the qPCR reaction or to exclude it as a source of the observed inhibition.

In addition, other metals can be detected, primarily chromium, as it comprises 16-18% of 440C stainless steel and is a known PCR inhibitor by causing mtDNA damage via crosslinking (Thompson et al. 2000). Previous experiments had determined that samples processed with stainless steel components showed significantly higher levels of inhibition than those processed with polycarbonate. In addition to the quantification of calcium in these samples, ICP-OES was utilized in order to detect chromium and iron, two other metals suspected to be introduced via the pulverization process.

2.9.1. Inhibition Assay to Determine Calcium Concentrations that Result in qPCR Inhibition

To measure the amount of calcium present in mtDNA extracts using ICP-OES, it was necessary to first obtain an estimate of the amount of calcium that may be present in the mtDNA extracts. An inhibition assay was developed to assess calcium inhibition in the qPCR quantification assay used in this study, as well as to determine at what concentrations of Ca^{2+} inhibition would be detected. A stock solution of calcium was prepared in a solution of hydrochloric acid (0.084 M Ca^{2+} pH adjusted to 7.03) from $CaHPO_4$ and serial dilutions were created from this stock solution.

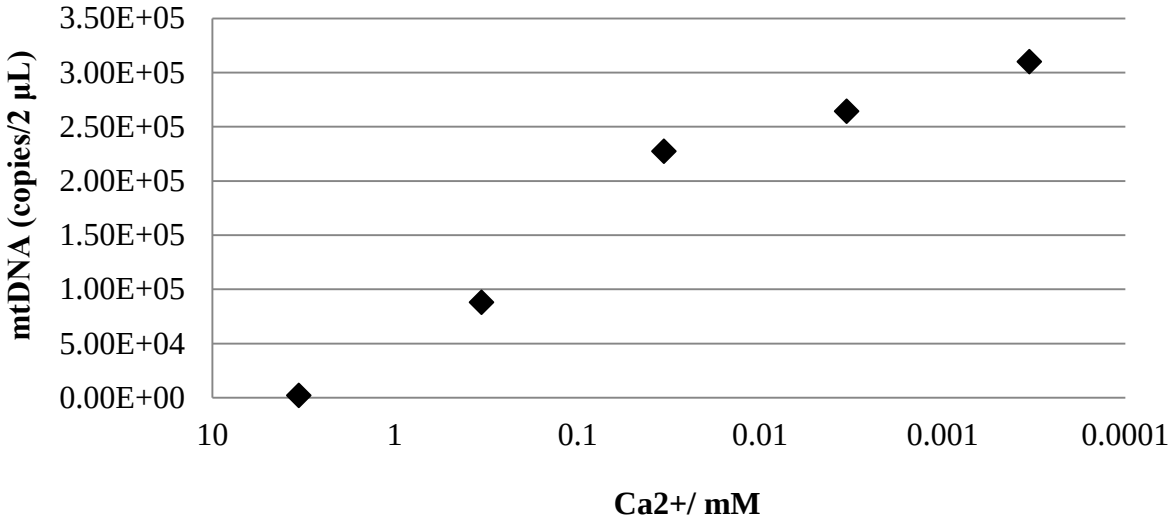

Figure 2.15: A calcium inhibition assay in which calcium in the form of CaHPO4 was added to a known concentration of mtDNA (HL60) ranging from final concentrations of 3.36 mM to 0.336 µM.

Commercially available DNA (HL60 derived from human promyelocytic leukemia cells) at a known concentration was treated with varying concentrations of divalent calcium (3.36 mM to 0.336 µM). The calcium preparation and template mtDNA were added separately to the 96 well plate in 1 µL volumes, respectively. The samples were then quantified using real time PCR following the method described in Kavlick et al. for mtDNA quantification (2011). Complete inhibition of the IPC occurred at a Ca^{2+} concentration of 3.36 mM (Table 2.1) along with a 99% decrease in mtDNA quantity, and a 17% decrease in mtDNA recovery was observed at a Ca^{2+} concentration as low as 3.36 µM (Fig. 2.15). Inhibition by calcium has been observed as low as 0.2 µM (Opel et al. 2010).

Table 2.1: IPC C_T values with known concentrations of added divalent calcium

Ca^{2+}	**Mean IPC C_T**	**IPC C_T 95% CI**
3.36 mM	Undetermined	N/A
336 uM	25.55	0.2
36.6 uM	25.58	0.6
3.36 uM	26.22	4
0.336 uM	25.00	0.5
0	26.89	4
Control	**Mean IPC C_T**	**IPC C_T 95% CI**
No HL60, 3.36 mM Ca^{2+}	32.52	0
NTC	25.03	0.1

2.9.2. Bone Sample Processing

A human rib was cut into cross sections and half of the cross sections were pulverized with stainless steel and half with polycarbonate SPEX impactor bar and end caps. The samples were then processed following the procedure outlined in section 2.2.

2.9.3. Preparation of ICP-OES Calcium Standards

Calcium standards were prepared using concentrated HNO_3 diluted to a final concentration of 7% in MBG H_2O. A 150 mL solution of 7% HNO_3 was prepared from a concentrated HNO_3 and used to dilute the standards. A stock solution of 404.78 ppm was prepared by dissolving 0.07 g $CaHPO_4$ in 7 % nitric acid at a final volume of 50 mL. Seven standards were prepared from the stock solution, ranging in concentrations of 404.78 ppm to 40.480 ppb.

2.9.4. Sample Prep ICP-OES

For the real-time PCR quantification, it was necessary to use 6 µL of the purified mtDNA extract, because a total sample volume of at least 1.5 mL is required for ICP-OES analysis. Hence, 15 samples were pooled to a total volume of 810 µL. The pooled samples were adjusted to a final volume of 1.5 mL with MBG H_2O and 150 µL of 70% HNO_3 to a final concentration of 7%.

2.9.5. Quantification and Detection of Metals in Bone Extracts Pulverized with Polycarbonate or Stainless Steel

In an attempt to detect metals in bone extracts and quantify the amounts of calcium present, samples from a human rib were pulverized using either polycarbonate or stainless steel end caps and impactor bar and then processed according to the protocol outlined in section 2.2.

2.5.9.1. Direct quantification of calcium using ICP-OES. The samples processed with stainless steel failed to amplify in the qPCR reaction and all IPC C_T values were undetermined (Fig. 2.16). Samples processed with polycarbonate also showed very high levels of inhibition. The internal positive control was undetermined for 14/15 samples processed in this study. A tremendous variation in mtDNA recovery was observed between the samples that were successfully quantified. This variation had been observed previously with very inhibited bone samples.

An interesting observation was obtained with one sample during this particular study. Sample LR11315.A4 was quantified and exhibited a mtDNA concentration much higher than those observed from the other samples that were processed and was the only sample to not show an undetermined internal positive control (Fig. 2.17). The processing of this sample appeared to have affected the levels of inhibition in the final extract. After the lysis incubation, 300 µL of

Qiagen buffer AL, which maintains the proper pH and contains chaotropic salts to facilitate the binding of mtDNA to the spin column, was added to most samples. However, an operator error with a repeating pipetter resulted in a larger volume (>900 µL) of buffer AL delivered to the sample LR11315.A4. The lysate was equally divided into two aliquots and incubated with the other samples.

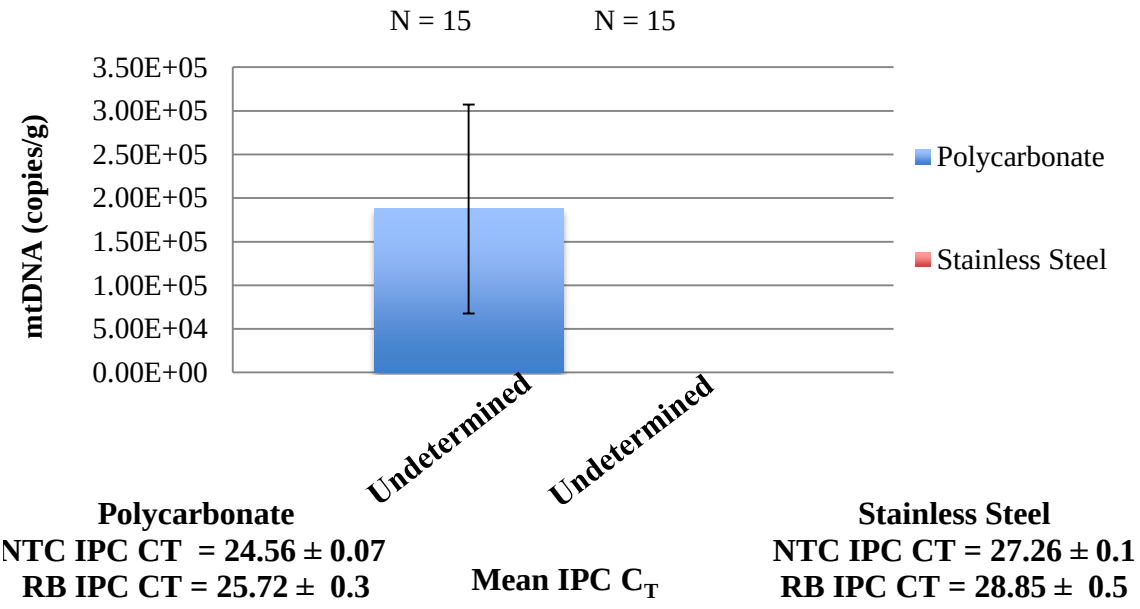

Figure 2.16: Comparison of mtDNA recovered (copies/g) when either polycarbonate or stainless steel SPEX components were used to pulverize samples from the same human rib. Samples pulverized with stainless steel failed to quantify and all IPC C_T values were undetermined. Sample LR11315.A4 has been excluded. Error bars represent 95% CI.

After the incubation, 400 µL of ethanol was added to each sample, including both aliquots of sample LR11315.A4. The lysate was added to the spin column at a volume of 500 µL, centrifuged, and the flow through discarded. This was repeated a total of 4 times for sample LR11315.A4 until all the lysate had been added to the spin column and centrifuged out. Sample LR11315.A4 was then processed in the same manner as the other samples during the remaining steps of the procedure. It is possible that the larger volume of buffer AL changed the pH and ionic strength and therefore increased binding of mtDNA to the column. The increased volume may have also washed away potential inhibitors. Sample LR11315.A4 was the only sample to not have a completely inhibited IPC C_T, which suggests that the increased volume allowed for more efficient removal of inhibitors.

