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There are approximately 4,400 sets of unidentified human remains recovered each year, nearly a quarter of which are not identified within the year following recovery. Obtaining genetic information through DNA testing of bone samples has become a critical element to identifying missing persons and recovered human remains.

DNA is preserved within the structure of bone for vast amounts of time, surviving environmental and microbial insults, yet bone is one the most challenging sample types encountered by forensic scientists. This is due to the resilient structure of bone and the prevalence and variety of materials which co-isolate with DNA during extraction and function as inhibitors of the polymerase chain reaction (PCR). Bone-associated PCR inhibitors include native components and environmental materials, acquired as a consequence of the porous composition of bone. Quality assurance requirements governing DNA testing laboratories do not mandate direct evaluation of the product of the DNA extraction process; coupled with poor characterization of PCR inhibitors, the forensic community has not adequately demonstrated the efficiency of methods used to extract DNA from bone samples. The primary hypothesis is failure of PCR-based testing of DNA from skeletal remains is frequently encountered due to inefficient extraction methods and PCR inhibition.

This dissertation project has: 1) demonstrated an approach for identifying and characterizing putative PCR inhibitors, emphasizing those originating from the mineral

contents of bone; and, 2) assessed the efficiency of current methods used for extracting DNA from bone samples, in terms of quality and quantity of the recovered template. Control genomic DNA, bone samples from adjudicated forensic cases obtained from the University of North Texas Center for Human Identification, and cadaver bone samples obtained from the Willed Body Program at University of North Texas Health Science Center were used for experiments. Laboratory experiments included: DNA extraction, analysis of DNA fragmentation, quantification of DNA, amplification of short tandem repeat (STR) forensic loci, genetic analysis, and elemental analyses that were conducted in collaboration with the University of North Texas Department of Chemistry and Forensic Science Program.

PHYSICAL AND BIOCHEMICAL FACTORS AFFECTING

THE RECOVERY AND ANALYSIS OF DNA

FROM HUMAN SKELETAL REMAINS

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PHYSICAL AND BIOCHEMICAL FACTORS AFFECTING THE RECOVERY AND ANALYSIS OF DNA FROM HUMAN SKELETAL REMAINS

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INTRODUCTION

Statement of the Problem

More than twenty years of scientific advancements have supported the continuous improvement of laboratory procedures used for testing deoxyribonucleic acid (DNA) obtained from skeletal remains; this has led to human identification efforts that successfully obtain full DNA profiles for a large percent of samples tested. Despite these improvements, no human identification project to date has achieved 100 percent success on all skeletal remains tested. Existing evidence demonstrates DNA is preserved within skeletal remains, long after the soft tissues of the body have decomposed, even though the microscopic structure and chemical composition of the bone become significantly altered by the burial environment [1-4]. Native components of bone, such as calcium and collagen, and materials introduced into the bone from exposure to the burial environment can be co-isolated with DNA during routine laboratory processing and function as inhibitors of polymerase chain reaction (PCR)-based DNA testing [5-6]. Published works that aim to demonstrate the efficiency of a DNA extraction method or make comparisons between multiple methods often use bone samples that are of non-human origin, human samples that have been exposed to unknown variables, or forensic casework samples. The use of nonspecific, highly-variable samples make it impossible to determine if poor PCR-based results are due to initial sample quality or if routine extraction methods fail to adequately isolate DNA, contribute to

template degradation, or co-isolate sufficient non-DNA materials to inhibit PCR. Controlled studies, using standard materials and human bone samples exposed to known variables, will help develop methods that optimize recovery and purification of DNA. These studies will provide the scientific foundation necessary for the future development of protocols, specific to human bone samples, to maximize the DNA recovery from bone samples and improve the percent of full profiles obtained.

Human Genetic Identification

Forensic applications of DNA typing was first reported in 1985 using DNA extracted from bloodstains, vaginal samples, and sperm, and by 1991, the forensic DNA community was performing DNA extractions from human skeletal remains for identification purposes [7-8]. Short tandem repeat (STR) markers were first characterized for identification applications, and demonstrated to be consistent between different tissue types within a single individual [9]. These markers became the foundation for contemporary human genetic identification applications, both in routine forensic casework and in cases of missing persons or identified human remains.

Forensic DNA testing consists of several steps: isolation of DNA, quantification of DNA, amplification of DNA markers used for testing, analysis of genetic DNA, and analysis of the typing results, developing what is referred to as a "DNA profile." The objective of DNA isolation is to extract the DNA from the biological sample; this often includes purification and concentration of the DNA molecules [7]. Quantification of the amount of DNA in a sample is important, as the chemistry kits used to amplify DNA markers work best when the DNA is present at a specified concentration range for the system [10]. Amplification of genetic markers is accomplished using the polymerase chain reaction (PCR), which targets the markers of interest within DNA molecules, copies them exponentially and labels each copied DNA fragment with a fluorescent tag [11]. This

enables detection during genetic analysis, when DNA fragments are separated by capillary electrophoresis (CE) due to differences in size and charge of the molecule [11]. Data analysis, which separates the DNA fragments of similar sizes by the fluorescent label, allows the development of the DNA profile, which is the collection of the results obtained at the markers targeted during amplification [11].

Laboratories performing forensic DNA testing in the U.S. are required to comply with a set of standards that govern all aspects of laboratory operations and outline the critical elements to include in their quality assurance program. These *Quality Assurance Standards for Forensic DNA Testing Laboratories* (QA Standards) define methodology as "the analytical processes and procedures used to support a DNA typing technology" and specifically include those used for extraction. Methods are to be evaluated through validation studies, so that the efficacy and reliability are known, before being used in forensic casework analysis [12]. The measure by which these standards are applied to DNA extraction has generally been limited to results obtained from PCR-based DNA tests.

One of the challenges in working with evidentiary materials is the lack of knowledge of the sample history and exposure to variables that can affect the quality of a sample, prior to DNA extraction. Because knowledge of the existence of these challenges is prevalent, the QA Standards require stability studies to be performed during validation to ensure that the results of testing will not change as a consequence of environmental exposure degradation, or the presence of non-DNA materials that co-isolate with DNA and may inhibit PCR-based DNA testing. Many of the existing PCR inhibition studies are completed during the course of validating of multiplex amplification systems (or kits) commonly employed in human genetic identity testing. These studies provide experimental data to evaluate the performance of the multiplex amplification kit and its respective

buffering system to a limited selection of PCR inhibitors. The design of these studies varies greatly between manufacturers, even between different multiplex amplification systems produced by a single manufacturer, and virtually no scientific evidence is presented that supports the biological relevance of the range of concentrations employed for any particular inhibitor [10,13-14]. The results of these studies are presented without reference to their biological context, as most PCR inhibitors have not been characterized in a manner that demonstrates a relevant range of concentrations at which they co-isolate with DNA.

DNA Recovery from Human Skeletal Remains

Human skeletal remains have been shown to preserve DNA for more than 7,000 years [15]. The remarkable preservation of DNA is credited to the complex structure of the bone matrix, capable of protecting it from a variety of chemical and enzymatic diagenic processes that would eventually lead to degradation of the DNA molecule [16]. This structure also makes bone one of the most challenging biological sample types, both in terms of its structural stability and its ability to acquire materials that can inhibit the polymerase chain reaction (PCR) [6,15-17].

Bone is a composite sample matrix with a hierarchical physical structure. The basic structural components of bone are the Type I collagen fibrils; these are comprised of heterogeneous triple-helix proteins that contain and are surrounded by plate-like hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) mineral crystals. These 1.5-3.5 nm fibrils cluster to form larger arrangements of collagen fibers (50-70 nm) and bundles (150-250 nm). The collagen fibers first form primary (also, non-lamellar or woven) bone during development and after injury, characterized by random orientation of the collagen fibers. These fibers eventually become arranged in parallel layers or sheets, called lamellae, in secondary (also, lamellar or mature) bone. The lamellae arrange to form higher order microstructures and interstitial lamellae, the combination of which forms the

observable macrostructure of bone [18]. The layered lamellae also provide housing for the osteocytes, the most likely source of DNA from bone samples. Osteocytes are present at approximately ten-fold the number of the next most prominent cell; during development they become trapped within the bone layers, whereas the other cell types remain primarily on the bone's surface [19]. The osteocyte is housed inside a thin layer of unmineralized collagen matrix within the layers of lamellae and maintains physical connections to neighboring osteocytes, which allow communication between cells through gap junctions and an interstitial fluid network [20]. In vivo, the average life-span of an osteocyte is 25 years and while the proportion of dead osteocytes increases with age, survival of the osteocyte population is greater than 25% in individuals more than 80 years old [21].

Published works have shown that DNA preservation in human remains is not well correlated to either perceived condition of the bone sample, time since death, nor the predicted rates of DNA degradation based on the stability of DNA in normal physiological conditions [17,22-26]. It has been suggested that unanticipated preservation of DNA is the result of the DNA molecule becoming adsorbed to the hydroxyapatite (HA) after the osteocyte has died. A crucial factor in preserving DNA within skeletal remains is the burial environment; DNA survival increases in environments with low humidity, low temperatures and the absence of microorganisms [16-17,23-26]. The alteration and decomposition of bone in the burial environment is a process called diagenesis. There are three primary mechanisms contributing to this process, including: chemical deterioration of the organic (collagen) fraction, chemical deterioration/alteration of the inorganic (mineral) fraction, and microbial attack that degrades both fractions in small patches. These mechanisms are not mutually exclusive processes, and the environmental conditions of the burial site will determine the rate at which each of these will occur.

These processes result in changes to the bone structure, including: decrease in the amount of organic material, increase in the structural crystallinity, and changes to the porosity and mineral constituents of the bone. When a bone sample is introduced to a burial environment, dissolution and reprecipitation of minerals occurs because the sample is not in thermodynamic equilibrium with the environment. The greater the initial difference in mineral content between the sample and environment, the more significant the extent of the alteration and the rate at which it occurs [1].

Fossilized bones do not reach equilibrium with the environment and it is suggested that reduced porosity of the bone from deposition of mineral is responsible for limiting the thermodynamic exchange. The mechanisms by which bones are believed to incorporate metal cations in a burial environment are: adsorption to mineral crystals (which may require displacement of the surface calcium), direct or coupled ion substitution and growth of new mineral crystals (phosphate combined with a non-calcium ion) [2]. Recent studies have indicated these changes occur much earlier in the diagenic process and are relevant to modern remains, in addition to fossilized bones. Non-human bone samples exposed on the soil surface for less than two years developed new crystal growth on their surfaces and after 15 years were reported to have "suffered extensive mineralogical and chemical alteration" [3]. Experiments which used human bone samples that were buried in a natural environment for less than two years, to assess whether changes in the mineral concentration could aid the determination of time since deposition, demonstrated substantial concentration increases for several metals present in the soil [4].

Early applications of DNA extracted from skeletal samples were reported by both the archaeological and forensic DNA communities in 1991 [8,17]. The first forensic protocols promoted the use of chemicals to decrease the amount of mineral present in the skeletal samples, prior to attempting amplification of DNA markers [8]. In 1993, studies evaluated the procedures

used for extraction of DNA from human skeletal remains and reported improved results without decalcification, and demonstrated that DNA was being lost in the buffer washes during the removal of mineral content from the bone samples [27]. Fourteen years later, complete demineralization of the bone sample into solution was shown to provide improved results over extraction procedures that do not employ a demineralization step [28]. This demineralization procedure is the basis for the DNA extraction procedures currently used by many forensic DNA testing laboratories.