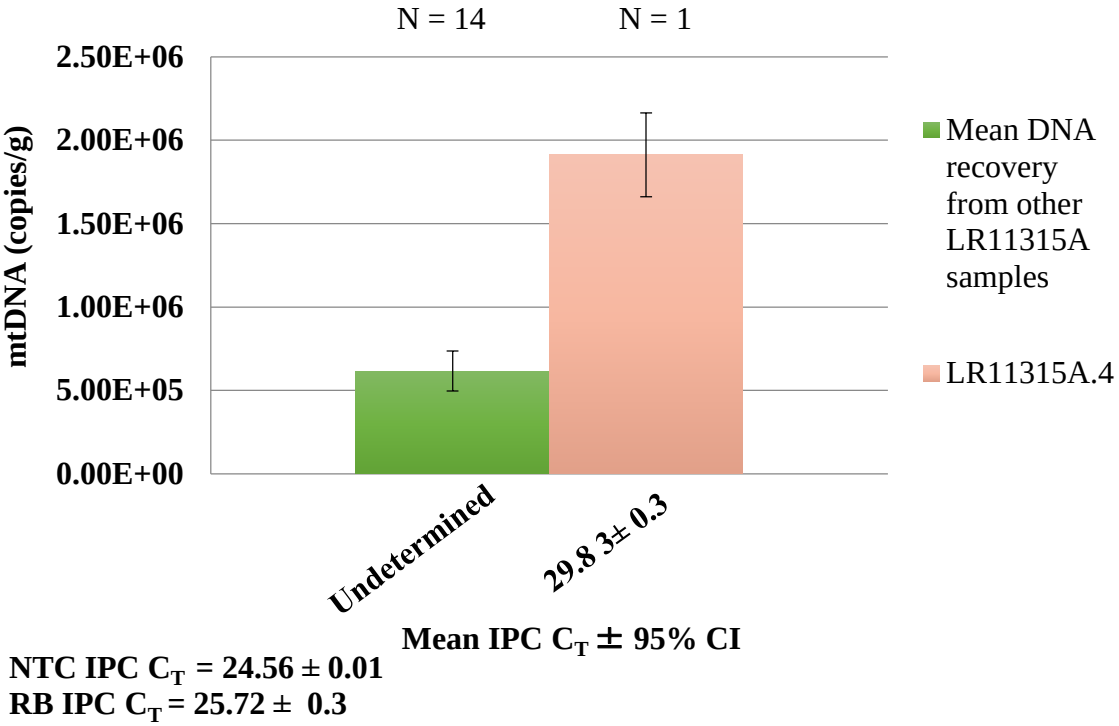

Figure 2.17: mtDNA recovered (copies/g) from a human rib when polycarbonate SPEX components were used. Sample LR11315A.4 showed significantly greater recovery of mtDNA than all other samples and an IPC C_T that was not undetermined. Error bars represent 95% CI.

Table 2.2: mtDNA recovered (copies/g) when either polycarbonate or stainless steel SPEX components were used as well as the quantified calcium concentrations in each sample

Pulverization Component	# samples completely inhibited	Ca^{2+} (ppm)	Ca^{2+} (mM)	Mean mtDNA (copies/g)	95% CI (copies/g)
Stainless Steel	15/15	1.32	0.033	0	0
Polycarbonate	5/15	1.33	0.033	3.08×10^5	2.5×10^5

Calcium was detected and quantified at the wavelength of 315.887 nm on the ICP-OES in concentrations of 1.32 and 1.33 ppm (0.033 mM) in samples processed with polycarbonate and stainless steel, respectively (Fig. 2.18). Previous work by Opel et al. demonstrated that divalent calcium can cause PCR inhibition at concentrations as low as 0.2 μM (2010). The calcium inhibition assay performed showed a 17% decrease in mtDNA recovery in the presence of 3.36 μM Ca^{2+}. Chromium and iron were not detected by the instrument, so if they were present, it was at a concentration below the detection limit of the instrument of approximately 0.0002 and 0.0001 ppm, respectively (Perkin Elmer® 2013). Because the calcium concentrations are nearly the same in samples processed with polycarbonate and samples processed with stainless steel, the difference in inhibition levels and mtDNA recovery from the qPCR data cannot be attributed to the presence of calcium alone. This suggests that while calcium is present in the samples and causing inhibition, the use of stainless steel pulverization components is contributing to the inhibition observed in these bone samples.

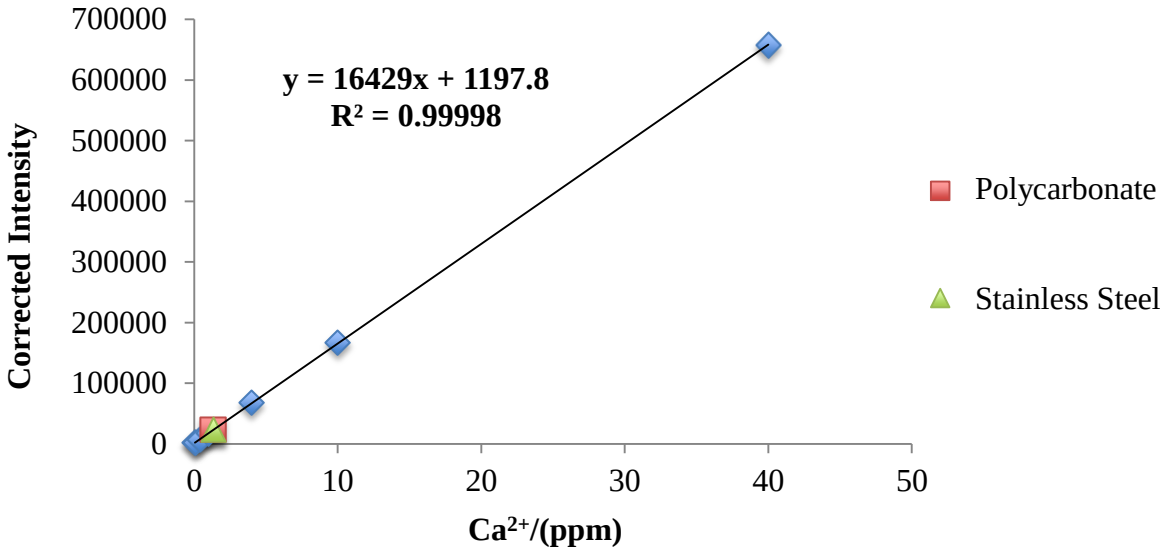

Figure 2.18: ICP-OES calcium standard curve at the wavelength of 315.887 nm and corrected intensities for samples processed with polycarbonate or stainless steel.

2.9.6. qPCR of Known mtDNA Concentration Added to Bone Extract

To better understand the nature of the inhibition observed in bone extracts, known concentrations of HL60 DNA were added to the bone extract to examine the effects of the bone extract itself on the ability of the mtDNA template to amplify. In addition, calcium was quantified in these mtDNA extracts. The samples were processed following the method outlined in section 2.2 and the ICP-OES standards prepared as in section 2.9.3. Bone extracts were prepared for ICP-OES analysis as described in section 2.9.4.

2.9.6.1. Sample preparation for inhibition study. All of the extracted mtDNA samples were pooled into a 15 mL conical tube, and then several 54 µL aliquots were removed and the remaining volume used in the ICP-OES run and prepared as seen in section 2.9.4.

To two of the 54 µL aliquots, 6 µL of HL60 mtDNA was added for a final concentration of 10 pg/ µL (roughly 15,000 copies/ 2 µL), and to the other two, HL60 mtDNA at a final concentration of 1.67 pg/ µL (roughly 2,500 copies/ 2 µL). The last two of the 54 µL aliquots of bone extract had 6 µL of MBG H_2O added to serve as negative controls. The six aliquots containing mtDNA was then quantified using the qPCR assay outlined in section 2.2.4.

2.9.6.2. ICP-OES and qPCR Results from adding known concentrations of mtDNA to bone extract. The qPCR data for the pooled mtDNA extracts indicated moderate levels of inhibition (Fig. 2.19) and calcium was quantified at 1.962 ppm (0.04895 mM). Despite a higher calcium concentration than the previously processed rib samples overall (1.334 ppm), lower levels of inhibition were observed and higher amounts of mtDNA amplification were obtained (Fig. 2.19).

The average quantity of mtDNA recovered from the two aliquots that contained no HL60 was 2,494.44 ± 186.25 copies/2 µL. Moderate levels of inhibition were observed with a mean IPC C_T value of 27.9 ± 0.15 as compared to the NTC IPC C_T of 25.39 ± 0.13. This baseline quantity was compared to the observed quantities when HL60 was physically added to bone extract. To assess the levels of inhibition in each sample, the expected quantification values were calculated by numerically adding the baseline quantity of mtDNA from the pooled bone extract to the positive control mtDNA quantities.

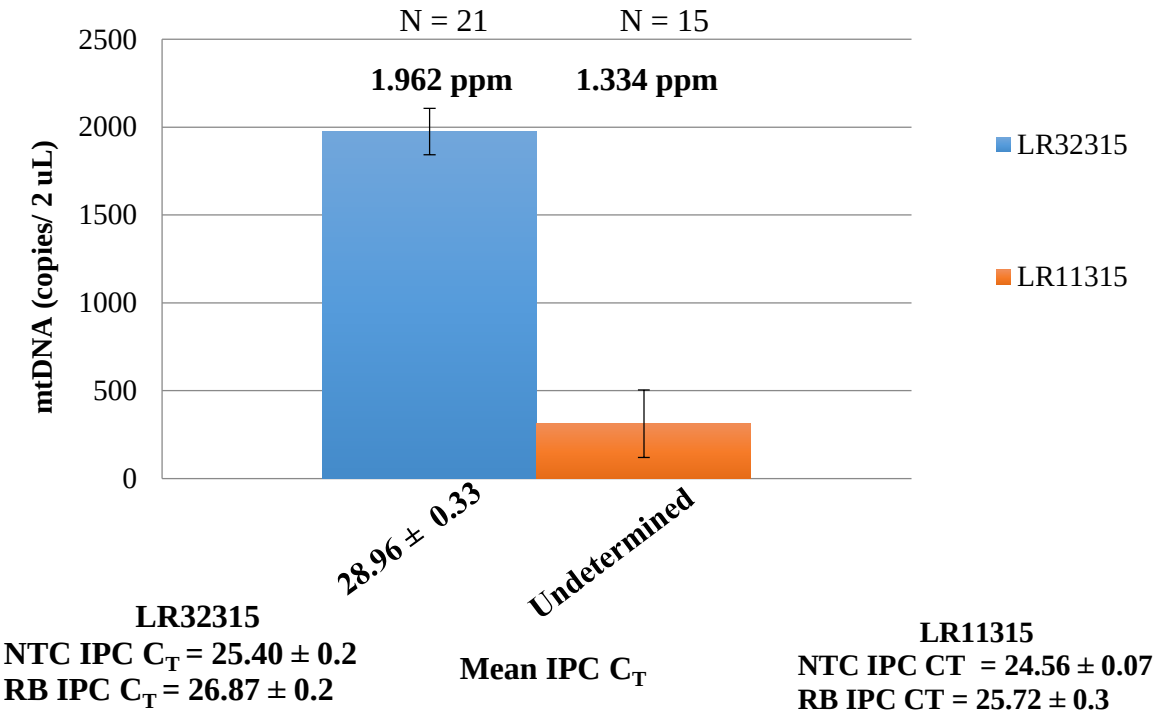

Figure 2.19: Comparison of mtDNA recovery and calcium concentration between two rib samples processed identically. Samples processed from the rib LR32315 showed significantly greater mtDNA recovery at a confidence level of 95% than samples processed from LR11315, $t(33) = 10.25$, $p < 0.001$ despite the fact that 1.962 ppm of divalent calcium was measured in samples from LR32315 as compared to 1.334 ppm of calcium from samples processed from LR11315. Error bars represent ± 1 standard deviation.

The difference between the expected and observed HL60 quantities in the 10 pg/µL samples was not found to be significant at the 95% confidence level in that the confidence levels overlapped (Fig. 2.20). In the samples containing 1.67 pg/µL of HL60, the difference between the expected and observed mtDNA quantities was also found to be insignificant at the 95% confidence level in that the confidence levels overlapped (Fig. 2.20).

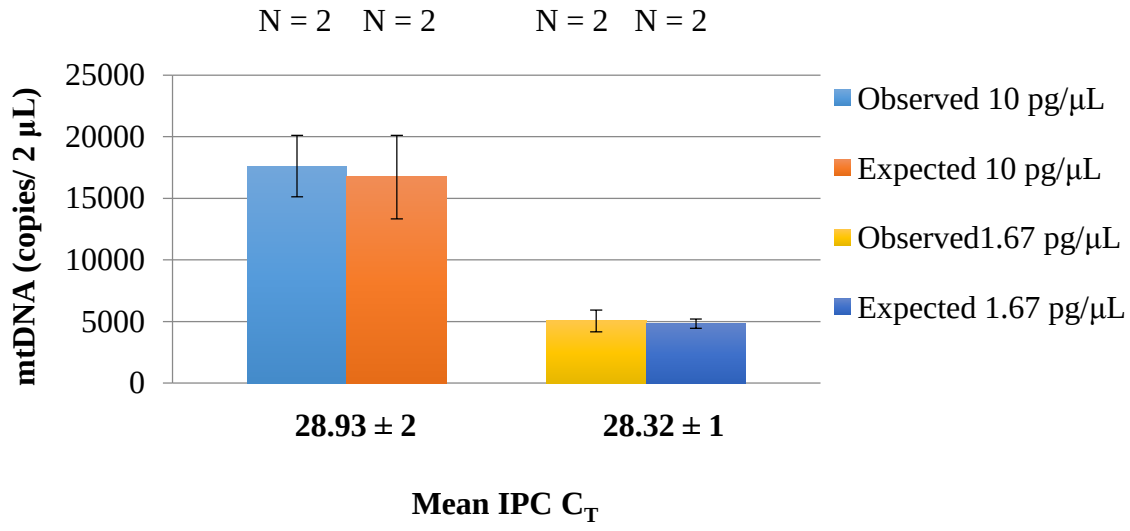

Figure 2.20: Comparison of corrected qPCR data compared to the expected values. Expected values are the positive control quantities plus the quantity observed in the samples of 0 pg/ µL HL60. Error bars represent ± 95% CI.

2.10. Amplification of HVI and HVII

The purpose of an optimized extraction method is to provide enough amplifiable DNA to perform downstream procedures such as amplification and sequencing. To see if the mtDNA target used in the quantification assay was a general predictor of amplification success, HVI and HVII were amplified from bone extracts. Several purified extracts from bone and their associated reagent blanks were selected to be amplified with human-specific mtDNA primer sets and the PCR products quantified on the Agilent Technologies® 2100 Bioanalyzer® using the Agilent Technologies® DNA 1000 Kit™. Amplification of HVI was done using the primers A1 (L 15997, 5'-CAC CATTAG CAC CCA AAG CT-3') and B1 (H 16391, 5'-GAG GAT GGT CAA GGG AC-3'). HVII was amplified using the primers C1 (L 048, 5'-CTC ACG GGA GCT

CTC CAT GC-3') and D1 (H 409, 5'-CTG TTA AAA GTG CAT ACC GCC-3'). For PCR, an ABI Model 9700 thermal cycler was utilized with the following cycling parameters: 95°C for 9 min, then 36 cycles of 95°C for 10 s, 60°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 10 min (FBI 2010).

Table 2.3: Master mix calculations for amplification of HVI and HVII from mtDNA extracted from human bones (FBI 2010)

Reagent	µL/Reaction	Final Concentration
DNA Template	10	-
MBG H_2O	4	-
GeneAMP® 10x PCR buffer	2.5	1 x
Primer Pair 10 uM each	1.5	0.6 uM
BSA (1.6 µg/µL)	2.5	0.16 µg/µL
dNTP mix 10 mM	0.5	0.2 mM
$MgCl_2$ 25 mM	1.5	2.5 mM
Amplitaq Gold® DNA polymerase (5 U/µL)	1	5 U
Total Volume	25	-

2.10.1. Amplification of HVI and HVII of mtDNA Extracted from Human Bone

A correlation between the quantity of mtDNA from the qPCR and the amount of amplified mtDNA as assessed from the Bioanalyzer® run was observed (R^2 =0.77716). In general, samples that had a significant amount of inhibition when analyzed with qPCR showed lower concentrations of amplified mtDNA quantified with the Bioanalyzer®. Samples that quantified with the mtDNA assay used in this study also showed successful amplification of the HVI and HVII regions.

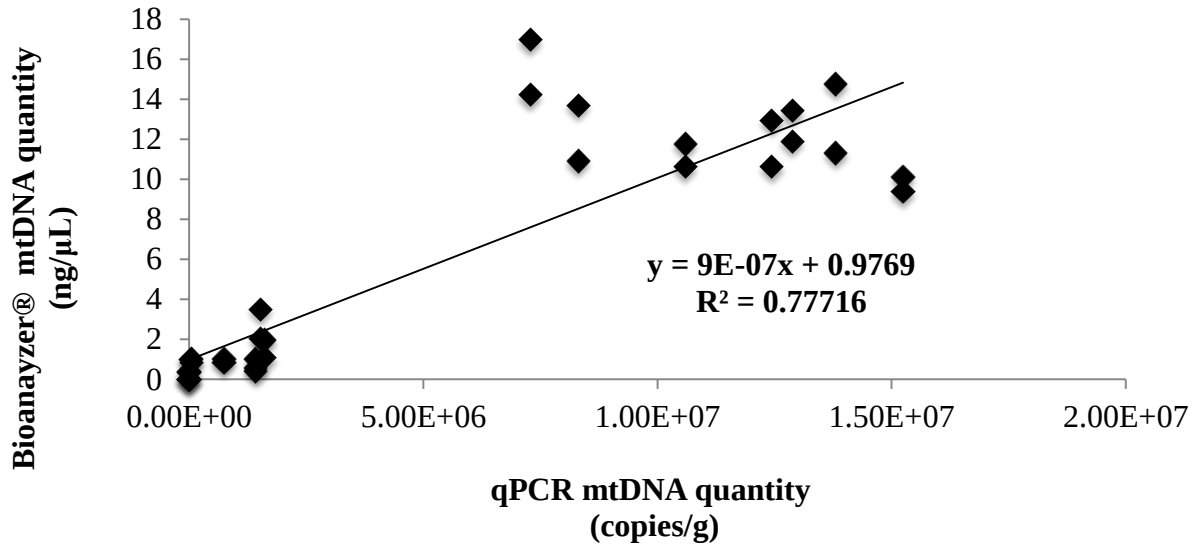

Figure 2.21: Correlation between the quantity of mtDNA observed from qPCR and the Bioanalyzer®.

2.11. Quantification of Nuclear DNA from Purified Bone Extracts

To see if samples containing large quantities of mtDNA also contained amplifiable nuclear DNA, selected samples of purified bone-extracted DNA were quantified for nuclear DNA using the Life Technologies™ Quantifiler® HP and Trio Quantification Kit (Thermo Fisher Scientific Corporation, Carlsbad, CA). The Quantifiler® HP assay contains four nuclear DNA targets: a short and long autosomal PCR amplicon of 80 and 214 bases in length, respectively, a Y chromosome target of 75 bases in length, and 130 base long internal positive control. It is a 5' endonuclease assay that uses TaqMan® probes. The ratio of the large amplicon over the small amplicon describes what is known as the degradation index, which provides information of how degraded the extracted DNA may be. A third target on the Y chromosome can be used to determine the sex of the individual. The assay was run on an Applied Biosystems® 7500 Real-Time PCR instrument and analyzed with HID HIG Real-Time PCR Analysis Software v1.2 Real-Time PCR Analysis Software v1.2.

2.11.1. Nuclear DNA Quantification from Human Rib

The difference in effectiveness of each lysis buffer was not found to be significant at the 95% confidence level rib samples pulverized with either polycarbonate or stainless steel end caps and impactor bar (Table 2.4). The IPC C_T values of the samples were similar to the IPC C_T values of the non-template controls indicate that little to no inhibition was occurring in the samples. However, the differences between samples processed with stainless steel pulverization components and polycarbonate suggests that undetectable inhibition is still occurring. The difference in recovered DNA quantity between samples pulverized with stainless steel or polycarbonate was significant at the 95% confidence level (large autosomal target $t(32) = 4.54$ $p < 001$, small autosomal target $t(31) = 4.73$ $p < 001$, Y target $t(32) = 3.53$, $p < 0.001$) (Fig. 2.22).

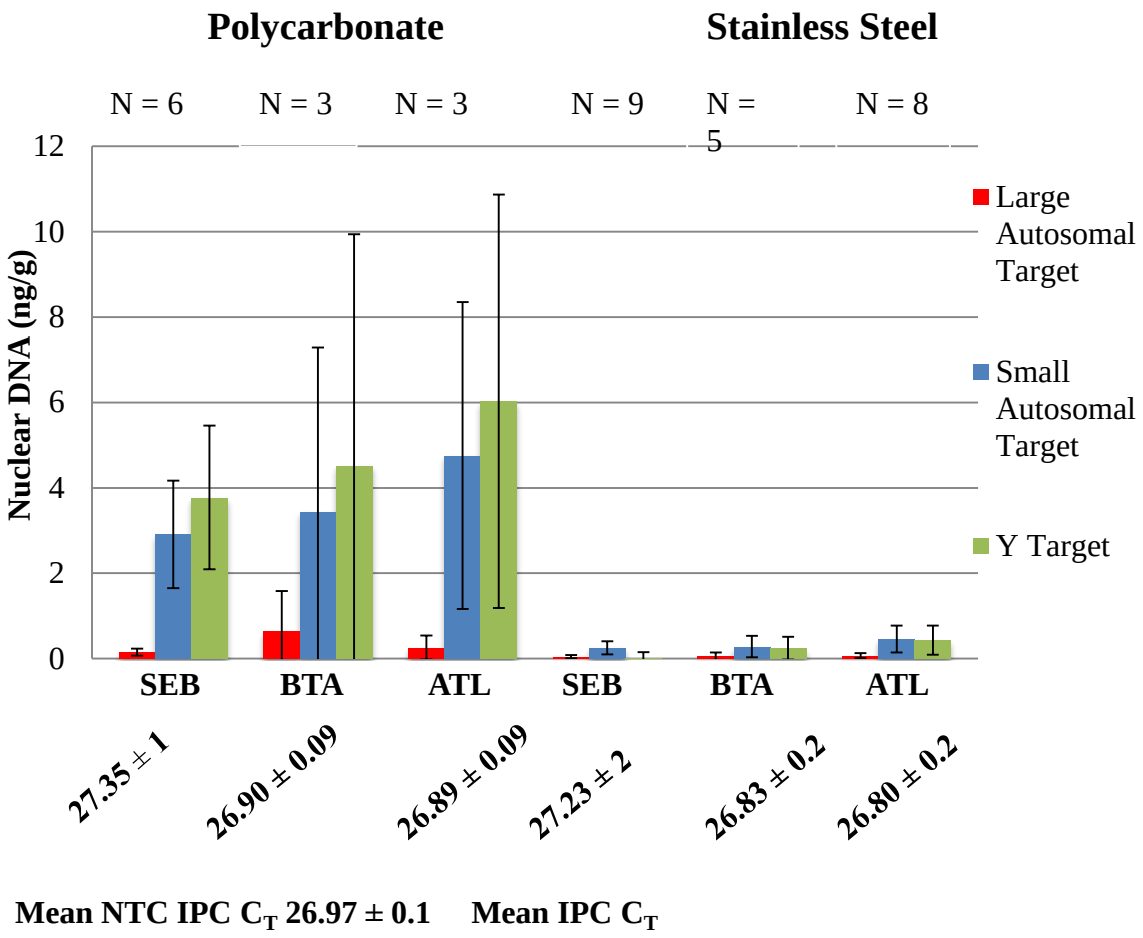

Figure 2.22: Nuclear DNA quantification data from human rib samples. Error bars represent ± 95% CI.

Dilution studies with mtDNA from the same human rib suggest that when samples were diluted, no significant difference in DNA recovery was observed whether the samples were processed with stainless steel or polycarbonate. This suggests that inhibition is still occurring in the samples although not detected by a relative increase in the IPC C_T.