Despite the extensive development history that has attempted to optimize DNA extraction from bone samples, more than two decades of experiments employing DNA extraction from bone samples have documented the inhibitory effect of numerous materials that co-isolate with DNA during extraction. The materials include endogenous calcium and collagen, as well as environmentally-introduced humic substances, and Maillard products of reducing sugars on PCR [15,17,27,29-30]. Two recent studies have added metals, native to the environment and introduced by human cultural practices, to the list of potential PCR inhibitors associated with human remains [5,31]. Despite evidence that PCR inhibition is common with DNA extracted from bone samples, PCR success is most often used to determine the success of the extraction [5,15,17,27,29-31]. Under this assumed correlation, amplification serves as an indirect indicator of extraction efficiency, and PCR failure from bone-derived samples is often explained as the result of DNA degradation, a condition that presently lacks remediation.

Quantification of DNA

The QA Standards require quantification of DNA, after extraction, from evidentiary materials [12]. Quantification is performed using quantitative PCR (qPCR), a technique that measures the fluorescence in a sample after each PCR cycle and compares the change in fluorescence over the number of cycles to the fluorescence measurements obtained for a series of

external calibration standards [32]. Commercially available qPCR kits used by forensic DNA testing labs usually contain a minimum of two assays: a human-specific assay and an internal PCR control (IPC) assay, consisting of a synthetic DNA sequence that co-amplifies in the same tube as the unknown sample. Reduction or failure of the IPC assay may indicate the presence of PCR inhibitors in a sample [32].

When a DNA extract from a bone sample fails to yield a detectable amount of human DNA after quantification with a qPCR assay, it is often concluded the DNA has suffered from degradation if the IPC assay performed as expected. This conclusion fails to take into account the variety of PCR inhibitors known to co-isolate with DNA from bone samples, many of which have inhibitory mechanisms that may go undetected by the IPC assay. There is a significant difference between "no amplifiable template" and "no template present" in a sample. A sample with no amplifiable DNA template may have DNA of sufficient quality for PCR that is inhibited. Types of inhibitory mechanisms that may go undetected by the IPC assay include ones that prevent isolation of DNA or sequesters it in solution, preventing access to it during PCR [33]. These are legitimate possibilities for bone samples containing metals, which have been implicated in adduct-formation directly with DNA, produce substantial crosslinks between DNA and proteins, and can even form soluble, high molecular weight complexes with humic substances and DNA [34-37].

Amplification and Analysis of DNA

Amplification using PCR is performed to copy only the markers of interest and label them for subsequent analysis. The genetic location, referred to as a *locus*, on a single strand of DNA is copied two times for each PCR cycle [38]. Commercially available DNA typing chemistry kits type between 16 and 24 markers, including Amelogenin (AMEL), a sex-determining marker and the remaining markers are STR loci. The loci typed by the AmpFLSTR® Identifiler® PCR

Amplification Kit (Identifiler®) and Identifiler® Plus PCR Amplification Kit (Identifiler® Plus) from Applied Biosystems and the PowerPlex® 16 HS and PowerPlex® Fusion Systems from Promega Corporation are detailed in **Table 1**. This table also includes the chromosomal location of each locus and the repeated unit of nucleic acid sequence for the STR markers.

The collection of results from these typing kits yields a DNA profile. With the exception of identical twins, a DNA profile is unique to an individual and is a valuable tool for human identification applications. An advantage to the small sizes of the STR loci targeted by these kits is that it is often possible to obtain at least a partial DNA profile from damaged DNA samples. One of the limits of this technology is the susceptibility of PCR-based testing systems to the presence of PCR inhibitors. The developmental validation reports for these systems included inhibition assessments for hematin for Identifiler®, hematin and humic acid for Identifiler® Plus, hematin, humic and tannic acids for the PowerPlex® 16 HS System, and hematin, humic and tannic acids, and EDTA for the PowerPlex® Fusion System [10,13,39-40].

Table 1
Human genetic identity loci targeted by commercial STR testing kits, including chromosomal locations, repeat motif (5' to 3'), and dyes used to label the loci in the kits used for this project.

Locus	Chromosomal Location	Repeat Motif (5´to 3´)	Identifiler® and Identifiler® Plus	PowerPlex® 16 HS	PowerPlex® Fusion
AMEL	Xp22.1–22.3,Y	N/A	PET®	TMR	FL
DYS391	Y	TCTA	-	-	TMR-ET
CSF1PO	5q33.1	AGAT	6-FAM™	JOE	JOE
D1S1656	1q42	TAGA Complex	-	-	FL
D2S1338	2q35	TGCC/TTCC	VIC®	-	JOE
D2S441	2p14	TCTA	-	-	FL
D3S1358	3p21.31	TCTA Complex	VIC®	FL	FL
D5S818	5q23.2	AGAT	PET®	JOE	TMR-ET
D7S820	7q21.11	GATA	6-FAM™	JOE	TMR-ET
D8S1179	8q24.13	TCTA Complex	6-FAM™	TMR	CXR-ET
D10S1248	10q26.3	GGAA	-	-	FL
D12S391	12p12	AGAT/C Complex	-	-	CXR-ET
D13S317	13q31.1	TATC	VIC®	JOE	FL
D16S539	16q24.1	GATA	VIC®	JOE	JOE
D18S51	18q21.33	AGAA	NED™	FL	JOE
D19S433	19q12	AAGG Complex	NED™	-	CXR-ET
D21S11	21q21.1	TCTA Complex	6-FAM™	FL	TMR-ET
D22S1045	22q12.3	ATT	-	-	CXR-ET
FGA	4q28	TTTC Complex	PET®	TMR	CXR-ET
Penta D	21q22.3	AAAGA	-	JOE	JOE
Penta E	15q26.2	AAAGA	-	FL	FL
TH01	11p15.5	AATG	VIC®	FL	TMR-ET
TPOX	2p25.3	AATG	NED™	TMR	TMR-ET
vWA	12p13.31	TCTA Complex	NED™	TMR	TMR-ET

NA = not applicable; TMR = carboxy-tetramethylrhodamine; FL = fluorescein;
JOE = 6-carboxy-4´,5´-dichloro-2´,7´-dimethoxyfluorescein
Highlighted rows indicate the core loci selected for the Federal Combined DNA Index System (CODIS); chromosomal location and repeat motifs were obtained from STRbase [41].
The dyes used to label the primers targeting each locus was obtained from the respective kit user manuals and technical bulletins [42-45].

Specific Aims

The **rationale** of the proposed project is that extraction efficiency determines the quality of DNA isolates and ultimately, the success of PCR-based DNA testing. The primary **hypothesis** is failure of PCR-based testing of DNA from skeletal remains is frequently encountered due to PCR inhibition caused by endogenous and environmentally-introduced metals and inefficient extraction methods. The immediate goals are to demonstrate an approach for identifying and characterizing putative PCR inhibitors and determine the efficiency of current methods used for extracting DNA from bone samples. The long term goal of this project is to increase the number of individuals identified from skeletal remains by providing a comprehensive understanding of the major factors which can affect the success of PCR-based DNA testing. The objectives which support these goals are to:

1. **Demonstrate comprehensive characterization of established PCR inhibitors, hematin and humic acid, and establish the inhibitory potential of metal ions.**
2. **Elucidate the inhibitory effect of endogenous and environmentally-introduced metals, present in human skeletal remains, on PCR-based DNA testing.**
3. **Evaluate the effect of pre-extraction processing on DNA extracted from bone samples.**

The data obtained from this project will advance understanding of the physical and biochemical factors that can affect PCR-based DNA testing of human skeletal remains. These data sets will also illustrate factors that can improve the success of forensic testing, which will ultimately serve to increase the identification rates from DNA testing of human skeletal remains.

Significance

The National Crime Information Center (NCIC) Missing Person and Unidentified Person Statistics reports 84,136 missing and 8,045 unidentified person cases as of December 31, 2013 [46]. The total number of recovered human remains in the United States that have never been identified is estimated to be at least 40,000 [47]. These alarming numbers evidence what has been described as "a mass disaster over time" in the United States [48]. Identification of recovered human remains is a critical step toward closing missing persons' investigations and providing closure to the friends and families of missing individuals. Confirmation of a decedent's identity can be challenging due to a significant passage of time since a person disappeared, lack of appropriate antemortem records, or geographic distance between the found remains and where the missing persons' report was filed [49]. Improvements in DNA testing of human skeletal remains has had a dramatic impact on the most challenging identification cases, where traditional methods cannot confirm identity or are not available for use [49].

Results from the following experiments make several important contributions toward demonstrating the significance of the extraction process to human identification efforts and illustrate factors which can impact the success of forensic PCR-based testing of skeletal remains. Routine laboratory practices for DNA extraction from bone samples will be improved, ultimately serving to increase the identification rate of human remains. This project may also serve to substantiate the development of policies that specifically address both the methodology used to identify forensically-relevant inhibitors and those governing the validation of forensic DNA extraction protocols.

CHAPTER II.

COMPARATIVE EFFECTS OF HEMATIN AND HUMIC ACID INHIBITION OF FORENSIC MULTIPLEX SHORT TANDEM REPEAT SYSTEMS AND POLYMERASE CHAIN REACTION ENHANCERS

II.1. Introduction

Substances that inhibit the polymerase chain reaction (PCR) may be endogenous to the sample source material, or become introduced to the sample through the depositional environment or laboratory handling [33]. Due to the highly variable nature of evidentiary samples, studies evaluating the performance of forensic DNA testing systems in the presence of inhibitory substances are required and are typically conducted during validation of the system [10,12-13]. Models of PCR inhibition are often used for completion of these studies and include materials such as hematin and humic acid [10,13].

Ferriprotoporphyrin hydroxide (alkaline hematin) is an Fe^{3+}-containing metalloporphyrin, present in bloodstains that is formed as a degradation product from the oxidation of hemoglobin [50]. Since the late 1980's, hematin has been believed to contaminate DNA extracts from bloodstain samples and suggested to inhibit the enzyme responsible for PCR product formation, *Taq* polymerase [51-54]. Crystalized hematin is solubilized in 0.1 N NaOH solutions before preparation of inhibited DNA samples, as a common practice [10,13,55].

Humic acids are a class of molecules formed as a degradation product of organic material found in soil [56]. The mass-to-charge ratio of humic acids is similar to that of DNA, making it

likely that extraction and purification methods targeting DNA will also co-isolate humic acids [57]. Experiments aiming to identify the mechanism of PCR inhibition suggest that humic acid limits the amount of available DNA template through sequence-specific binding [54]. Inhibition experiments using humic acid are typically prepared using a Tris-EDTA buffer (TE^{-4}) with a low EDTA concentration [10,13,58].

Contemporary short tandem repeat (STR) DNA testing systems, including AmpFLSTR® Identifiler® Plus (Applied Biosystems, Foster City, CA) and the PowerPlex® 16 HS System (Promega Corporation, Madison, WI), were designed with advanced buffering systems to mitigate the effect of PCR inhibitors, such as hematin and humic acid [11,44]. These kits claim an increased tolerance to the presence of inhibitors over the prior generation of kits, which include AmpFLSTR® Identifiler® (Applied Biosystems) and the PowerPlex® 16 System (Promega Corporation) [11,44]. Previous attempts at overcoming the effects of PCR inhibitors have included sample dilution and treatment with other chemicals that have been reported to enhance the performance of the PCR [33].

There are a variety of commercial products available that are reported to enhance results from PCR-based genetic testing in the presence of inhibitors. STRboost™ (Biomātrica Inc., San Diego, CA) was selected because it was designed specifically to improve results of STR testing [59]; and, bovine serum albumin (BSA), due to its prevalent use for the treatment of inhibited samples and established efficacy in a variety of genetic testing contexts [60]. STRboost™ contains <10% 1 M Tris-HCL and "trade secret" numbers 081763 and 090046 [61]. This proprietary reagent has been shown to improve STR results from samples containing hematin and humic acid [62]. BSA is carrier protein that can bind to materials that inhibit PCR, reducing or preventing the

inhibition; it is also included in the AmpFLSTR® PCR Reaction Mix at 4 μg per 25 μL amplification and at an undisclosed concentration in the PowerPlex® HS 5X Master Mix [11,13].