Table 2.4 ANOVA statistical analysis of lysis buffer and pulverization component performance with human rib

Pulverization component	**Nuclear target**	**df (between,within)**	**F value**	**p value**
Polycarbonate	Large Autosomal	2,9	1.40	0.30
	Small Autosomal	2,9	0.57	0.59
	Y	2,9	0.44	0.66
Stainless Steel	Large Autosomal	2,19	1.91	0.16
	Small Autosomal	2,19	1.91	0.18
	Y	2,19	2.11	0.15

2.11.2. Nuclear DNA Quantification from Human Femur

When samples from a human femur were quantified for nuclear DNA, it was observed that the IPC C_T values of the bone samples were similar to the IPC C_T values of the non-template controls, indicating that little to no inhibition was occurring in the amplification of the samples. However, the differences between samples processed with stainless steel pulverization components and polycarbonate was significant at the 95% confidence level from the large and small autosomal targets but not for the Y targets (large autosomal target $t(47) = 2.34$, $p = 0.02$, small autosomal $t(47) = 2.34$ $p = 0.02$, Y target $t(47) = 1.92$, $p = 0.06$) (Fig. 2.23). Again, as in samples processed from ribs, it is likely that inhibition is still occurring in samples processed with stainless steel, but the effects may not be as dramatic with smaller targets. In addition, the overall performances of the lysis buffers is similar to what is observed from the mtDNA quantification data (Fig. 2.23).

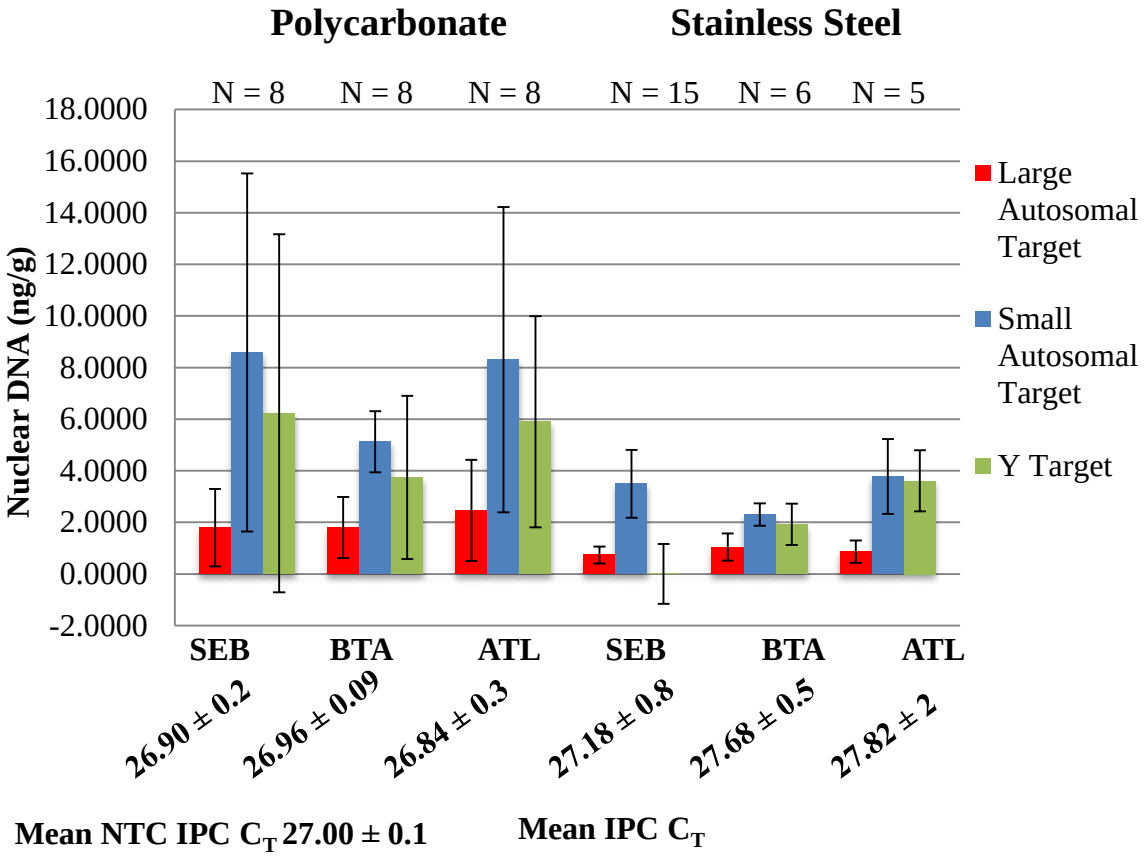

Figure 2.23: Nuclear DNA quantification data from human femur samples. Error bars represent ± 95% CI.

The difference in effectiveness of each lysis buffer was not found to be significant at the 95% confidence level between samples from a femur processed with either stainless steel or polycarbonate impactor bar and end cap (Table 2.5).

Table 2.5: ANOVA statistical analysis of lysis buffer and pulverization component performance with human femur

Pulverization component	Nuclear target	df (between,within)	F	p value
Polycarbonate	Large Autosomal	2,21	0.23	0.80
	Small Autosomal	2,21	0.40	0.68
	Y	2,21	0.55	0.59
Stainless Steel	Large Autosomal	2,23	0.98	0.39
	Small Autosomal	2,26	1.45	0.25
	Y	2,23	1.49	0.25

CHAPTER 3: DISCUSSION

3.1. Evaluation of Inhibition in Bone Samples

Bone tissue is considerably more resistant to degradation than other bodily tissues and as a result is often the only physical evidence available for the identification of human remains. The unique composition of bone makes it resistant to breakdown but also poses associated challenges with DNA extraction, resulting in tremendous difficulty for the forensic typing community. The mineral and collagen matrix creates a physical barrier, and the components of this bone matrix are known to be PCR inhibitors (Ye et al. 2004, Loreille et al. 2007). The environment from which the bone is found potentially provides another source of PCR inhibition, as well as any chemical treatments performed post mortem to preserve the bone (Ye et al. 2004, Kalmár et al. 2000).

It was found in this study that the coextraction of PCR inhibitors apparently caused the greatest variation in the amount of mtDNA recovered within the same bone types. However, it is unlikely that the inhibition observed originates from a single source. This work presents some preliminary observations and suggestions for future work in enhancing mtDNA recovery from bones and reducing the coextraction of PCR inhibitors. In addition, it is suggested that future work focus on elucidating the sources of inhibition present utilizing a variety of technologies, including spectrometric analysis.

3.1.1. Evaluation of Lysis Buffer Performance and Different Types of Bone

Initial efforts focused on the use of different lysis buffers in a standard extraction protocol (FBI 2010). The effectiveness of the three different lysis buffers evaluated in this study varied considerably when different bone types were examined. The femur used in this study routinely yielded higher quantities of mtDNA than the ribs studied, regardless of the lysis buffer

used. Traditionally, weight-bearing long bones have been regarded as the optimum choice for DNA recovery, likely due to better DNA preservation (Mundorff et al. 2009). Additional studies suggest that higher quantities of DNA can be recovered from metatarsals and phalanges. It is possible that cancellous bone in some situations may contain more cellular material associated with increased vascularization (Miloš et al. 2007); however, this appears dependent upon the preservation of the actual bone (Prinz et al. 2007, Mundorff et al. 2009). The bones in this study contained no visible cellular tissue and were processed with hydrogen peroxide; therefore, the DNA was likely better preserved in the dense cortical bone of the femur.

Overall, at the 95% confidence level, no difference in effectiveness of different lysis buffers was observed in the recovery of mtDNA per gram of bone material for femur or rib samples. However, the effectiveness of each lysis buffer varied considerably depending on the particular bone sampled. mtDNA recovery and inhibition varied greatly with some bone samples but not in others. In addition, the region sampled in the bone affected mtDNA recovery and the amount of inhibition observed.

To further understand the inhibition occurring in bone extracts, samples from a femur and two ribs were diluted in a series and the mtDNA quantified. The result of these dilution studies was that when femur and rib samples were diluted and re-quantified, it was found that at a 1:10 dilution, no difference in the quantity of recovered mtDNA with each lysis buffer could be observed at the 95% confidence level, suggesting that the difference in performance of each lysis buffer was due to the interaction between the lysis buffer and inhibitors present in the mtDNA extract and not a true difference in actual quantity of recovered mtDNA.

Analyzing the apparent mtDNA recovery in neat samples and 1:2 dilutions illustrated a difference in the performance of each lysis buffer when detectable inhibition was present. In

cases where rib samples showed high levels of inhibition, lysis buffer ATL was not found to be as effective as buffers SEB and BTA, which contain the divalent metal chelating agents EDTA and EGTA, respectively. The dilution series performed with a large rib resulted in no apparent mtDNA recovery at the neat and 1:2 dilutions. However, at the 1:10 dilution, it was possible to quantify mtDNA from the extract. Moreover, the mtDNA recovery from the rib samples diluted 1:10 was not found to be significantly different at the 95% confidence level than in samples treated with the lysis buffers SEB or BTA. Furthermore, no difference was observed in the sample IPC C_T values relative to the non-template controls, indicating no difference in the performance of each lysis buffer when inhibitors are sufficiently diluted.

A similar trend was observed in a smaller rib. Neat samples processed with buffer ATL exhibited no apparent mtDNA recovery, but did show mtDNA recovery occurring at the 1:2 and 1:10 dilutions. As seen in the other rib samples, no difference in the performance of the three lysis buffers was observed at the 1:10 dilution at the 95% confidence level. In addition to differences in mtDNA recovery, this variation in lysis buffer effectiveness was illustrated by the sample IPC C_T values. For rib samples processed with buffer ATL, the sample IPC C_T values were undetermined in all neat samples and either undetermined or elevated relative to the NTC IPC C_T in 1:2 dilutions. At the 1:10 dilution all samples had an IPC C_T value that was not different from the NTC IPC C_T values, where no inhibition is expected. It is noteworthy that the sample IPC C_T values were on average one cycle lower than observed in the reagent blanks. This suggests that the source of inhibition detected in the reagent blanks is something that can be diluted out.

Femur samples that were diluted and re-quantified showed no difference in the performance of each buffer relative to one another and no change in the IPC C_T values. In

addition, the IPC C_T values observed in the 1:10 dilution fell below the IPC C_T values of the reagent blanks and were similar to those observed in the NTCs. This suggests that inhibition is occurring in the reagent blanks as the IPC C_T values were not only a cycle higher than observed in the NTCs but also in diluted samples. This inhibition appeared to be caused by reagent carryover from the extraction process and may be an additional source of inhibition in bone extracts.

The chemistry and reagents used throughout the extraction method may also be a source of inhibition, as seen in the elevated reagent blank IPC C_T values as compared to NTCs. This increase in the IPC C_T from potential reagent carryover is troubling because it makes it more difficult to assess inhibition using this qPCR assay. The increase in IPC C_T of the reagent blank indicates that low levels of inhibition are present, which may be carryover reagents used in the extraction process. In addition, pH may be a factor, not only in the binding of DNA to the column, but also in that any reagent carryover that alters the pH of the final eluted DNA may affect the pH of the qPCR reaction and performance of the polymerase. It is possible that the bone lysate, including carryover EDTA from the demineralization step, may be affecting the pH of the samples and consequently affecting the binding affinity to the spin column.

Because buffer ATL was generally effective on femur samples but not as much with inhibited rib samples, a chelating agent, EDTA, was added to a final concentration of 10 mM in an attempt to improve its performance. However, the addition of 10 mM EDTA caused a reduction in the effectiveness of buffer ATL, possibly by affecting the pH or also potentially sequestering divalent magnesium cations in the downstream PCR reaction. Divalent magnesium is a critical cofactor for DNA polymerase and with insufficient magnesium present, it will appear as if samples are inhibited. Extremely high levels of inhibition were observed in the neat

samples. However, when samples were diluted 1:10, there was no difference in the recovery of mtDNA between lysis buffers. This indicates that the addition of EDTA is not likely interfering with the ability of the mtDNA to bind to the column, but may be interfering with the magnesium concentration and qPCR.

It is possible that the elevated C_T values observed in reagent blanks may be from EDTA carryover from the demineralization process. However, no difference was observed in the reagent blank IPC C_T values when different lysis buffers were used. More washes may be necessary after the demineralization incubation. If this inhibition is the result of EDTA carryover from the demineralization step, this could be ruled out by running a reagent blank that does not undergo the demineralization incubation step.

3.1.2. Pulverization Method Effects on Inhibition

Preliminary data with lysis buffers suggested that inhibition was the cause of the variation observed in many samples. In this study, a slight difference in lysis buffer effectiveness was observed in samples with low levels of inhibition. Through these experiments, a slight discoloration of the bone powder was sometimes observed, and what appeared to be metal debris was present in the spin column following purification. It was suspected that metal that chipped off of the stainless steel end caps and impactor bar during pulverization in the freezer mill may be causing some inhibition in samples. Polycarbonate end caps and impactor bar were utilized in an attempt to remove this potential source of inhibition.

The use of stainless steel end caps and impactor bar with the freezer mill for the pulverization of bone fragments into a powder was found to introduce PCR inhibitors into the bone powder that remained in the bone extract. This inhibition was observed as an increase of the IPC C_T relative to the non-template control IPC C_T, as well as a significant decrease in

recovered mtDNA as compared to samples from the same bone material that were pulverized with polycarbonate materials. Therefore, It is possible to avoid this introduction of inhibitors by utilizing polycarbonate end caps and impactor bars in the pulverization process. Chromium and iron were not detected by ICP-OES, so it is inconclusive if they are causing the observed inhibition.

3.1.3. Bone Powder Quantity and Inhibition

mtDNA extracts from bone often contain PCR inhibitors from the environment, the extraction process, and even from the bone itself. Divalent calcium ions from the mineral matrix of osseous tissue are known to inhibit PCR; however, it is unknown if calcium is present in the mtDNA extract after the entire extraction process and is therefore causing inhibition. In an attempt to reduce the levels of inhibition observed in samples believed to be caused by calcium, the sample input of bone powder for each extraction was reduced by one half, from 0.1 g to 0.05 g. If the inhibition occurring in samples was in fact due to the mineral matrix, in particular, divalent calcium ions, then it was expected that decreasing the ratio between bone powder to chelating agent in the demineralization incubation would reduce inhibition seen in samples. The results showed no difference in the levels of inhibition detected by the IPC $C_{T,}$ and no difference in the recovery of mtDNA per gram of bone material. This further suggests that calcium is not a major source of inhibition in the samples that were processed in this study.

3.1.4. Assessment of Inhibition by Calcium with ICP-OES

Samples processed with either stainless steel or polycarbonate were both quantified with qPCR to assess the levels of inhibition and then analyzed with ICP-OES to measure the amount of calcium present. Even though the qPCR results indicated extremely high levels of inhibition in samples processed with stainless steel and less inhibition in samples processed with

polycarbonate, the quantities of calcium measured in both samples were similar, further suggesting that the stainless steel is a key source of inhibition, more so than the previously suspected divalent calcium. However, because inhibition was still detected in samples processed with polycarbonate, the stainless steel is evidently not the only source of PCR inhibitors in bone tissue.

When additional samples from a human rib were processed and then quantified, the qPCR data indicated moderate levels of inhibition, although much less than that observed from the previous rib studied. When compared to the samples processed from a different rib, the recovered quantities of mtDNA were different. However, the more inhibited samples contained 1.334 ppm (0.033 mM) of calcium and the less inhibited samples contained 1.962 ppm of calcium (0.049 mM). The inhibition assay performed with $CaHPO_4$ showed a 17% decrease in mtDNA recovery at a concentration of 33.6 μM. This suggests that calcium, although present in samples and likely contributing to some inhibition observed, is not the major contributing source of inhibition in purified mtDNA extracts from bone using these extraction methods.

3.2. A Preliminary Look at Different Purification Systems

No significant difference in mtDNA recovery was observed at the 95% confidence level when Prepfiler® was used to purify the mtDNA from bone lysate and compared side by side with QIAamp®. However, Prepfiler® has its own manufacturer protocol for processing bone samples and the use of it in the extraction method used in this study may not have been optimized for bone samples. It is not possible to properly assess the performance of the Qiagen QIAamp® spin columns and Prepfiler® magnetic bead-based purification systems until the conditions for each have been optimized for bone tissue. It was observed that the IPC C_T of the reagent blanks of samples processed with Prepfiler® were one cycle threshold lower on average

than those processed with QIAamp®. In addition, the sample IPC C_T values for samples processed with Prepfiler® were also on average one cycle lower than those processed with QIAamp®. The bead-based system may be more efficient in the removal of inhibitors of PCR. On average, the quantity of recovered mtDNA from samples processed with Prepfiler® was significantly lower than that observed with samples processed with QIAamp® at the 95% confidence level; however, it may be possible to improve the performance of Prepfiler® through modifications of the method used in this study. Because Prepfiler® was more effective in the removal of inhibitors than QIAamp®, additional work with Prepfiler® should be considered.

3.3. Variation Caused by Kit Chemistry, pH, and Ionic Strength

In addition, adjusting the volume of buffer AL added to bone samples should be evaluated in an effort to enhance mtDNA recovery and removal of inhibitors with the use of Qiagen QIAamp® spin column purification system. With DNA extraction from bone, the increased substrate volume (300 µL from the lysis reaction in addition to the volume of the bone material in the reaction after the demineralization step) may be rendering the 300 µL of buffer AL insufficient in maintaining proper pH and ionic strength for the DNA to bind efficiently to the spin column. The bone material itself may be affecting the pH of the reaction and consequently the ability of the DNA to bind to the spin column. Previous studies have shown that reagents added to the bone material during the lysis incubation, such as EDTA and DTT, have been shown to raise the pH and affect DNA binding (Dukes et al. 2012). Adjusting the volume of buffer AL may be enough to lower the pH as well as maintain a more optimal ionic strength for DNA binding in the presence of the chaotropic salts. Inhibition of the IPC was reduced in a rib sample that was treated with approximately three times the suggested volume of buffer AL. This may have occurred because the altered salt concentration and lower pH affected

the retention of proteins and other inhibitors from the column.

The quantities of HL60 control DNA added to the purified and quantified bone extract showed no signs of inhibition in the presence of bone powder extract. However, the bone extract itself does contain inhibitors, as seen in the quantification data and elevated IPC C_T values. This could indicate that the mtDNA already present in the extract buffered the added HL60 from the effects of inhibitors. This dosage effect suggests a possible inhibitor mechanism of binding to or interacting directly with the bone-derived mtDNA.

3.4. Nuclear DNA Quantification from Bone Samples

Nuclear DNA quantified from bone samples yielded results similar to those observed with mtDNA quantification. Dilution studies with mtDNA from the same human rib suggest that when samples were diluted, no significant difference in DNA recovery was observed regardless of whether the samples were processed with stainless steel or polycarbonate. This suggests that inhibition is still occurring in the samples although not detected by a relative increase in the IPC C_T.

3.5. Future Directions for Research

The process of optimizing a DNA extraction method for bone material should be attempted from the bottom up, beginning with the sampling technique and procedure. The quantities of mtDNA recovered from different bone samples varied considerably based upon where the sampling was performed within the bone itself. In the rib samples and femur samples the mtDNA recovery varied by several orders of magnitude based on where the bone was cut. Unfortunately, this phenomenon was not anticipated and locations of the bone where samples were taken were not catalogued thoroughly. Moreover, previous work has shown inconsistent results as to determining optimum sampling sites as mtDNA concentration within bone tissue

can be hard to predict. The densest bone does not necessarily always yield the most amplifiable mtDNA (Barta et al. 2014). The recovery of mtDNA from bone varies considerably within the same bone based on sampling. In challenging bone samples, it may not always be feasible to select an optimum sampling site, such as with very small fragments of bone. The lack of consistency in predicting optimum sampling sites also makes this difficult.

Inhibitors present in purified mtDNA extract from bone can be due to the structure and composition of the bone, from contamination from the pulverization process, as well as potentially from carryover of reagents used in the extraction process. In addition, inhibition may be introduced from the environment in which the bone is recovered. These studies have been performed on bones that were obtained commercially, and hence were not exposed to soil, contamination, or harsh chemical treatment (cleaned with dermestid beetle maceration followed by hydrogen peroxide bleaching). Hence, these results provide insight into the sources of inhibition from the bone material itself and/or the extraction process, and not added from the environment. This is critical to elucidate which inhibitors are inherently present in bone material.

Calcium, while present in extracts, is not likely a significant source of inhibition in the samples. Stainless steel has been observed to increase the levels of inhibition detected and therefore the use of polycarbonate is highly recommended to decrease inhibition in samples. Still, it is likely that there are additional sources of inhibition, and it is may be important to consider the organic content of bone tissue. Collagenases could be introduced into the lysis incubation, in addition to increasing the quantity of proteinases utilized.

The greatest challenge that occurred in this work was dealing with the presence of PCR inhibitors. The focus of this work shifted from optimizing the use of different kit based

chemistries to elucidating the types of inhibitors present that will affect downstream amplification. Because the bones used in this study were gently cleaned (beetle maceration) and not exposed to harsh environmental conditions, it was possible to gain insight as to the sources of inhibition from the bone itself as well as from the methods used to extract the mtDNA.

The data collected in this study suggests that calcium, although present and detectable in mtDNA extracts from bone, is not likely a significant source of PCR inhibitors in bone samples. Increasing the ratio between EDTA and bone powder was ineffective in enhancing mtDNA recovery via the removal of inhibitors. Furthermore, in the preliminary data collected using the ICP-OES, there was not an apparent relationship between calcium concentration and the levels of inhibition observed.

Additional substances naturally occurring in bone can be evaluated by the addition of proteases, possibly increasing the concentration of proteinase K added to the reaction or the addition of a collagenase. Collagen is highly prevalent in bone tissue and will not be removed during the demineralization step. Increasing the ratio between bone powder and EDTA failed to decrease the inhibition observed, which may suggest that any inhibitors found in the bone powder are organic in nature. Because the samples used in that experiment were pulverized with polycarbonate, stainless steel carryover was clearly not a source of inhibition present.

The reagents used throughout the extraction process are also a source of low levels of PCR inhibitors. This was observed in each experiment by assessing the IPC C_T of the reagent blanks. The IPC C_T values of the reagent blanks were consistently one cycle higher relative to the NTC IPC C_T values, which is a difference on a scale of an order of magnitude. Even in fairly robust bone samples, such as many femur cross section samples, this increase in the IPC C_T was observed. Because of the large volume and high concentration of EDTA used in the

pulverization step, it is possible that some residual EDTA carried over into the purified mtDNA extract. In addition, EDTA or EGTA is also present in two of the lysis buffers used in this work. EDTA can inhibit DNA polymerase by sequestering Mg^{2+}, which is a necessary cofactor. However, it is less likely that EDTA from the lysis buffer is carrying over to the purified extract and more likely from the demineralization step, which is rich in EDTA. This is likely due to the fact that reagent blanks for samples processed with lysis buffer SEB, BTA, and ATL showed no difference in the amount of inhibition observed of the IPC C_T. Should EDTA or EGTA from the lysis buffers SEB or BTA, respectively, be carried over, then one might expect that greater inhibition should have been observed in the reagent blanks.