This was a trial study to determine the utility of expanding the analysis of STR data obtained from inhibited samples beyond the number of alleles and/or percent of expected profile, using well-characterized inhibitors, and to evaluate the potential of PCR additives or enhancers to improve results. The purpose of this study was to evaluate the effects of hematin and humic acid inhibition on two generations of forensic multiplex STR DNA testing systems with and without the inclusion of PCR enhancers. Genetic results from samples treated with hematin and humic acid were obtained from AmpFLSTR® Identifiler® and the PowerPlex® 16 HS System, without treatment and with separate inclusion of STRboost™ and BSA. These results were analyzed to determine whether enhancement of either system improves results obtained in the presence of hematin and humic acid inhibition. The evaluation was conducted by statistically analyzing the peak height and peak area data to provide a comprehensive understanding of the effect of PCR inhibition on the quality of genetic results obtained from inhibitor-treated, control DNA samples.

II.2. Materials and Methods

II.2.1 DNA sample preparation

Hematin-treated samples were prepared using solutions of porcine hematin (Sigma-Aldrich, St. Louis, MO) solubilized in 0.1 N sodium hydroxide (NaOH) solution (FisherChemical, Fairlawn, NJ). A stock solution was prepared at 85 μM and dilutions were prepared using the stock solution and 0.1 N NaOH.

Humic acid-treated samples were prepared using technical grade humic acid (Sigma-Aldrich) solubilized in TE^{-4} buffer (10 mM Tris-HCL, pH 8.0, and 0.1 mM EDTA, pH 8.0). A stock solution was prepared at 507 ng/μL and dilutions were prepared using TE^{-4} buffer.

DNA samples containing hematin and humic acid were prepared by combining 2 µL of 10 ng/µL human genomic 9947A control DNA (Promega Corporation, Madison, WI) to each 148 µL aliquot of inhibitor solution. This was sufficient to obtain a DNA concentration of 1 ng in 7.5 µL of DNA-inhibitor solution. The concentration of inhibitor in solution was diluted as a consequence of the addition of DNA, and again, after addition to PCR reagents for STR reactions. The naming convention used to describe inhibited samples reflects the concentration of inhibitor present in the STR reactions (**Table 2** and **Table 3**).

Table 2
The DNA samples are labeled with the naming convention used to describe the approximate hematin concentration present in each 25 µL STR reaction. The approximate concentrations of hematin in the inhibitor solution, combined with DNA in solution and in the STR reactions are included.

Final Concentration of Hematin (µM) in:			
Inhibitor Solution	Description	DNA Sample	STR Reaction
0.0085	Colorless	0.0083	0.0025
0.085	Colorless	0.083	0.025
0.85	Colorless	0.83	0.25
8.5	Faint green	8.3	2.5
85	Green	83	25

Table 3
The DNA samples are labeled with the naming convention used to describe the approximate humic acid concentration present in each 25 µL STR reaction. The approximate concentrations of humic acid in the inhibitor solution, combined with DNA in solution and in the STR reactions are included.

Final Concentration of Humic Acid (ng/µL) in:			
Inhibitor Solution	Description	DNA Sample	STR Reaction
12.5	Colorless	12.4	3.70
37.5	Faint yellow	37.0	11.1
113	Light yellow	111	33.3
338	Dark yellow	333	100
507	Light brown	500	150

II.2.2 DNA amplification

All DNA amplifications were performed in duplicate using AmpFLSTR® Identifiler® PCR Amplification Kit (Applied Biosystems, Foster City, CA) and PowerPlex 16 HS® System (Promega Corporation), according to the manufacturer's instructions for 25 µL PCR reaction setup and thermocycling parameters [42,44]. Preliminary amplifications were conducted using positive and negative control samples as a quality control assay for the forensic STR typing systems. These sample sets included an untreated 9947A DNA positive control sample and a positive control sample treated with 2.5 µL of 50 µg/µL bovine serum albumin (BSA); yielding a final concentration of 5 µg/µL in the PCR reaction.

Experimental amplifications included a separate set of positive and negative control samples for each chemistry condition tested. The master mix used for the AmpFLSTR® Identifiler® amplification setup included 10.5 µL of AmpFLSTR® PCR Reaction Mix, 5.5 µL of AmpFLSTR® Identifiler® Primer Set, and 0.5 µL AmpliTaq Gold® DNA Polymerase (Applied Biosystems) per sample. A 15.0 µL aliquot of this master mix was added to each PCR amplification tube, before adding 7.5 µL of DNA-inhibitor solution. The final 2.5 µL added to each PCR tube consisted of amplification grade water for samples without enhancer treatment, STRboost™ (Biomātrica Inc., San Diego, CA) or 50 µg/µL BSA [59-60]. Amplification was performed using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems), in 9600 emulation mode, using the following parameters: 95°C hold for 11 minutes; 28 cycles of 94°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute; 60°C hold for 60 minutes, and 4°C hold until samples removed from the thermocycler and stored refrigerated.

The master mix used for the PowerPlex® 16 HS System amplification setup included 7.5 µL of amplification grade water, 5.0 µL of PowerPlex® HS 5X Master Mix, and 2.5 µL of

PowerPlex® 16 HS 10X Primer Pair Mix per sample. A 15.0 µL aliquot of this master mix was added to each PCR amplification tube, before adding 7.5 µL of DNA-inhibitor solution. The final 2.5 µL added to each PCR tube consisted of amplification grade water for samples without enhancer treatment, STRboost™, or 50 µg/µL BSA. Amplification was performed using a GeneAmp® PCR System 9700 thermocycler, in 9600 emulation mode. An initial 96°C hold for 2 minutes was followed by two cycling groups, the first consisted of 10 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 70°C for 45 seconds; and the second consisted of 22 cycles of 90°C for 30 seconds, 60°C for 30 seconds, and 70°C for 45 seconds. These cycles were followed by a final 60°C hold for 30 minutes, and 4°C hold until samples removed from the thermocycler and stored refrigerated.

II.2.3 Analysis and genotyping

Amplified products were analyzed by capillary electrophoresis, according to the manufacturer's instructions for each amplification system [42,44]. Genetic analysis of Identifiler®-amplified samples was performed after combining 1.0 µL of product or allelic ladder with 25 µL master mix that included a ratio of 24 µL Hi-Di™ Formamide and 1.0 µL of GeneScan™ 500 LIZ® Size Standard per sample (Applied Biosystems). Capillary electrophoresis of Identifiler®-amplified samples was conducted using a 15 kV, 5 second injection and POP-4™ Polymer on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Genetic analysis of PowerPlex® 16 HS-amplified samples was performed after combining 1.0 µL of product or allelic ladder with 25 µL master mix that included 24 µL Hi-Di™ Formamide and 1.0 µL of Internal Lane Standard 600 size standard (Promega). Capillary electrophoresis of PowerPlex® 16 HS-amplified samples was conducted using a 15 kV, 3 second injection and POP-4™ Polymer on an ABI Prism 310 Genetic Analyzer.

The STR profiles, including individual allele peak heights and areas, were analyzed with GeneMapper® ID Software v.3.2 (Applied Biosystems). A 25 RFU detection threshold was used throughout this study; this low level was used to maximize the number of allele calls that could be obtained from samples, in order to assess the effect of each inhibitor over as much of the full concentration range tested as possible.

II.2.4 Statistical analyses

Duplicate samples were assessed for concordance using Chi-square tests; the matched alleles between samples were paired for analysis (*i.e.* AMEL-X peaks in both samples with the sample inhibitor concentration and amplification conditions). Average allele counts between duplicates, expressed as a percent of the expected number of alleles in the 9947A STR profile for each system, were used to compare results between chemistry combinations. Individual allele peak heights and areas, as functions of inhibitor concentrations, were assessed by regression analysis; logarithmic transformation was employed to improve the uniformity of the distribution of data points. The linear dependence of allele peak heights and peak areas to the inhibitor concentration was examined through Pearson's correlation coefficients; these coefficients were also used to assess concordance between chemistry combinations for the same experimental treatments. All tests were conducted with a significance level (α) of 5%, where applicable.

II.3. Results

Control samples performed as expected for all AmpFLSTR® Identifiler® and PowerPlex® 16 HS chemistry combinations; positive control samples yielded complete 9947A DNA profiles and no alleles were obtained from negative control samples (*data not shown*).

II.3.1 Concordance between duplicate samples

Individual allele peak heights between duplicate samples were concordant. There was no significant difference between paired samples, as determined by the Chi-square tests. All p-values were greater than 0.999, using the following parameters: AmpFLSTR® Identifiler®, *n allele pairs* = 26, *df* = 25; and, PowerPlex® 16 HS, *n allele pairs* = 25, *df* = 24.

II.3.2 Average allele counts, as a percent of the 9947A DNA profile

The average number of alleles obtained for each pair of inhibitor-treated samples was expressed as a percent of the total number of expected alleles for the 9947A STR profile (hematin, **Fig. 1**; humic acid, **Fig. 2**). No alleles were obtained for any hematin-treated samples amplified with AmpFLSTR® Identifiler®, and only one hematin-treated sample amplified with the PowerPlex® 16 HS System failed to produce a full profile. This 0.25 μM hematin-treated sample, amplified with the PowerPlex® 16 HS System without enhancer treatment, only yielded 22 out of 25 alleles; however, all other hematin-treated samples yielded complete profiles, even at higher hematin concentrations.

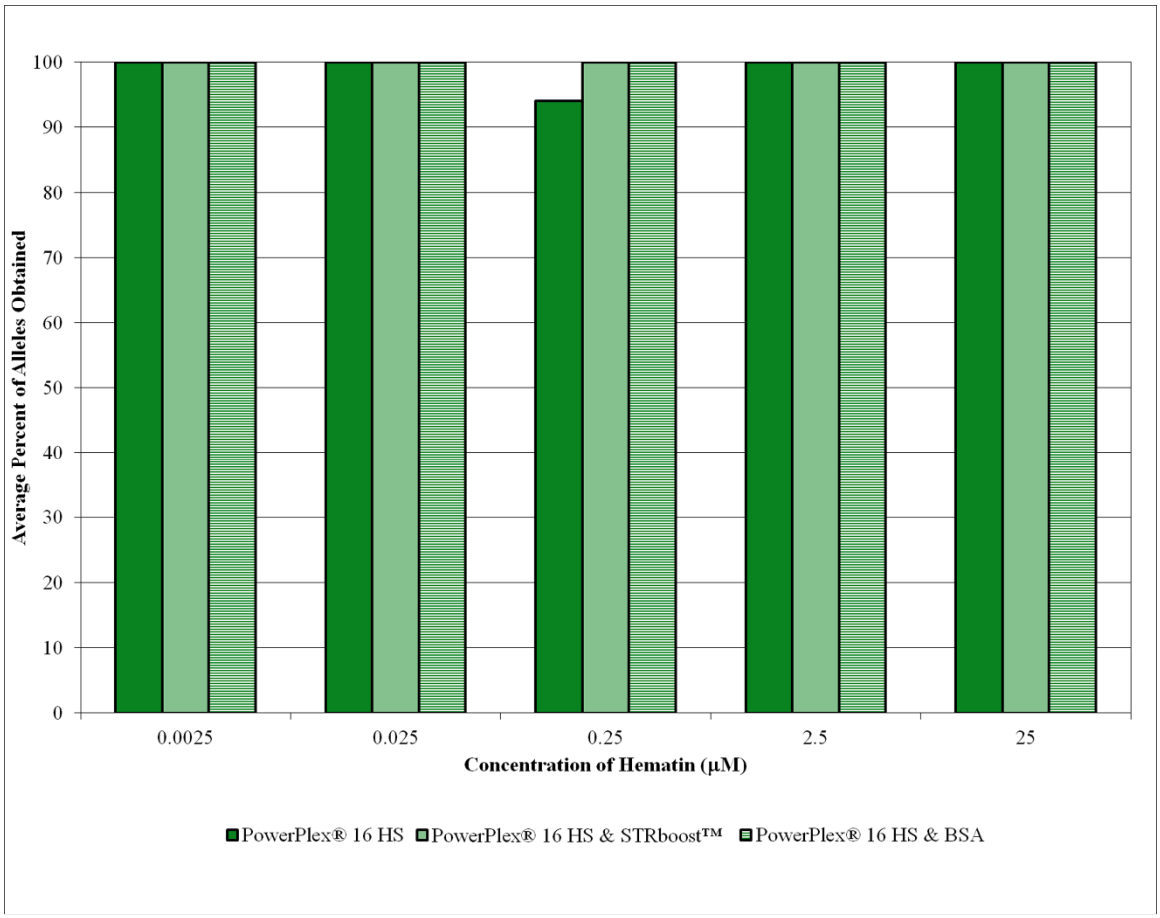

Fig. 1. Duplicate, hematin-treated 9947A DNA samples amplified with the PowerPlex® 16 HS System were analyzed and the total alleles obtained were counted. The number of alleles for each duplicate pair were averaged and are expressed as a percent of the expected number of alleles for the complete 9947A STR profile (100% is equal to 25 alleles).