The increase in volume of Qiagen buffer AL in the beginning of the extraction step may be key in enhancing mtDNA recovery. The increased mtDNA recovery as well as the decrease in the IPC C_T, suggests that the conditions for the mtDNA to bind to the silica spin column may be suboptimal for this method. The large volume of EDTA may affect the pH, as may the DTT or proteinase K. It is possible that increasing the volume of buffer AL brought the pH back to the optimum level. Previous work from Dukes et al. suggested that pH was a factor in their work, in which the addition of sodium acetate lowered the pH and enhanced the mtDNA recovery (2012). This should be explored further in future work. In addition, the larger sample volume may be diluting the chaotropic salts needed to facilitate binding to the column. Adding a greater volume of buffer AL may have raised the ionic strength of the solution to the appropriate concentration required for optimum binding.

WORKS CITED

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. 1981. Sequence and organization of the human mitochondrial genome. Nature. [Internet]. [cited 2014 April 10]. 290(5806):457-465. Available from: http://www.researchgate.net/publication/232762992_Sequence_and_organization_of_the_human_mitochondrial_genome doi: 10.1038/290457a0

Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. 1999. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet. [Internet]. [cited 2014 April 10]. 23(2):147. Available from: http://www.nature.com/ng/journal/v23/n2/full/ng1099_147.html doi:10.1038/13779

Alaeddini R. 2012. Forensic implications of PCR inhibition—a review. Forensic Sci Int Genet. [Internet]. [cited 2014 February 2]. 6(3):297–305. Available from: http://www.sciencedirect.com/science/article/pii/S1872497311001797 doi:10.1016/j.fsigen.2011.08.006

Alaeddini R, Walsh SJ, and Abbas A. 2010. Forensic implications of genetic analyses from degraded DNA—a review. Forensic Sci Int Genet. [Internet]. [cited cited 2014 Feb 2]. 4(3):148–157. Available from: http://www.sciencedirect.com/science/article/pii/S1872497309001483 doi:10.1016/j.fsigen.2009.09.007

Bandelt HJ, Brandstatter AK, Richards MB, Yao YG, Logan I. 2014. The case for the continuing use of the revised Cambridge Reference Sequence (rCRS) and the standardization of notation in human mitochondrial DNA studies. J Hum Genet. [Internet]. [cited 2014 April 10]. 59(2):66-77. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24304692 doi: 10.1038/jhg.2013.120

Banfalvi G. 1986. Structural organization of DNA. Biochem Educ. [Internet]. [cited 2015 May 10]. 14(2):50-59. Available from http://onlinelibrary.wiley.com/doi/10.1016/0307-4412(86)90060-9/abstract doi: 10.1016/0307-4412(86)90060-9

Barta JL, Monroe C, Crockford SJ, Kemp BM. 2014. Mitochondrial DNA preservation across 3000-year-old northern fur seal ribs is not related to bone density: Implications for forensic investigations. Forensic Sci Int. [Internet]. [cited 2015 July 6]. 239(2014);11-18. Available from: http://public.wsu.edu/~bmkemp/publications/pubs/Barta%20et%20al%202014-FSI.pdf

Berglund EC, Kiialainen A, and Syvanen AC. 2011. Next-generation sequencing technologies and applications for human genetic history and forensics. Investig Genet. [Internet]. [cited 2014 Feburary 3]. 2:23. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3267688/ doi: 10.1186/2041-2223-2-23

Bessettii J. 2007. An introduction to PCR inhibitors [Internet]. Madison (WI): Promega Corporation; [cited 2015 May 15]. Available from: http://www.promega.com/~/media/files/resources/profiles%20in%20dna/1001/an%20introduction%20to%20pcr%20inhibitors.pdf http://www.promega.com/~/media/files/resources/profiles%20in%20dna/1001/an%20introduction%20to%20pcr%20inhibitors.pdf

Boss CB and Fredeen KJ. 2004. Concepts, instrumentation and techniques in inductively coupled plasma optical emission spectrometry third edition [Internet]. Shelton (CT): Perkin Elmer®; [cited 2015 May 24]. Available from http://www.perkinelmer.com/CMSResources/Images/44-159043GDE_Concepts-of-ICP-OES-Booklet.pdf

Budowle B, Wilson MR, DiZinno JA. 1999. Interpretation guidelines for mitochondrial DNA sequencing. Proc Tenth Int Symp Hum Identif.

Butler JM. 2011. Advanced Topics in Forensic DNA Typing: Methodology. Waltham (MA): Academic Press.

Butler JM, Shen Y, McCord BR. 2003. The development of reduced size STR amplicons as tools for analysis of degraded DNA. J Forensic Sci. [Internet]. [cited 2015 May 10]. 48(5):1054-1064. Available from: http://www.cstl.nist.gov/strbase/pub_pres/Butler2003d.pdf

Coble MD, Just RS, O'Callaghan JE, Letmanyi IH, Peterson CT, Irwin JA, Parsons TJ. 2004. Single nucleotide polymorphisms over the entire mtDNA genome that increases the power of forensic testing in Caucasians. Int J Legal Med. [Internet]. [cited 2014 April 10]. 118(3):137–146. Available from: http://cstl.nist.gov/strbase/pub_pres/Coble_IJLM2004.pdf DOI 10.1007/s00414-004-0427-6

Clarke B. 2008. Normal Bone Anatomy and Physiology. Clin J Am Soc Nephrol. [Internet]. 3:S131–S139. [cited 2014 February 3]. Available from: http://cjasn.asnjournals.org/content/3/Supplement_3/S131.full.pdf+html doi: 10.2215/CJN.04151206

Crick F. 1970. Central dogma of molecular biology. Nature. [Internet]. [cited 2015 May 10]. 227:561-563. Available from: http://www.nature.com/nature/focus/crick/pdf/crick227.pdf

Dukes JM, Williams AL, Massey CM, Wojtkiewicz PW. 2012. Technical Note: Bone DNA Extraction and Purification Using Silica-Coated Paramagnetic Beads. Am J Phys Anthropol. [Internet]. [cited 2015 Feb 11]. 148(3):473-482. Available from: http://onlinelibrary.wiley.com/doi/10.1002/ajpa.22057/abstract doi: 10.1002/ajpa.22057

Edson SM, and Christensen AF. 2013. Field Contamination of Skeletonized Human Remains with Exogenous DNA. Forensic Sci. [Internet]. [cited 2014 Feb 2]. 58(1):206–209. Available from: http://onlinelibrary.wiley.com/doi/10.1111/j.1556-4029.2012.02270.x/abstract doi: 10.1111/j.1556-4029.2012.02270.x

Esser KH, Marx WH, Lisowsky T. 2006. MaxXbond: first regeneration system for DNA binding silica matrices. Nature Method. [Internet]. [cited 2014 Feb 2]. 3(1):1–2. Available from: http://www.nature.com/nmeth/journal/v3/n1/full/nmeth845.html doi:10.1038/nmeth845

Farrell BS, Best M, Brown R, Richardson T, Sennette LA, Steele A, Weissman C, Westheimer A, Willems M. 2013. DOD's POW/MIA mission: top-level leadership attention needed to resolve longstanding challenges in accounting for missing persons from past conflicts. GAO-13-619 Missing Persons. United States Government Accountability Office Report to Congressional Committees. [Internet]. [cited 2015 April 4]. Available from: http://www.gao.gov/products/gao-13-619

Farell EM, Alexandre G. 2012. Bovine serum albumin further enhances the effects of organic solvents on increased yield of polymerase chain reaction of GC-rich templates. BMC Res Notes. [Internet]. [cited 2015 May 9]. 5:257. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3466135/ doi: 10.1186/1756-0500-5-257

(FBI) Federal Bureau of Investigation. (US). 2010. DNA Analysis Unit II Mitochondrial DNA Analysis Protocol [Print]. Quantico (VA): Federal Bureau of Investigation (US); [cited 2014 April 8].

Fernández-Martínez R, Caballero R, Guillen C, Gutierrez MT, Rucandio MI. 2005. Application of ICP-OES to the determination of CuIn(1−x)Ga(x)Se2 thin films used as absorber materials in solar cell devices. Anal Bioanal Chem. [Internet]. [cited 2015 March 22]. 382(2):466-470. Available from: http://link.springer.com/article/10.1007%2Fs00216-004-2997-z

Filippini G. 2010. Human Skeletal Remains: Development of DNA Extraction and Typing Methods [PhD thesis]. University of Verona. [Internet]. [cited 2014 Feb 2]. Available from: http://www.univr.it/documenti/AllegatiOA/allegatooa_5790.pdf

Frackman S, Kobs G, Simpson D, Storts D. 1998. Betaine and DMSO: enhancing agents for PCR. Promega Notes. [Internet]. [cited 2015 May 9]. 65:27. Available from: http://www.promega.com/~/media/files/resources/promega%20notes/65/betaine%20and%20dmso-%20enhancing%20agents%20for%20pcr.pdf

(NLM) US National Library of Medicine®. 2015. Genetics Home Reference: What is DNA? [Internet]. Bethesda (MD): National Institutes of Health (US); [cited 2015 May 12]. Available from: http://ghr.nlm.nih.gov/handbook/basics/dna

Gilbert MT, Willerslev E, Hansen AJ, Barnes I, Rudbeck L, Lynnerup N, Cooper A. 2003. Distribution patterns of postmortem damage in human mitochondrial DNA. Am J Hum Genet. [Internet]. [cited 2014 Feb 3]. 72(1):32-47. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC420011/ doi: 10.1086/345378

Gong JK, Arnold JS, Cohn SH. 1964. Composition of trabecular and cortical bone. Anat Rec. [Internet]. [cited 2014 July 8]. 149(3):325-331. Available from: http://onlinelibrary.wiley.com/doi/10.1002/ar.1091490303/abstract DOI: 10.1002/ar.1091490303

Ha¨nni C, Brousseau T, Laudet V, Stehelin D. 1995. Isopropanol precipitation removes PCR inhibitors from ancient bone extracts. Nucleic Acids Res. [Internet]. [cited 2015 May 9]. 23(5):881-882. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC306775/

Heid CA, Stevens J, Livak KJ, Williams PM. 1996. Real time quantitative PCR. Genome Res. [Internet]. [cited 2015 May 10]. 6(10):986-994. Available from: http://genome.cshlp.org/content/6/10/986.long doi: 10.1101/gr.6.10.986

Hellmann A, Rohleder U, Schmitter H, Wittig M. 2001. STR typing of human telogen hairs – a new approach. Int J Legal Med. [Internet]. [cited 2015 May 15]. 114(4-5):269-273. Available from: http://download.springer.com/static/pdf/769/art%253A10.1007%252Fs004140000175.pdf?originUrl=http%3A%2F%2Flink.springer.com%2Farticle%2F10.1007%2Fs004140000175&token2=exp=1433793563~acl=%2Fstatic%2Fpdf%2F769%2Fart%25253A10.1007%25252Fs004140000175.pdf%3ForiginUrl%3Dhttp%253A%252F%252Flink.springer.com%252Farticle%252F10.1007%252Fs004140000175*~hmac=6bd73d15c286b5c5263bf1985f94a83eb76c9ab64d1354047b50d5d0f20d457b

Henke W, Herdel K, Jung K, Schnorr D, Loening, SA. 1997. Betaine improves the PCR amplification of GC - rich DNA sequences. Nucleic Acids Res. [Internet]. [cited 2015

May 11]. 25(19):3957-3958. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC146979/pdf/253957.pdf

Holland MM, Parsons TJ. 1999. Mitochondrial DNA sequence analysis – validation and use for forensic casework. Forensic Sci Rev. [Internet]. [cited 2014 Feb 8]. 11(1):21–50. Available from: http://www.mitotyping.com/cms/lib7/PA08000101/Centricity/Domain/9/HollandParsons1999.pdf

Hucklenbroich J, Scherer M, inventors; Qiagen Gmbh, assignee. 2013 Jan 24. Method for selective isolation and purification of nucleic acids. US patent 0023656 A1. [Internet]. [cited 2014 Feb 16]. Available from: http://www.google.com/patents/US20130023656

Kalmár T, Bachrati CZ, Marcsik A, and Raskó I. 2000. A simple and efficient method for PCR amplifiable DNA extraction from ancient bones. Nucleic Acids Res. [Internet]. [cited 2015 May 15]. 28(12):67. Available from: http://www.nature.com/nprot/journal/v2/n7/full/nprot.2007.247.html doi:10.1038/nprot.2007.247

Kavlick MF, Lawrence HS, Merritt RT, Fisher C, Isenberg A, Robertson JM, Budowle B. 2011. Quantification of human mitochondrial DNA using synthesized DNA standards. J Forensic Sci. [Internet]. [cited 2014 Feb 16]. 56(6):1457-1463. Available from: http://onlinelibrary.wiley.com/doi/10.1111/j.1556-4029.2011.01871.x/abstract DOI: 10.1111/j.1556-4029.2011.01871.x

King CE, Debryune R, Kuch M, Schwarz C, Poinar H. 2009. A quantitative approach to detect and overcome PCR inhibition in ancient DNA extracts. Biotechniques. [Internet]. [cited 2015 May 9]. 47(5):941-949. Available from: http://www.biotechniques.com/multimedia/archive/00072/BTN_A_000113244_O_72782a.pdf doi 10.2144/000113244

Kreader CA. 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl Environ Microbiol. [Internet]. [cited 2015 May 9]. 62(3):1102–1106. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC167874/pdf/621102.pdf

Lindahl T. 1993. Instability and decay of the primary structure of DNA. Nature. [Internet]. [cited 2014 Feb 2]. 362(6422):709–715. Available from http://www.nature.com/nature/journal/v362/n6422/abs/362709a0.html doi:10.1038/362709a0

Loreille OM, Diegoli TM, Irwin JA, Coble MD, Parsons TJ. 2007. High efficiency DNA extraction from bone by total demineralization. Forensic Sci Int Genet. [Internet]. [cited

2014 Feb 7]. 1(2):191-195. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1872-4973(07)00063-4 doi: 10.1016/j.fsigen.2007.02.006.

Melton T. 2014. Digging Deep: Next Generation Sequencing for Mitochondrial DNA Forensics. Forensic Magazine [Internet]. [cited 2014 May 28]. Available from http://www.forensicmag.com/articles/2014/01/digging-deep-next-generation-sequencing-mitochondrial-dna-forensics

Melton T, Holland C, Holland M. 2012. Forensic Mitochondrial DNA Analysis: Current Practice and Future Potential. Forensic Sci Rev. [Internet]. [cited 2014 May 28]. 24:101-22. Available from http://www.mitotyping.com/cms/lib7/PA08000101/Centricity/Domain/1/FSR_Paper,_2012-2.pdf

Miloš A, Selmanovic A, Smajlovic L, Huel RL, Katzmarzyk C, Rizvic A, Parsons TJ. 2007. Success rates of nuclear short tandem repeat typing from different skeletal elements. Croat Med J. [Internet]. [cited July 2 2015]. 48:486–493. Available from http://www.scopus.com/record/display.url?eid=2-s2.0-34548218334&origin=inward&txGid=0A8A37185F92219E0777CEDE6E28639D.FZg2ODcJC9ArCe8WOZPvA%3a19

Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol. [Internet]. [cited 2015 May 16]. 1:263-273. Available from http://sites.chapman.edu/2014fao-biol-208-lab/files/2014/09/mullis_etal_cshl_1986-15gkz13.pdf

Mundorff A, Bartelink EJ, Mar-Cash E. 2009. DNA Preservation in skeletal elements from the World Trade Center Disaster: recommendations for mass fatality management. J Forensic Sci. [Internet]. [cited on 2015 July 2]. 54(4);739-745. Available from http://onlinelibrary.wiley.com/doi/10.1111/j.1556-4029.2009.01045.x/epdf

NCI (National Cancer Institute) (US). [Date unknown]. SEER Training Modules. Structure of bone tissue [Internet]. Bethesda (MD): US National Institutes of Health; [Cited 2014 Feb 5]. Retrieved from http://training.seer.cancer.gov/anatomy/skeletal/tissue.html

NLM (US National Library of Medicine®). 2015. Genetics home reference: what is DNA? [Internet]. Bethesda (MD): National Institutes of Health (US); [cited 2015 May 13]. Available from http://ghr.nlm.nih.gov/handbook/basics/dna

Opel KL, Chung D, McCord BR. 2010. A Study of PCR inhibition Mechanisms Using Real Time PCR. J Forensic Sci. [Internet]. [cited 2014 July 13]. 55(1):25-33. Available

from http://sites.chapman.edu/2014fao-biol-208-lab/files/2014/09/mullis_etal_cshl_1986-15gkz13.pdf

SPEX® SamplePrep. 2008. Operating manual – 6770 Freezermill®. [Internet]. [cited 2015 March 5]. Available from http://www.gbcpolska.pl/ul/mlynki/pdfy/6770_manual_version_6_06_en.pdf

Pääbo S. 1989. Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proc Natl Acad Sci USA. [Internet]. [cited 2014 Feb 2]. 86(6):1939–1943. Available from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC286820/

Perkin Elmer®. 2013. Atomic spectroscopy: a guide to selecting the appropriate technique and system. [Internet]. [cited 2015 July 3]. Available from http://www.perkinelmer.com/CMSResources/Images/44-74482BRO_WorldLeaderAAICPMSICPMS.pdf

Prinz M, Carracedo A, Mayr WR, Morling N, Parsons TJ, Sajantila A, Scheithauer R, Schmitter H, Schneider PM. 2007. DNA Commission of the International Society for Forensic Genetics (ISFG): Recommendations regarding the role of forensic genetics for disaster victim identification (DVI). Forensic Sci Int Genet. [Internet]. [cited 2015 July 2]. 1(1);3-12. Available from http://www.sciencedirect.com/science/article/pii/S1872497306000032

NLM (US National Library of Medicine®). 2014. Polymerase chain reaction (PCR) [Internet]. Bethesda (MD): National Center for Biotechnology Information; [cited 2015 May 12]. Available from http://www.ncbi.nlm.nih.gov/probe/docs/techpcr/

Life Technologies™. 2015. qPCR vs. Digital PCR vs Traditional PCR. [Internet]. [cited 2015 May 11]. Available from http://www.lifetechnologies.com/us/en/home/life-science/pcr/real-time-pcr/qpcr-education/qpcr-vs-digital-pcr-vs-traditional-pcr.html

Life Technologies™. 2012. PrepFiler® and PrepFiler® BTA Forensic DNA Extraction Kits. [Internet]. [cited 2014 April 3]. Available from https://www3.appliedbiosystems.com/cms/groups/applied_markets_support/documents/generaldocuments/cms_096102.pdf

Life Technologies™. 2011. PrepFiler® BTA Lysis Buffer Safety Data Sheet. [Internet]. [cited 2014 April 3]. Available from https://tools.lifetechnologies.com/content/sfs/msds/2011/4463559_MTR-NALT_EN.pdf.

Qiagen®. 2012. QIAamp® DNA Mini and Blood Mini Handbook. [Internet]. [cited 2014 April 3]. Available from https://www.qiagen.com/us/resources/resourcedetail?id=67893a91-946f-49b5-8033-394fa5d752ea&lang=en

Qiagen®. 2013. Material Safety Data Sheet Buffer ATL. [Internet]. [cited 2014 March 11]. Available from http://www.qiagen.com/data/Support/MSDS/US/Buffer_ATL_US7.pdf

Ramanaji M. 2014. Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) [Internet]. Hyderabad (TS):Centre for Materials and Electronics Technology (IN); [Cited 2015 May 21]. Available from http://www.rohs-cmet.in/content/icp-oes.dd

Reeve MA, inventor; Amhersham International plc, assignee. 1997 Oct 28. Precipitating polymers. US patent 5,681,946 C1. [Internet]. [cited 2014 April 3]. Available from https://patentimages.storage.googleapis.com/pdfs/US5681946.pdf

Ritter N. 2007. Missing persons and unidentified remains: the nation's silent mass disaster. NIJ J. [Internet]. [cited 2015 May 20]. 256:2-7. Available from https://www.ncjrs.gov/pdffiles1/nij/jr000256.pdf

Saiki R, Gelfand D, Stoffel S, Scharf S, Higuchi R, Horn G, Mullis K, Erlich H. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Sci. [Internet]. [cited 2015 May 17]. 239(4839): 487–491. Available from http://www.mun.ca/biology/scarr/Saiki,_Mullis_et_al_1988_Science_239,487.pdf

Satoh M, Kuroiwa T. 1991. Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. Exp Cel Res. [Internet]. [cited 2014 April 3]. 196(1):137–140. Available from http://linkinghub.elsevier.com/retrieve/pii/0014-4827(91)90467-9

Scholz M, Giddings I, Pusch CM. 1998. A polymerase chain reaction inhibitor of ancient hard and soft tissue DNA extracts is determined as human collagen type I. Anal Biochem. [Internet]. [cited 2015 May 9]. 259(2):283-286. Available from http://ac.els-cdn.com/S0003269798926768/1-s2.0-S0003269798926768-main.pdf?_tid=1e78b768-0e18-11e5-b781-00000aab0f6c&acdnat=1433793425_b65797395 db1bd9f495a4c34b8b8dcd1 doi:10.1006/abio.1998.2676

Takahashi M, Kato Y, Mukoyama H, Kanaya H, Kamiyama S. 1997. Evaluation of five polymorphic microsatellite markers for typing DNA from decomposed human tissues-correlation between the size of the alleles and that of the template DNA. Forensic Sci. Int. [Internet]. [cited 2014 Feb 2]. 90(1–2):1–9. Available from http://www.sciencedirect.com/science/article/pii/S0379073897001291 doi:10.1016/S0379-0738(97)00129-1

Tilstone B. 2007. DNA Analyst Training [Internet]. Largo (FL): NFSTC Science Serving Justice® (US); [cited 2014 April 3]. Available from http://projects.nfstc.org/pdi/

Thompson SL, McColl SM, Manning FCR. 2000. Detection and quantitation of chromium induced DNA damage using PCR. Biochem Soc T. [Internet]. [cited 2015 July 2]. 28(5);A169. Available from:

Vallone PM, Hill CR, Butler JM. 2008. Demonstration of rapid multiplex PCR amplification involving 16 genetic loci. Forensic Sci Int Genet. [Internet]. [cited 2015 May 15]. 3(1):42-45. Available from: http://www-06.all-portland.net/bst/028/a169/028a169a.pdf

Vass AA. 2001. Beyond the grave understanding human decomposition. Microbiol Today. [Internet]. [cited 2014 Feb 2]. 28:190-193. Available from http://www.archeo.uw.edu.pl/zalaczniki/upload617.pdf

Watson JD, Crick FHC. 1953. Molecular structure of nucleic acids. Nature. [Internet]. [cited 2015 May 10]. 4356(171):737-738. Available from http://www.nature.com/nature/dna50/watsoncrick.pdf