The humic acid-treated samples amplified with AmpFLSTR® Identifiler® chemistry combinations yielded full profiles from samples treated with 3.70 ng/µL humic acid for samples that had no enhancer treatment and those containing STRboost™; however, both of these amplification chemistry combinations had a single allele drop out from one of the two duplicate samples at 11.1 ng/µL humic acid. Identifiler® amplifications without enhancer yielded a single allele at 33.3 ng/µL humic acid and no alleles were obtained at higher concentrations. No alleles were obtained from samples amplified with STRboost™ at ≥ 33.3 ng/µL humic acid.

Allelic dropout was observed in samples treated with 3.70 ng/µL humic acid and amplified with the inclusion of BSA, one and three alleles from the duplicate samples; however, amplifications containing BSA continued to yield results at higher humic acid concentrations. Six to eight alleles dropped out of samples at 11.1 and 33.3 ng/µL humic acid, and 22 to 24 alleles were lost at a humic acid concentration of 100 ng/µL. A single allele was obtained from one sample amplified with Identifiler® containing BSA and containing 150 ng/µL humic acid.

Complete 9947A profiles were obtained for nearly all humic acid-treated samples amplified with the PowerPlex® 16 HS System chemistry combinations. Allelic dropout was only observed in the samples treated with the 150 ng/µL humic acid and amplified without enhancer treatment, a total of three and five alleles dropped out from those duplicate samples.

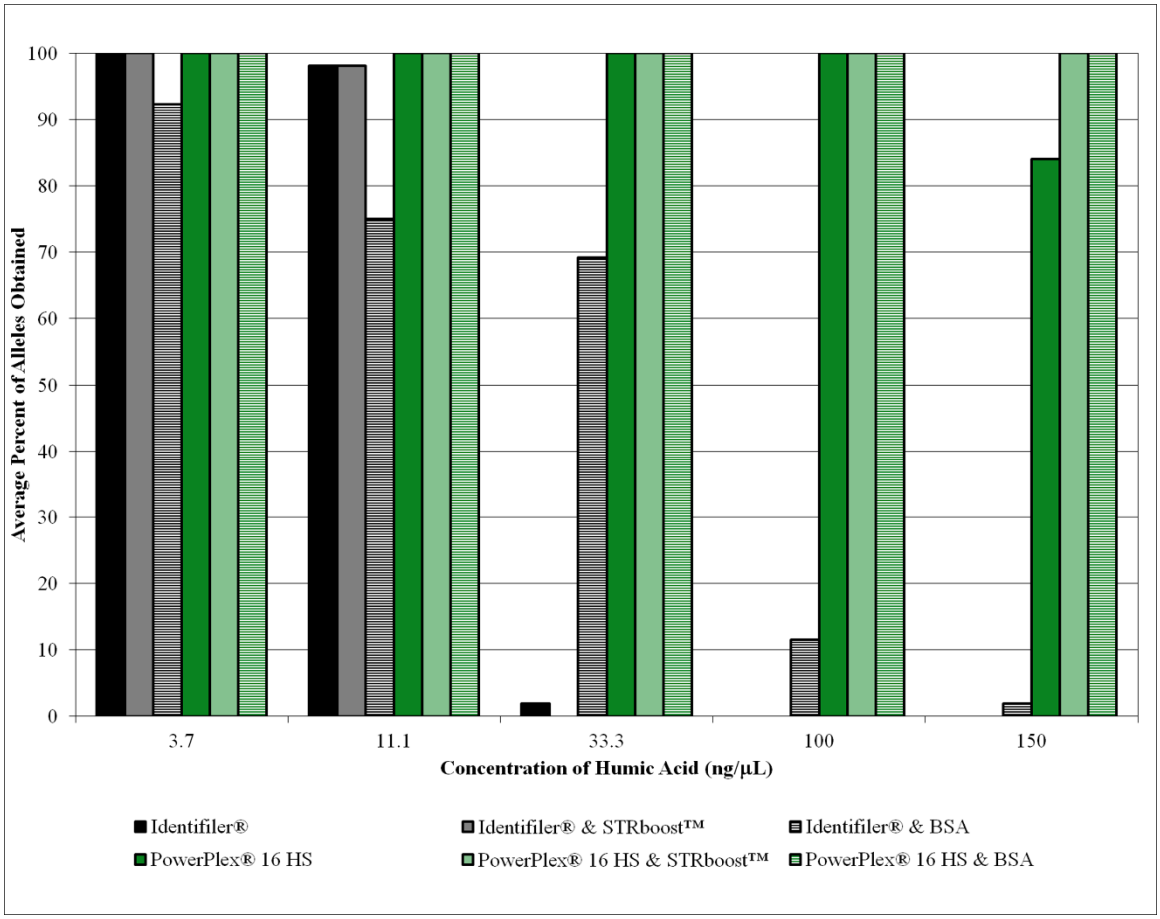

Fig. 2. Duplicate, humic acid-treated 9947A DNA samples amplified with Identifiler® and the PowerPlex® 16 HS System were analyzed and the total alleles obtained were counted. The number of alleles for each duplicate pair were averaged and are expressed as a percent of the expected number of alleles for the complete 9947A STR profile (100% is equal to 26 alleles for Identifiler®-amplified samples and 25 alleles for the PowerPlex® 16 HS System).

II.3.3 Hematin-treated samples amplified with AmpFLSTR® Identifiler®

No results were obtained from any of the hematin-treated samples amplified with AmpFLSTR® Identifiler® chemistry combinations. All sample sets were re-amplified in duplicate and amplification of hematin-treated samples failed, again. Additional amplifications were not performed due to resource limitations.

II.3.4 Humic acid-treated samples amplified with AmpFLSTR® Identifiler®

The peak height relative fluorescent units (RFU) obtained for the individual alleles obtained for all AmpFLSTR® Identifiler® amplifications of humic acid-treated samples are depicted in **Fig. 3**. Expected alleles below the 25 RFU detection threshold were manually-assigned values of zero and were excluded from calculation of mean values and determination of the ranges. Similar results were obtained for humic acid treated samples amplified with AmpFLSTR® Identifiler® without enhancer treatment and enhanced with STRboost™; mean peak heights for samples containing 3.70 ng/mL humic acid were 419 and 462 RFU, respectively, with ranges of approximately 120 to 1000 RFU. This was also the case with samples treated with 11.1 ng/µL humic acid, where mean values were 280 and 295 RFU and ranges of approximately 30 to 700-800 RFU for samples amplified with AmpFLSTR® Identifiler® without enhancer treatment and enhanced with STRboost™, respectively. In contrast samples amplified with AmpFLSTR® Identifiler® and enhanced with BSA yielded mean RFU values of 242 (range 29-651) and 226 (range 25-571) for the 3.70 and 11.1 ng/µL humic acid-treated samples. Because of the reduction in number of alleles obtained at concentrations of 33.3 and above, the maximum allele heights are

better representation of the overall profile: Identifiler® without enhancer treatment amplification of 33.3 ng/µL humic acid treated samples was 28 RFU (one allele total); Identifiler® and enhanced with BSA amplification of 33.3 ng/µL humic acid treated samples was 531 RFU (36 alleles total), Identifiler® and enhanced with BSA amplification of 100 ng/µL humic acid treated samples was 81 RFU (6 alleles total), Identifiler® and enhanced with BSA amplification of 150 ng/µL humic acid treated samples was 30 RFU (one allele total).

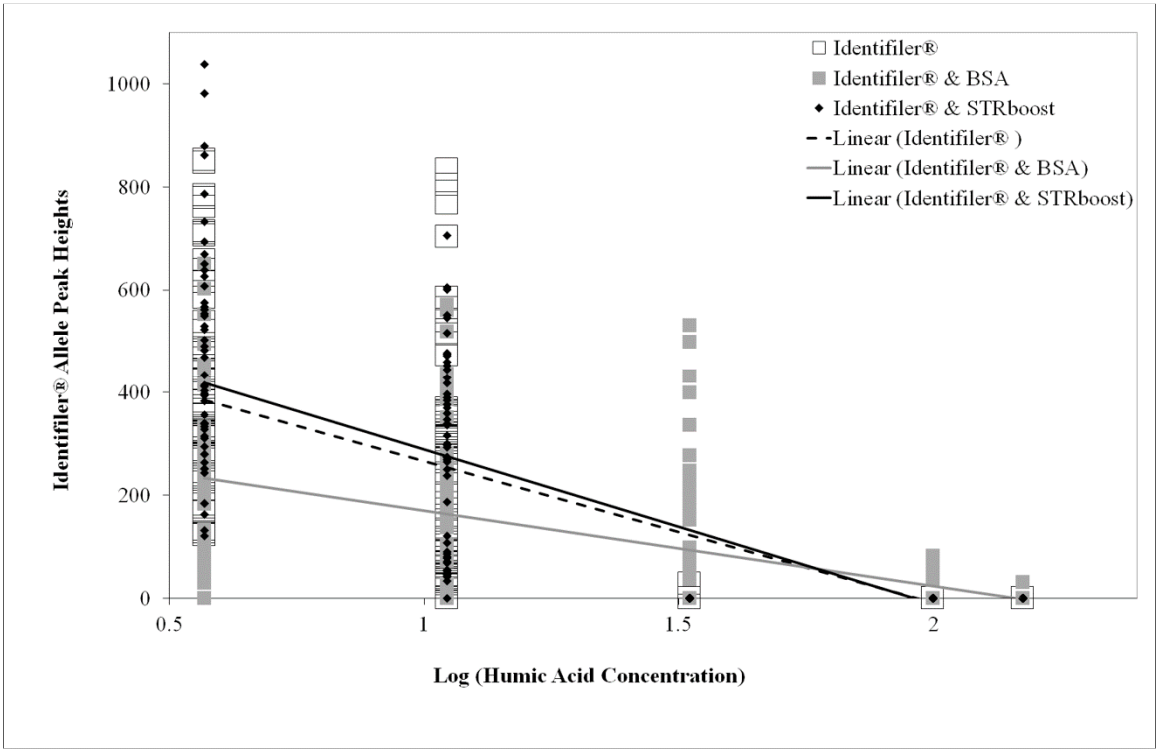

Fig. 3. Duplicate, humic acid-treated 9947A DNA samples, amplified with Identifiler® were analyzed and the individual peak heights were plotted in a scatter diagram. The allele peak height data for samples amplified with Identifiler® without enhancer treatment (open square, dashed black line), Identifiler® enhanced with BSA (gray square, solid gray line), and Identifiler® with STRboost™ (black diamond with solid black line) are illustrated in the scatter diagram.