Weyant RS, Edmonds P, Swaminathan B. 1990. Effect of ionic and nonionic detergents on the Taq polymerase. Biotechniques. [Internet]. [cited 2015 May 9]. 9(3):308–9. Available from http://www.ncbi.nlm.nih.gov/pubmed/2223070

Wilson IG. 1997. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol. [Internet]. [cited 2015 May 9]. 63(10):3741–3751. Available from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC168683/pdf/633741.pdf

Ye J, Ji A, Parra EJ, Zheng X, Jiang C, Zhao X, Hu L, Tu Z. 2004. A simple and efficient method for extracting DNA from old and burned bone. J Forensic Sci. [Internet]. [cited 2015 May 3]. 49(4):754-9. Available from http://www.ncbi.nlm.nih.gov/pubmed/153171902 1487284

APPENDICES

Appendix A: Examining the Effectiveness of Three Difference Lysis Buffers and Interactions with Different Bone Types

Table A1: A comparison of quantities of mtDNA recovered from human femur and human rib using three different lysis buffers

Bone Type	Lysis Buffer	Mean (copies/g)	95% CI (copies/g)
Femur	BTA	1.78×10^7	1×10^7
	SEB	1.98×10^7	9.9×10^6
	ATL	2.58×10^7	1.5×10^7
Bone Type	**Lysis Buffer**	**Mean (copies/g)**	**95% CI (copies/g)**
Rib	BTA	1.42×10^6	2×10^6
	SEB	4.02×10^6	5×10^6
	ATL	5.26×10^5	1×10^6

Appendix B: Comparison of Stainless Steel and Polycarbonate Pulverization Components

Table B1: A compilation of data from different bone types and lysis buffers from samples processed with polycarbonate or stainless steel pulverization components.

Pulverization Component	Mean (copies/g)	95% CI (copies/g)
Stainless Steel	4.62×10^6	1.8×10^6
Polycarbonate	2.07×10^8	2.3×10^7

Table B2: Quantities of mtDNA recovered from human femur using three different lysis buffers and either stainless steel or polycarbonate freezer mill end caps and impactor bar

SPEX component	Lysis Buffer	Mean (copies/g)	95% CI (copies/g)
Polycarbonate	BTA	2.98×10^7	1×10^7
	SEB	3.71×10^7	8×10^6
	ATL	4.95×10^7	2×10^7
SPEX component	**Lysis Buffer**	**Mean (copies/g)**	**95% CI (copies/g)**
Stainless Steel	BTA	8.17×10^6	$4x\ 10^6$
	SEB	8.23×10^6	5×10^6
	ATL	1.00×10^7	6×10^6

Table B3: A comparison of quantities of mtDNA recovered from a large human rib when processed with either polycarbonate or stainless steel and diluted ten-fold

SPEX Component	**Dilution**	**Mean (copies/2 µL)**	**95% CI (copies/2 µL)**
Stainless Steel	Neat	0	0
Polycarbonate	Neat	2,480.64	4×10^2
Stainless Steel	1:10	54.05	1×10^2
Polycarbonate	1:10	178.71	1×10^2

Appendix C: Bone Sample Mass Effects on mtDNA Recovery from Human Femur

Table C1: Mean quantities of recovered DNA from small rib using three different lysis buffers and either 0.1g or 0.05 g bone powder

Mean Sample Mass (g)	Lysis Buffer	Mean (copies/g)	95% CI (copies/g)
0.0506	SEB	3.70×10^7	4×10^6
	BTA	3.24×10^7	8×10^6
	ATL	3.95×10^7	3×10^6
	Total	3.63×10^7	4×10^6
0.0965	SEB	3.71×10^7	1×10^7
	BTA	2.98×10^7	2×10^7
	ATL	4.95×10^7	2×10^7
	Total	3.88×10^7	1×10^7

Appendix D: Dilution of Samples to Observe the Behavior of Lysis Buffers with Inhibitors

Table D1: Observed DNA recovery for femur samples treated with lysis buffer BTA, SEB, or ATL, compared to the 1:10 dilution quantities and the interpolated expected value

Lysis Buffer	Neat Observed (Copies/2 µL)[a]	Neat Expected (Copies/2 µL)[a]	1:10 (Copies/2 µL)	% Difference from Expected Value[b]
BTA	5.57×10^4	4.66×10^4	4.66×10^3	1.64
SEB	9.76×10^4	5.60×10^4	5.60×10^3	4.26
ATL	1.01×10^5	7.38×10^4	7.38×10^3	2.69
Lysis Buffer	**1:2 Observed (Copies/2 µL)[a]**	**1:2 Expected (Copies/2 µL)[a]**	**1:10 (Copies/2 µL)**	**% Difference from Expected Value[b]**
BTA	2.64×10^4	2.33×10^4	4.66×10^3	1.34
SEB	4.65×10^4	2.84×10^4	5.60×10^3	6.60
ATL	4.82×10^4	9.63×10^4	7.38×10^3	3.04

[a]The quantities of mtDNA recovered from the 1:10 were multiplied by ten and five, respectively to estimate the actual quantity of mtDNA present in neat and 1:2 diluted samples
[b]The difference is shown as a percent difference of the observed neat quantity and the expected quantities

Table D2: Observed DNA recovery for large rib samples treated with lysis buffer BTA, SEB, or ATL, compared to the 1:10 dilution quantities and the interpolated expected value.

Lysis Buffer	Neat Observed (Copies/2 µL)	Neat Expected (Copies/2 µL)[a]	1:10 (Copies/2 µL)	% Difference from Expected Value[b]
BTA	2.76×10^3	2.21×10^3	2.21×10^2	24.90
SEB	1.78×10^3	1.97×10^3	1.97×10^2	-9.63
ATL	0	1.98×10^3	1.98×10^2	-100
Lysis Buffer	**1:2 Observed (Copies/2 µL)**	**1:2 Expected (Copies/2 µL)[a]**	**1:10 (Copies/2 µL)**	**% Difference from Expected Value[b]**
BTA	2.85×10^3	1.10×10^3	2.21×10^2	74.21
SEB	3.57×10^3	1.09×10^3	1.97×10^2	67.22
ATL	0	9.91×10^2	1.98×10^2	-100

[a]The quantities of mtDNA recovered from the 1:10 were multiplied by ten and five, respectively to estimate the actual quantity of mtDNA present in neat and 1:2 diluted samples
[b]The difference is shown as a percent difference of the observed neat quantity and the expected quantities

Table D3: Observed DNA recovery for small rib samples treated with one of three lysis buffers compared to the 1:10 dilution quantities and the interpolated expected value

Lysis Buffer	Neat Observed (Copies/2 µL)	Neat Expected (Copies/2 µL)[a]	1:10 (Copies/2 µL)	% Difference from Expected Value[b]
BTA	1.22×10^3	7.09×10^2	7.09×10^1	-72.23
SEB	9.29×10^2	4.76×10^2	4.77×10^1	-95.04
ATL	0	6.11×10^2	6.11×10^1	100

Lysis Buffer	1:2 Observed (Copies/2 µL)	1:2 Expected (Copies/2 µL)[a]	1:2 (Copies/2 µL)	% Difference from Expected Value[b]
BTA	2.18×10^2	3.55×10^2	7.09×10^1	-25.56
SEB	2.64×10^2	2.38×10^2	4.76×10^1	8.50
ATL	1.01×10^2	3.05×10^2	6.11×10^1	66.84

[a]The quantities of mtDNA recovered from the 1:10 were multiplied by ten and five, respectively to estimate the actual quantity of mtDNA present in neat and 1:2 diluted samples

[b]The difference is shown as a percent difference of the observed neat quantity and the expected quantities

Appendix E: Addition of a Chelating Agent to Buffer ATL

Table E1: A comparison of neat and 1:10 diluted mean DNA yields from rib samples using one of four different lysis buffers

Lysis Buffer	Neat Mean (Copies/2 µL)	Neat 95% CI (Copies/2 µL)	1:10 Dilution Mean (Copies/2 µL)	1:10 Dilution 95% CI (Copies/2 µL)
BTA	1.62×10^4	2×10^4	4.14×10^3	2×10^2
SEB	3.04×10^4	6×10^3	5.06×10^3	7×10^2
ATL	6.98×10^3	1×10^4	4.85×10^3	4×10^2
ATL+10 mM EDTA	1.45×10^3	3×10^3	4.41×10^3	1×10^3

Appendix F: Preliminary Work on Comparison of QIAamp® and Prepfiler®

Table F1: Preliminary comparison of DNA recovery from human femur cross sections processed using two different commercial purification systems

Pulverization Component	Lysis Buffer	Purification Method	Mean (copies/g)	95% CI (copies/g)
Stainless Steel	SEB	QIAamp®	9.22×10^6	2×10^6
Stainless Steel	SEB	PrepFiler®	6.93×10^6	2×10^6

Appendix G: Results Inhibition Study

Table G1: Quantities of mtDNA observed when different known quanities of HL60 DNA were added to purified DNA extract.

HL60 (pg/µL)	Observed Sample Mean mtDNA (copies/2µL)	IPC C_T	Expected (copies/2 µL)[a]
10 pg/µL	$1.76 \times 10^3 \pm 1.80 \times 10^3$	28.93 ± 1.71	$1.67 \times 10^4 \pm 2.45 \times 10^3$
1.67 pg/µL	$5.05 \times 10^3 \pm 6.43 \times 10^2$	28.32 ± 0.83	$4.81 \times 10^3 \pm 2.72 \times 10^2$
0 pg/µL	$2.49 \times 10^3 \pm 1.86 \times 10^2$	27.90 ± 0.15	$1.97 \times 10^3 \pm 3.08 \times 10^2$
NTC	Undetermined	25.3925 ± 0.125	n/a

[a] Expected quantities were calculated as the quantity of DNA from the positive control added to the mean of the samples containing 0 pg/µL of HL60

Appendix H: Nuclear DNA Quantification from Human Rib and Human Femur

Table H1: Nuclear DNA quantification data from human rib

Pulverization Component	Nuclear target	Buffer	Mean DNA (ng/g)	95% CI (ng/g)
Polycarbonate	Large Autosomal	SEB	0.15	0.1
		BTA	0.64	0.8
		ATL	0.26	0.3
	Small Autosomal	SEB	2.91	2
		BTA	3.44	3
		ATL	4.76	3
	Y	SEB	3.77	2
		BTA	4.52	5
		ATL	6.03	4
Stainless Steel	Large Autosomal	SEB	0.05	0.05
		BTA	0.06	0.1
		ATL	0.07	0.08
	Small Autosomal	SEB	0.25	0.2
		BTA	0.28	0.3
		ATL	0.46	0.5
	Y	SEB	0.22	0.2
		BTA	0.24	0.3
		ATL	0.43	0.5

Table H2: Nuclear DNA quantification data from human femur

Pulverization Component	Nuclear target	Buffer	Mean DNA (ng/g)	95% CI (ng/g)
Polycarbonate	Large Autosomal	SEB	1.79	2
		BTA	1.80	2
		ATL	2.47	3
	Small Autosomal	SEB	8.58	2
		BTA	5.13	2
		ATL	8.30	9
	Y	SEB	6.23	1×10^1
		BTA	3.74	5
		ATL	5.90	6
Stainless Steel	Large Autosomal	SEB	0.74	0.7
		BTA	1.04	0.7
		ATL	0.86	0.5
	Small Autosomal	SEB	3.50	3
		BTA	2.30	0.5
		ATL	3.78	2
	Y	SEB	3.24	2
		BTA	1.92	1
		ATL	3.61	1