Pearson's coefficients indicated a significant negative correlation between the concentration of humic acid and peak heights (**Table 4**, *lower diagonal*) and peak areas (**Table 4**, *upper diagonal*). A significant negative correlation was determined between allele length and allele peak heights obtained with AmpFLSTR® Identifiler® enhanced with BSA; however, allele

length was found only to have a significant negative correlation with peak areas obtained with AmpFLSTR® Identifiler® enhanced with STRboost™. There were significant positive correlations found when comparing the results obtained from each of the three chemistry combinations tested, indicating concordant results. Correlations were more significant when evaluating peak heights, as compared to peak areas; this may suggest peak areas obtained with AmpFLSTR® Identifiler® are less susceptible to the effects of humic acid inhibition than the allele peak heights.

Table 4
Allele peak heights (*lower diagonal*) and peak areas (*upper diagonal*) were independently analyzed with respect to the length of the allele in base pairs (bp), the concentration of humic acid used to treat the DNA samples, and the amplification system without enhancer treatment, enhanced with BSA, and enhanced with STRboost™. Significance at the 0.05 level for negative correlation is between -1.0 and -0.1217, and for positive correlation is between 0.1217 and 1.0 (*n alleles = 260, df = 258*). Significant values are indicated by bold typeface.

Height \ Area	1	2	3	4	5
1. Allele length (bp)	•	-6.9×10^{-17}	0.012	**-0.20**	-0.10
2. Humic acid concentration	-6.9×10^{-17}	•	**-0.74**	**-0.60**	**-0.80**
3. Identifiler®	-0.018	**-0.75**	•	**0.76**	**0.95**
4. Identifiler® & BSA	**-0.25**	**-0.59**	**0.75**	•	**0.75**
5. Identifiler® & STRboost™	-0.09	**-0.78**	**0.94**	**0.75**	•

II.3.5 Hematin-treated samples amplified with the PowerPlex® 16 HS System

The peak height RFU obtained for individual alleles obtained for all PowerPlex® 16 HS amplifications of hematin-treated samples are depicted in **Fig. 4**. Results from all samples, at all concentrations, and with each chemistry combination resulted in similar minimum, mean, and maximum peak height values. The difference between RFU values obtained for the amplification chemistry combinations was between 13 and 94 RFU for minimum allele peak height, 38 and 323 RFU for the mean allele peak height, and 131 and 1111 RFU for the maximum allele peak height. Samples amplified with the PowerPlex® 16 HS System enhanced with STRboost™ maintained the highest minimum, mean, and maximum RFU values for peak heights, except for the samples

treated with 25 mM hematin, where the highest maximum peak value of 2581 was obtained from a sample amplified with BSA as an enhancer.

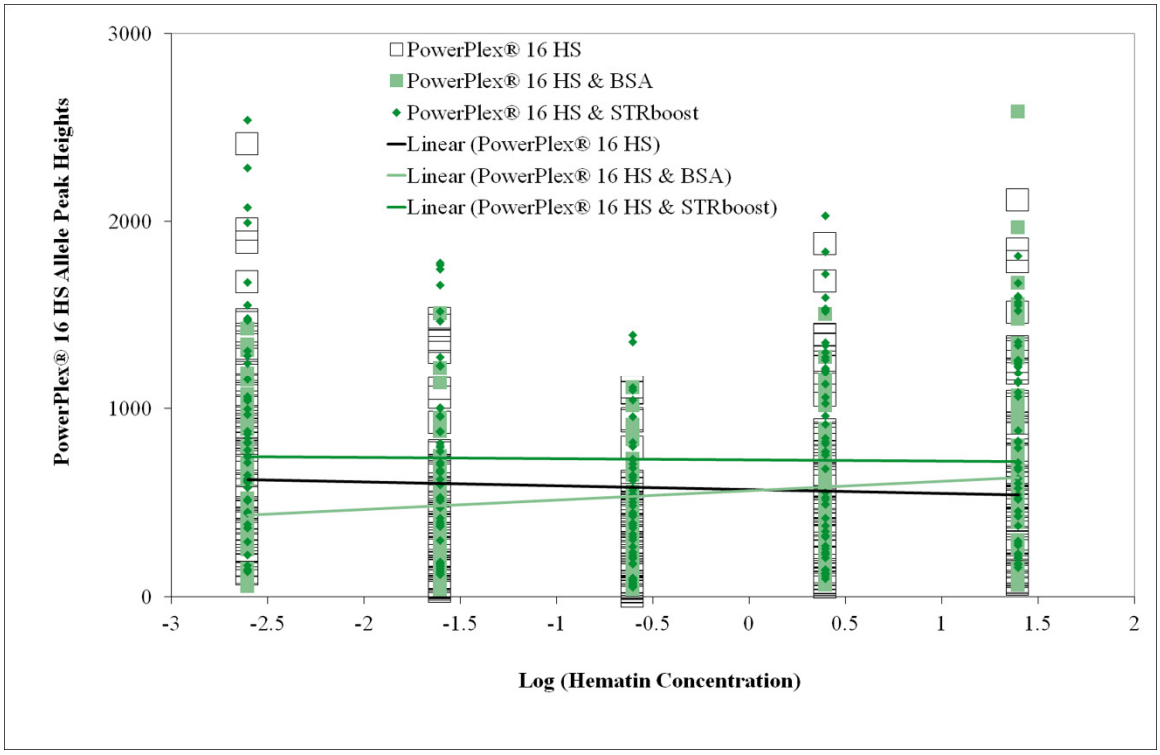

Fig. 4. Duplicate, hematin-treated 9947A DNA samples, amplified with the PowerPlex® 16 HS System were analyzed and the individual peak heights were plotted in a scatter diagram. The allele peak height data for samples amplified with PowerPlex® 16 HS without enhancer treatment (open square, black line), PowerPlex® 16 HS enhanced with BSA (light green square and line), and PowerPlex® 16 HS with STRboost™ (dark green diamond and line) are illustrated in the scatter diagram.

Pearson's coefficients indicated a significant negative correlation between the allele length and peak heights (**Table 5**, *lower diagonal*) and peak areas (**Table 5**, *upper diagonal*). Interestingly, a significant positive correlation was found between both the peak heights and peak areas obtained with the PowerPlex® 16 HS System enhanced with BSA and the concentration of hematin. There were also significant positive correlations when the results obtained from each of the three chemistry combinations tested were compared, indicating concordant results. Correlations were more significant when evaluating peak heights, as compared to peak areas; this

may suggest peak areas obtained with the PowerPlex® 16 HS System are less susceptible to the effects of hematin inhibition than the allele peak heights.

Table 5
Allele peak heights (*lower diagonal*) and peak areas (*upper diagonal*) were independently analyzed with respect to the length of the allele in base pairs (bp), the concentration of hematin used to treat the DNA samples, and the amplification system without enhancer treatment, enhanced with BSA, and enhanced with STRboost™. Significance at the 0.05 level for negative correlation is between -1.0 and -0.1241, and for positive correlation is between 0.1241 and 1.0 (*n alleles = 250, df = 248*). Significant values are indicated by bold typeface.

Height \ Area	1	2	3	4	5
1. Allele length (bp)	•	2.3×10^{-17}	**-0.31**	**-0.33**	**-0.25**
2. Hematin concentration	2.3×10^{-17}	•	0.019	**0.25**	0.08
3. PowerPlex® 16 HS	**-0.47**	-0.061	•	**0.81**	**0.90**
4. PowerPlex® 16 HS & BSA	**-0.52**	**0.17**	**0.83**	•	**0.81**
5. PowerPlex® 16 HS & STRboost™	**-0.49**	-0.016	**0.91**	**0.82**	•

II.3.6 Humic acid-treated samples amplified with the PowerPlex® 16 HS System

The peak height RFU obtained for the individual alleles obtained for all PowerPlex® 16 HS amplifications of humic acid-treated samples are depicted in **Fig. 5**. Samples amplified with the PowerPlex® 16 HS System without enhancer treatment yielded the highest minimum, mean, and maximum RFU values for samples containing 3.70, 11.1, and 33.3 ng/µL humic acid. In general, samples enhanced with BSA had higher minimum, mean, and maximum RFU values for samples containing 100 and 150 ng/µL humic acid. The sole exception was the maximum peak height obtained for samples containing 100 ng/µL humic acid was obtained from a sample amplified with STRboost™ enhancement, instead of BSA enhancement (2274 versus 1674).

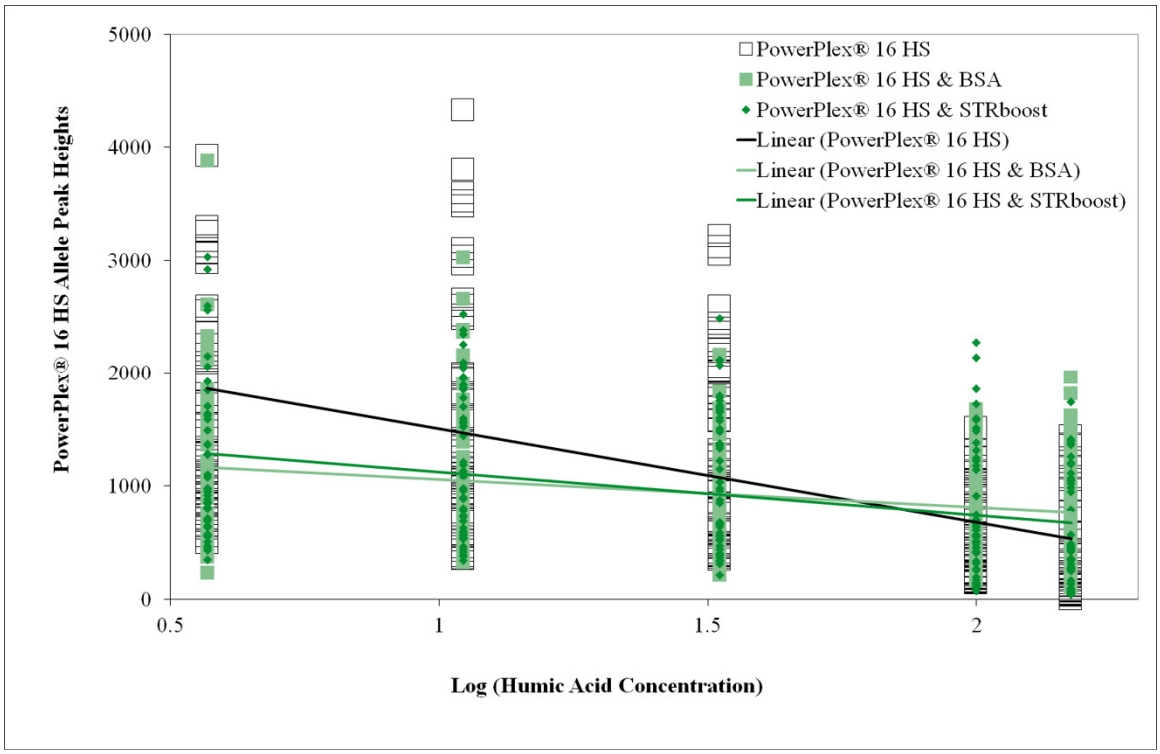

Fig. 5. Duplicate, humic acid-treated 9947A DNA samples, amplified with the PowerPlex® 16 HS System were analyzed and the individual peak heights were plotted in a scatter diagram. The allele peak height data for samples amplified with PowerPlex® 16 HS without enhancer treatment (open square, black line), PowerPlex® 16 HS enhanced with BSA (light green square and line), and PowerPlex® 16 HS with STRboost™ (dark green diamond and line) are illustrated in the scatter diagram.

Pearson's correlation coefficients identified a significant negative correlation between the concentration of humic acid with peak heights (**Table 6**, *lower diagonal*) and peak areas (**Table 6**, *upper diagonal*). Significant negative correlations were also present between allele length with both peak heights and peak areas. Comparisons yielding positive correlations were between results obtained from each of the three chemistry combinations tested, indicating concordant results. Correlations were more significant when evaluating peak heights, as compared to peak areas; this may suggest peak areas obtained with the PowerPlex® 16 HS System are less susceptible to the effects of humic acid inhibition than allele peak heights.

Table 6
Allele peak heights (*lower diagonal*) and peak areas (*upper diagonal*) were independently analyzed with respect to the length of the allele in base pairs (bp), the concentration of humic acid used to treat the DNA samples, and the amplification system without enhancer treatment, enhanced with BSA, and enhanced with STRboost™. Significance at the 0.05 level for negative correlation is between -1.0 and -0.1241, and for positive correlation is between 0.1241 and 1.0 (*n alleles = 250, df = 248*). Significant values are indicated by bold typeface.

Height \ Area	1	2	3	4	5
1. Allele length (bp)	•	-2.0×10^{-17}	**-0.17**	**-0.19**	**-0.19**
2. Humic acid concentration	-2.0×10^{-17}	•	**-0.55**	**-0.25**	**-0.37**
3. PowerPlex® 16 HS	**-0.43**	**-0.52**	•	**0.69**	**0.80**
4. PowerPlex® 16 HS & BSA	**-0.56**	**-0.25**	**0.73**	•	**0.78**
5. PowerPlex® 16 HS & STRboost™	**-0.52**	**-0.35**	**0.83**	**0.82**	•

II.4. Conclusions and Discussion

It would be inappropriate to make direct comparisons between the results obtained from the AmpFLSTR® Identifiler® PCR Amplification Kit and the PowerPlex® 16 HS System, as they represent different generations of DNA typing chemistries, and that was not the purpose of this study. This study was conducted in order to determine the utility of expanding the analysis of STR data obtained from inhibited samples beyond the number of alleles and/or percent of expected profile, and to evaluate the potential of PCR additives or enhancers to improve results. These results were analyzed to determine whether enhancement of either system improves results obtained in the presence of hematin and humic acid inhibition, through statistical analysis of the peak height and peak area data to provide a comprehensive understanding of the effect of PCR inhibition on the quality of genetic results obtained from inhibitor-treated, control DNA samples.

In contrast to other inhibition studies, the solvents used for inhibitor solution preparation (0.1 N NaOH and TE^{-4} buffer) were used instead of water to prepare subsequent dilutions to normalize any effect of the solvent across the range of inhibitor concentrations tested. As an unanticipated consequence of this approach, it was discovered that the concentration of NaOH used to solubilize hematin in these types of studies was sufficient to cause amplification failure of

all samples tested with the AmpFLSTR® Identifiler® PCR Amplification Kit. This was confirmed by evaluating the effect of varying concentrations of NaOH on the Identifiler® Kit, following repeated amplification failure of all hematin-treated samples. Stock 1 N NaOH was serially-diluted (1:10) to prepare three additional samples with concentrations (N) of 0.1, 0.01, and 0.001. Sufficient 10 ng 9947A DNA was added to include 1 ng of DNA in 7.5 µL of NaOH solution. Once combined with reagents in 25 µL PCR reactions, the NaOH concentrations (N) were diluted to 0.3, 0.03, 0.003, and 0.0003. These amplifications were performed according to the manufacturer's instructions, as described previously, without inhibitor or enhancer treatments. The results of these experiments in the context of their preparation are included in **Table 7**.

Table 7
The concentration of NaOH used to experimentally treat 9947A DNA, prior to amplification (*first row*), and the resulting concentration of NaOH in the PCR reaction (*second row*) are displayed. The results from this titration assay include the total number of 9947A STR profile alleles (*third row*) and are expressed as a percent of the expected number of alleles for the complete 9947A STR profile, 100% is equal to 26 alleles for Identifiler® (*fourth row*).

Concentration of NaOH solution with 1 ng DNA in 7.5 µl	1 N	0.1 N	0.01 N	0.001 N
Concentration of NaOH in 25 µL PCR	0.3 N	0.03 N	0.003 N	0.0003 N
Number of Alleles Obtained	0	0	10	26
Percent of 9947A STR Profile	0 %	0 %	39 %	100 %

The failure to obtain results from hematin-treated samples amplified with AmpFLSTR® Identifiler® is likely the result of the NaOH concentration used in sample preparation than that of hematin, based on existing studies demonstrating callable alleles developed from Identifiler® amplification of hematin-treated DNA samples [10-11,58]. Stability studies conducted during the developmental validation of Identifiler® demonstrated observable inhibition, in the form of reduced peak heights, at 12 µM and complete sample failure at 22 µM or more [10]. Those results were reproduced in subsequent studies, which obtained full profiles at 10 µM and severe inhibition at 20 µM and support the conclusion that sample failure was not a consequence of the hematin

concentration, but a result of the NaOH concentration used for sample preparation [58]. The results of the dilution series of NaOH, amplified with AmpFLSTR® Identifiler® without enhancer treatment, indicate that a NaOH solution of 0.01 N was sufficient to cause partial amplification failure and 0.1 N was sufficient to cause total amplification failure (**Table 7**). These results demonstrate the inhibitory capacity of NaOH on Identifiler® and elucidate the possibility that results of inhibition studies using water, instead of a uniform concentration of solvent for preparation of diluted inhibitor solutions, may be confounded by solvent effects. It is important to mention that 0.1 N NaOH has been used for preparation of hematin-inhibited DNA samples for testing the stability of STR testing systems since at least 2001, when the first commercially-available kits were validated for routine use [55].

The PowerPlex® 16 HS amplifications without enhancer treatment and enhanced with STRboost™ of hematin-treated samples did not yield a significant inhibitory effect as a consequence of hematin; PowerPlex® 16 HS amplification enhanced with BSA actually had a significant positive correlation between hematin concentration and allele peak heights and areas. These results were expected, as the PowerPlex® 16 HS System has been shown to produce full DNA profiles from DNA with up to 200 μM hematin [13]. The lack of a negative effect from the concentration of NaOH demonstrates the stability of the advanced buffering system, likely to increased pH, compared to the previous generation chemistry. Similar stability would be expected from the Identifiler® Plus PCR Amplification Kit.

Attempts at determining a biologically-relevant concentration range of hematin from the published literature were not successful. One study, completed by Akane *et al.*, measured hemoglobin derivatives in extracted and purified DNA [52]. Those results demonstrated <0.01% of alkaline hematin persisted following organic extraction and purification of DNA, making the

level of hematin expected to co-isolate with DNA negligible, and the authors concluded that hematin is not the material responsible for inhibition of PCR from bloodstain-derived DNA samples [52]. Additional time and resources were not dedicated to further resolving hematin-inhibited DNA samples, as a result of this finding.

Identifiler® amplification of humic acid-treated samples yielded similar results from samples amplified without enhancer treatment and samples enhanced with STRboost™. Enhancement with BSA did improve the peak height and areas obtained for these samples. This pattern was also observed for PowerPlex® 16 HS amplification of humic acid treated samples. STR results that were obtained with the PowerPlex® 16 HS System from humic acid-treated samples demonstrated a high tolerance to the presence of this inhibitor. Approximately 15 percent of expected alleles were lost due to allelic drop-out in samples amplified without enhancer treatment at 150 ng/μL, the highest concentration of humic acid tested, and full profiles were obtained from samples that were amplified with enhancer treatments. In addition, increased peak heights and areas were observed for both enhancer treatments. The results of amplifications performed without enhancer treatment exceed those reported in previous studies, which only obtained about 40 percent of expected alleles at 125 ng/μL humic acid [13]. The increase in allele intensity with STRboost™ was less than expected, based on previous reports [62]. Improvement as a consequence of enhancement with BSA, a binding protein, is not surprising because humic acid is theorized to inhibit PCR by binding the DNA template [54]. Tebbe and Vahjen measured the co-purified amount of humic acid with DNA samples extracted from soil using an organic extraction technique, purified with phenol-chloroform-isoamyl alcohol (PCIA), and precipitated with isopropanol. Their work demonstrated the possibility of co-isolating nearly 100 ng/μL of humic acid, the second to highest concentration tested in this study [56]. Future studies, using

higher concentrations of BSA to enhance STR testing of DNA samples containing humic acids are recommended.

Expanding the analysis of data sets that aim to assess the tolerance of amplification chemistries to PCR inhibitors preserves quantifiable results, typically lost when only allele counts or the percent of expected number of alleles are provided. Scatter diagrams with regression analysis provides a visual display of allele data; the results presented in this study have elucidated a pattern of behavior for inhibited STR profiles. As the concentration of inhibitor increases, the distribution of alleles (data points) for a given sample initially shows increased variation, followed by decreased variation, and finally, an overall reduction in the number of alleles. As the concentration of inhibitor increases over the range of tested, there is a trend of reduction in mean value for allele intensity (peak height and peak area) data. Each phase of this pattern is indicative of inhibition, even if full profiles are still obtained at a particular concentration. The correlation coefficients for all data sets indicate the reduction in allele peak heights and areas is primarily the result of inhibitor concentration, for both hematin and humic acid-treated samples, and the allele size in base pairs is a minor factor affecting the results. The data sets obtained from peak height data for hematin and humic acid-treated samples are more significantly reduced by the inhibitors than the peak area data sets for all chemistry combinations tested; however, the difference is minor.

It is recommended that the forensic DNA community further evaluate purported forensically-relevant inhibitors with contemporary extraction/purification chemistries to determine what materials are capable of co-purifying with DNA and determining the biologically-relevant concentration ranges, by comparing these co-purified ranges to the tolerances of the current generation STR typing kits.

CHAPTER III.

THE EFFECTS OF METAL ION POLYMERASE CHAIN REACTION INHIBITORS ON RESULTS OBTAINED WITH THE QUANTIFILER® HUMAN DNA QUANTIFICATION KIT

III.1. Introduction

Forensic DNA testing laboratories across the globe employ multiplex short tandem repeat (STR) typing kits for human genetic identification and forensic casework. These analyses are conducted through the use of commercially-available kits, such as Identifiler® Plus (Applied Biosystems®, Foster City, CA) and the PowerPlex® 16 HS System (Promega Corporation, Madison, WI). These kits are designed to co-amplify targets of 15 polymorphic STR loci plus Amelogenin, a sex determining marker [39,63]. Optimal performance of these kits requires adding DNA template to PCR reagents within the working range of the kit. If too little DNA is added to the reaction, it results in the preferential amplification of small loci, increased stochastic amplification, and a failure to obtain enough product to reach interpretation thresholds; conversely, too much DNA can lead to spectral overlap (pull-up) and incomplete adenylation [43]. These phenomena can cause interpretation challenges during analysis of multiplex STR data and may be avoided by adding a quantity of amplifiable DNA within the optimal working range of the kit. It is imperative to obtain an accurate quantification estimate of the DNA prior to STR amplification for optimal performance of these kits and ease of data interpretation.

There are several commercially-available chemistry systems that provide estimates of the amount of amplifiable DNA in a sample, most of which are designed for quantitative real-time PCR (qPCR)-based platforms. The qPCR-based quantification systems used by forensic DNA testing laboratories, such as the Plexor® qPCR System (Promega Corporation, Madison, WI), the Quantifiler® Human DNA Quantification Kit (Quantifiler kit; Applied Biosystems) and the Investigator® Quantiplex Kit (QIAGEN, Hilden, Germany), share conceptual similarities in their assay designs and physical kit components. Each system includes a human-specific probe that targets an evolutionarily conserved region of the human genome. While the physical design varies between manufacturers, these probes contain a fluorescent reporter dye whose fluorescence is altered as a consequence of target amplification and allow the amount of emitted fluorescent signal to be detected during each PCR cycle [64-66]. All qPCR-based quantification methods compare the fluorescent signal measured in a sample to a passive reference signal; this is a fluorescent component that does not change as a result of cycles of PCR. During absolute quantification, the cycle number of the PCR where there is a measureable difference between a sample's fluorescent signal and the passive reference signal is recorded as the quantification cycle (Cq); absolute quantification estimates are derived by comparing a sample's Cq to an external calibration curve from a dilution series of reference standards [32,67-68].

All qPCR assays are sensitive to factors that affect all types of PCR amplifications. Quantification results are estimates, not actual measurements, of the quantity of DNA in a sample and may not always reflect the true quantity present. Anything that changes the PCR conditions, such as the availability of reagents, pH or salt concentration, DNA polymerase function, or access to the DNA template, can impact the accuracy of the results [69]. Assessment of the individual

quantification reactions and detection of substances that can inhibit PCR is aided by the inclusion of an internal PCR control (IPC) in each of the aforementioned quantification systems [64-66].

Early studies aiming to identify the presence of a PCR inhibitor in a DNA sample were conducted using spiking experiments by either adding the DNA sample to the positive control reaction or by adding the positive control sample to the reaction containing the DNA sample. In either case, failure to obtain amplification results from the positive control sample would serve as confirmation of the presence of an inhibitory compound in the DNA sample [6,70]. The inclusion of IPCs into qPCR chemistry kits offers the same benefits provided by a positive control sample, evaluating reagent quality and instrument performance, as well as the possibility of identifying the presence of PCR inhibitors in a single, standardized reaction. The developmental validation studies and user manuals for these systems state that a negative IPC result with a negative human result is evidence of PCR inhibition and recommends treating the sample with dilution or additional purification; however, a positive IPC result with a negative human result is presented as "no human DNA detected," "confirmation of negative results," and "something that should instill confidence in the quantification results" [64-65,68].

Problems associated with the use of PCR-based assays for inhibitor detection and reliable quantification of inhibited samples have been presented in the scientific literature. It has been demonstrated that the effect of inhibitors on different PCR reactions is variable and there is no correlation between amplicon characteristics and the extent of inhibition, making it imperative to match the susceptibility to inhibitors between reference and experimental reactions to increase the accuracy and reduce the error of PCR-based quantification assays [71]. The potential of inhibitors to confound quantitative assessments of DNA quantity and increase the uncertainty of the values obtained has also been extensively studied [72]. Recognition of these issues has led to the

development of statistical methods that examine amplification efficiency in order to improve quantification estimates and the detection of the presence of inhibitors, including identification of kinetic outliers and multivariate analysis of the dynamic phase of amplification between samples and controls [73-75]. The developers of these methods acknowledge that certain types of inhibition may not be detected, even using these advanced mathematical analyses.

The IPC will detect a PCR inhibitor, assuming the inhibitor blocks requisite reagents, inactivates or interferes with the processivity of the polymerase, and in some cases, binds to the DNA template [54]. In order for this inhibitor-DNA complex to be detected by an IPC, there must be sufficient inhibitor in the reaction to affect the IPC template and the sample template. Metal ions have the potential to function as inhibitors of PCR-based STR assays and are present in a variety of forensically-relevant sample types, *e.g.*, bones [76]. Furthermore, metal ions can form soluble, high molecular weight complexes with humic substances in soils, have been implicated in adduct-formation with DNA, and can form crosslinks between DNA and proteins [5,34-35,37]. These interactions can reduce the efficiency of DNA extraction techniques and impair access to the DNA template during PCR. Metal ions, including calcium, can also competitively inhibit DNA polymerases [54,77]. If metal ion-template interactions prevent the DNA from being retained during extraction or are co-isolated as a complex, it is unlikely the inhibitor would be detected by an IPC assay because the inhibitor would not be available to interact with the IPC template. Due to the presence of metal ions in forensic samples and interactions with DNA that can reduce their detection by an IPC, it is important to elucidate their effects on qPCR assays.

The purpose of the following studies was to evaluate the ability of the Quantifiler kit to accurately estimate the concentration of amplifiable DNA in metal-treated DNA samples. Taking into consideration the potential challenges of attempting to quantify inhibited DNA samples, two

questions are addressed: 1) Are DNA samples that include metal ions accurately quantified? and, 2) Does the IPC detect the presence of metal ion PCR inhibitors? Because metal ions have the potential to interact directly with DNA and inhibit PCR, it is important to elucidate the reliability of results obtained from PCR-based methods in their presence and the ability of the IPC to differentiate a true negative from an inhibited sample.

III.2. Materials and Methods

III.2.1. DNA sample preparation

Samples were prepared using solutions of purified aluminum (Al), calcium (Ca), copper (Cu), iron (Fe), nickel (Ni), and lead (Pb) obtained in the form of certified analytical standards (High-Purity Standards, Charleston, SC). An aliquot of each standard was diluted to 21 mM and the pH adjusted (between 3.8 and 5.8) using 3 M NH_4OH and 1 M HCl. Dilutions of the metal stock solutions were created using UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA). Experimentally-treated DNA samples were prepared in duplicate by combining 9 µL of inhibitor solution and 1 µL of 1 ng/µL 2800M DNA (Promega). The naming convention used to describe the inhibited samples reflects the concentration of metal based on the initial units provided and not actual measurements after the addition of DNA; this convention does not reflect the final concentration in the quantification or STR amplifications (**Table 8**). The range of metal concentrations used to treat DNA samples was consistent with those previously determined to inhibit STR amplifications [76]. Duplicate positive and negative control samples were also tested. The final concentration of DNA in the inhibitor-treated and positive control samples was 0.1 ng/µL, chosen to reflect the optimal concentration of DNA to add to an STR amplification.

Table 8
The DNA samples are labeled with the naming convention used to describe the approximate metal concentration mixed with 1 ng DNA. The approximate concentrations of metal in the PCR reactions, after samples were combined with reagents required for each assay, are included.

Final Concentration of Metals (mM) in:		
DNA Sample	qPCR Reaction	STR Reaction
0.0025	0.0002	0.001
0.0150	0.0012	0.006
0.0750	0.0060	0.030
0.5000	0.0400	0.200
1.8250	0.1460	0.730
3.1250	0.2500	1.250
7.0000	0.5600	2.800
11.000	0.8800	4.400
14.750	1.1800	5.900
18.750	1.5000	7.500

III.2.2. DNA quantification

Amplification was performed using the Quantifiler kit. A master mix, consisting of 12.5 µL Quantifiler® PCR Reaction Mix and 10.5 µL Quantifiler® Human Primer Mix (Applied Biosystems) per sample, was prepared according to the manufacturer's instructions; 23 µL of master mix was distributed into the requisite wells of a 96-well optical reaction plate [32]. Total reaction volume for qPCR was 25 µL, which included the master mix plus 2 µL of DNA-metal sample at 0.1 ng/µL DNA.

Absolute quantification was performed using a 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions [32]. Amplification was performed in 9600 emulation mode using the following parameters: 95°C hold for 10 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data was collected during the 60°C stage for all cycles. The following data were obtained using SDS Software v.1.2 (Applied Biosystems): IPC quantification

cycle (Cq), human Cq, human DNA quantity, and the mean (μ) and standard deviation (σ) for duplicate samples, when available.

III.2.3. Analysis of DNA quantification results

Microsoft® Excel® 2013 (Microsoft Corporation, Redmond, WA) was used to calculate the following values: mean and σ for duplicate samples with manually-assigned Cq values; percent relative standard deviation (% RSD) for all sample pairs; mean Cq values for the pairs of Quantifiler® Human DNA and IPC; and, the difference in mean Cq values between metal-treated samples and their respective positive control samples (Δμ). It was also used to perform a regression analysis to compare the Quantifiler® Human and IPC Cq values to those obtained from the respective positive control Cq values. Samples with "undetermined" Cq values were manually assigned a Cq value of 40.00 to reflect the maximum number of cycles of PCR.

III.2.4. Preparation of copper-treated-6-carboxyfluorescein samples[1]

DNA quantification results of Cu-treated samples indicated the need for subsequent examination of potential interactions between the Cu metal solution and the dye used to label the Quantifiler® Human DNA probe, 6-carboxyfluorescein (Life Technologies) (personal communication with Life Technologies on June 11, 2013). Cu solutions, consistent with the range of concentrations used in the quantification reactions, were prepared with a uniform 2 nM concentration of 6-FAM dye (Invitrogen). Stock solutions and subsequent dilutions were prepared with an in-house preparation of 1X *Taq* DNA polymerase PCR buffer, 20 mM Tris-HCl, 50 mM KCl, and the pH adjusted to 8.4 using 3 M NaOH (GR ACS-grade; EMD Chemicals, Darmstadt, Germany).

[1] Preparation of copper-treated 6-carboxyfluorescein samples and fluorescence spectroscopy experiments were conducted by the Department of Chemistry at the University of North Texas.

III.2.5. Fluorescence spectroscopy[1]

Cu-treated 6-FAM solutions were placed in a quartz cuvette with a 1 cm optical path length. The excitation wavelength was 491 nm and the emission spectrum was recorded from 495 nm to 600 nm using a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian, Inc., Walnut Creek, CA). Three replicate measurements were recorded and a mean and σ were calculated for each sample.

III.3. Results

III.3.1. Estimated concentration of amplifiable DNA in metal-treated DNA samples

The standard curve parameters for each of the plates used in this study are listed in **Table 9**. The slope values are consistent with the typical range (-3.3 to -2.9) reported by the manufacturer [32], except for the Pb plate where the slope exceeded the lower-boundary of its range; however, this value was within the typical range observed in the laboratory. The intercepts reflect the expected Cq values for a 1 ng/μL DNA sample. These R^2 values indicate a close fit between the standard curve regression line and the individual data points for the standard reactions used to obtain DNA concentration estimates for experimental samples.

Table 9
Standard curve parameters, inclusive of all standard data points, for the Al, Ca, and Cu plate (Plate 1), the Fe and Ni plate (Plate 2), and the Pb plate (Plate 3).

Plate	Slope	Intercept	R^2
1	-3.132	28.52	0.998
2	-3.224	28.51	0.998
3	-3.947	29.43	0.998

The mean concentration of DNA in metal-treated samples, percent relative standard deviation for the mean (% RSD), and mean expressed as a percent of the DNA concentration in the positive control sample (%Pos) are listed in **Table 10** for Al, Ca, and Cu (Plate 1), **Table 11** for Fe and Ni (Plate 2), and **Table 12** for Pb (Plate 3). The results from each series of metal-treated

DNA samples produced a different dose-dependent pattern of inhibitory effect. At metal concentrations of 0.0025 and 0.0150 mM, five out of six samples had DNA concentration estimates < 25.0% RSD. As the concentration of metal was increased to 0.0750 mM, only two of six samples yielded < 25.0% RSD. At a concentration of 0.5000 mM, the quantity of DNA in Al-treated samples was undetermined, and only two of the other five samples had DNA concentrations < 25.0% RSD. Cu, Ni, and Pb, the only three metals tested at 1.8250 mM, all had < 25% RSD; however, they had mean DNA concentrations less than that of their respective positive control samples. At a concentration of 3.1250 mM metal, the quantity of DNA in Al- and Fe-treated samples was undetermined, Ca- and Ni-treated samples had > 25.0% RSD, and only the Cu- and Pb-treated samples had < 25% RSD.

Table 10

Samples treated with Al, Ca, and Cu. Mean concentration of DNA (ng/µL), percent relative standard deviation (% RSD), and mean (µ) expressed as a percent of the DNA concentration in the positive control sample (%Pos).

Metal Conc. (mM)	Al			Ca			Cu		
	µ (ng/µL)	% RSD	%Pos[a]	µ (ng/µL)	% RSD	%Pos[a]	µ (ng/µL)	% RSD	%Pos[a]
0.0025	0.084	17	94	0.12	31	134	0.12	3.2	139
0.0150	0.061	7.0	69	0.14	0.93	152	0.057	13	64
0.0750	0.024	51	27	0.079	13	89	0.086	1.0	97
0.5000	Undet.[b]			0.16	1.8	129	0.059	69	67
1.8250	NT			NT			0.080	5.4	89
3.1250	Undet.[b]			0.076	30	85	0.11	8.9	126
18.750	Undet.[b]			0.066	28	74	11	39	12234

[a] Positive control sample: µ = 0.089 ng/µL; 8.4% RSD

[b] Denotes samples with manually-assigned Cq values = 40.00

Undet. = undetermined; NT = not tested

Table 11

Samples treated with Fe and Ni. Mean concentration of DNA (ng/µL), percent relative standard deviation (% RSD), and mean (µ) expressed as a percent of the DNA concentration in the positive control sample (%Pos).

Metal Conc. (mM)	Fe			Ni		
	µ (ng/µL)	% RSD	%Pos[a]	µ (ng/µL)	% RSD	%Pos[a]
0.0025	0.076	24	93	0.089	16	110
0.0150	0.073	6.6	90	0.092	39	113
0.0750	0.056	45	69	0.093	33	115
0.5000	0.061	52	75	0.079	31	97
1.8250	NT			0.053	1.1	65
3.1250	Undet.[b]			0.018	50	23
18.750	Undet.[b]			Undet.[b]		

[a] Positive control sample: µ = 0.081 ng/µL; 7.9% RSD
[b] Denotes samples with manually-assigned Cq values = 40.00
NT = not tested; Undet. = undetermined

Table 12

Samples treated with Pb. Mean concentration of DNA (ng/µL), percent relative standard deviation (% RSD), and mean (µ) expressed as a percent of the DNA concentration in the positive control sample (%Pos).

Metal Conc. (mM)	Pb		
	µ (ng/µL)	% RSD	%Pos[a]
0.0025	0.15	6.3	136
0.0150	0.15	2.0	131
0.0750	0.16	32	144
0.5000	0.14	9.1	129
1.8250	0.065	11	59
3.1250	0.095	12	85
7.0000	0.017	31	15
11.000	Undet.[b]		
14.750	Undet.[b]		
18.750	Undet.[b]		

[a] Positive control sample: µ = 0.11 ng/µL; 23% RSD
[b] Denotes samples with manually-assigned Cq values = 40.00
Undet. = undetermined

III.3.2. Comparison of the Cq values between metal-treated DNA samples and untreated controls

The Cq values for the Quantifiler® Human DNA detector and the IPC detector for each experimentally-treated sample are compared to the mean Cq values obtained for the respective detector in the positive control samples (**Fig. 6**). A logarithmic transformation of the metal concentration used to treat DNA was used to improve the uniformity and linearity of data points for graphical display.

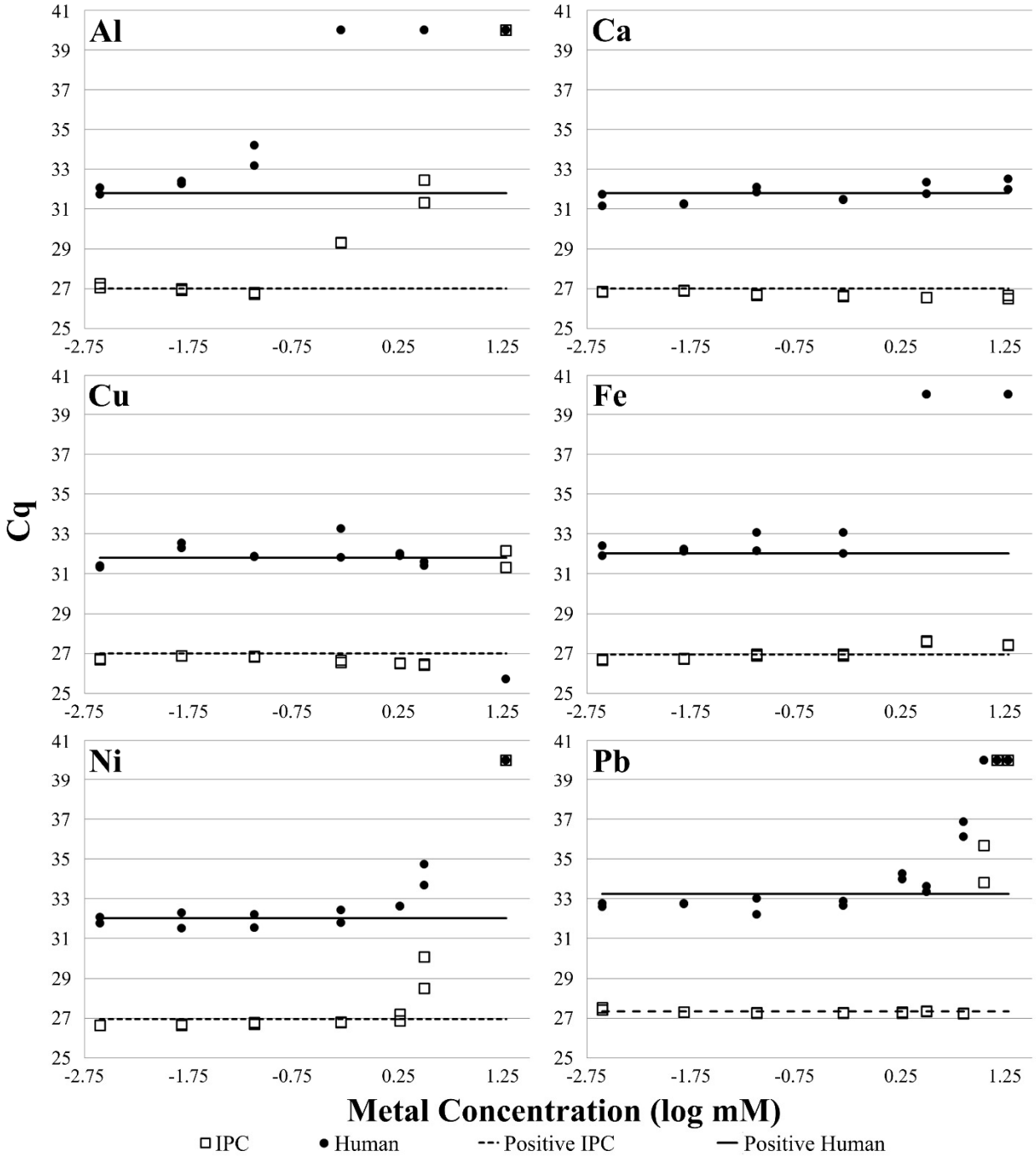

Fig. 6. Cq values obtained from metal-treated samples and controls quantified with the Quantifiler® Human DNA Quantification Kit. 2800M DNA samples were prepared in duplicate with metal solutions and quantified using the Quantifiler kit. Cq values were obtained from the IPC and human quantification assays and compared to the log of the metal concentration in the sample. Horizontal lines are provided as a visual reference for comparison between the Cq values obtained from metal-treated samples to those obtained from the positive control samples; the dashed reference lines indicate the Cq values obtained for the IPC assay and the solid reference lines indicate the Cq values obtained for the human assay.

The Δμ between Cq values was used to quantify the departure of an experimentally-treated sample from the respective positive control sample for both the IPC and Quantifiler® Human DNA detectors (**Table 13**). Samples with a Δμ of +/-1 were determined to be demonstrably affected by treatment with metal.

The level of Al sufficient to cause a shift of the IPC detector was 0.5000 mM. The mean Cq obtained for the Quantifiler® Human detector yielded a shift at a lower concentration than the IPC detector, 0.0750 versus 0.5000 mM Al. There were no Ca-treated samples that exhibited demonstrable Cq shifts for either the IPC or Quantifiler® Human detectors across the range tested: 0.0025 to 18.750 mM. Most samples treated with Cu were also not affected, with the exception of the samples treated with 18.750 mM Cu. The mean Cq values obtained for the IPC detector in Fe-treated samples did not indicate an inhibitory effect of Fe; however, the Quantifiler® Human detector exhibited a shift in mean Cq for samples treated with 3.1250 and 18.750 mM Fe. Shifts in mean Cq values for both detectors were observed for samples treated with 3.1250 mM and 18.750 mM Ni. The mean Cq values for the Quantifiler® Human detector had observable shifts in samples treated with 7.0000, 11.000, 14.75, and 18.750 mM Pb; however, a shift in the IPC Cq was only observed at 11.000, 14.75, and 18.750 mM Pb.

Table 13

The Δμ between Cq values was calculated for both the Quantifiler® Human DNA and IPC detectors and used to quantify the departure of a metal-treated sample from the respective positive control sample. Samples with a Δμ of +/-1 were determined to be demonstrably affected by treatment with metal (values in bold font).

Metal Conc. (mM)	Al		Ca		Cu		Fe		Ni		Pb	
	Human	IPC	Human	IPC	Human	IPC	Human	IPC	Human	IPC	Human	IPC
0.0025	0.10	0.15	-0.36	-0.16	-0.45	-0.28	0.11	-0.28	-0.12	-0.32	-0.56	0.12
0.0150	0.51	-0.06	-0.57	-0.10	0.61	-0.13	0.14	-0.23	-0.12	-0.30	-0.49	-0.04
0.0750	**1.88**	-0.24	0.17	-0.30	0.04	-0.17	0.58	-0.04	-0.16	-0.23	-0.63	-0.08
0.5000	**>8.19** [a]	**2.31**	-0.34	-0.36	0.74	-0.39	0.50	-0.04	0.08	-0.19	-0.47	-0.07
1.8250	Not tested		Not tested		0.16	-0.50	Not tested		0.59	0.06	0.88	-0.07
3.1250	**>8.19** [a]	**4.89**	0.25	-0.44	-0.31	-0.56	**>7.97** [a]	0.64	**2.18**	**2.32**	0.25	0.00
7.0000	Not tested		Not tested		Not tested		Not tested		Not tested		**3.26**	-0.11
11.000	Not tested		Not tested		Not tested		Not tested		Not tested		**>6.77** [a]	**7.40**
14.750	Not tested		Not tested		Not tested		Not tested		Not tested		**>6.77** [a]	**>12.66** [a]
18.750	**>8.19** [a]	**>13.01** [a]	0.44	-0.43	**-6.49**	**4.74**	**>7.97** [a]	0.47	**>7.97** [a]	**>13.04** [a]	**>6.77** [a]	**>12.66** [a]

[a] Denotes samples with manually-assigned Cq values = 40.00

III.3.3. Fluorescence spectroscopy results

Fluorescence emission measurements obtained from Cu-treated 6-FAM samples are displayed in **Table 14**. The characteristic emission peak of 6-FAM in *Taq* DNA polymerase PCR buffer was observed at 513 nm when excited at 491 nm. No significant increase in the fluorescence of 6-FAM in the presence of Cu metal was observed. Minimal variation was detected between samples across the range of Cu tested, with standard deviation values ranging from a minimum of 0.006 to a maximum of 0.04.

Table 14
Fluorescent emission measurements obtained from Cu-treated 6-FAM samples

Cu (mM)	Emission (a.u.)
0.0002	0.880 ± 0.03
0.0012	0.876 ± 0.006
0.0060	0.900 ± 0.04
0.0400	0.859 ± 0.009
0.1460	0.890 ± 0.03
0.2500	0.947 ± 0.01
0.5600	0.904 ± 0.01
0.8800	0.793 ± 0.03

III.4. Conclusions and Discussion

This study addressed two aspects of PCR-based DNA quantification using the Quantifiler kit, the accuracy of estimated DNA concentration in metal-treated samples and ability of the IPC detector to indicate the presence of metal ion PCR inhibitors. Comparing the estimated DNA concentration in metal-treated DNA samples with values obtained for positive control samples that were prepared with the same DNA concentration, conclusions can be drawn with respect to the accuracy of estimates for each of the six metals across the range of metal concentrations tested. In addition, the ability of the IPC included in the Quantifiler kit to detect the presence of metal ion PCR inhibitors can be evaluated using the instructions published in the Quantifiler® Kits User's

Manual that defines an IPC Cq of < 30 to be negative for the presence of PCR inhibitors, except in the case of samples whose human DNA quantity is > 10 ng/μL [32].

DNA samples treated with 0.0025 and 0.0150 mM Al were consistent with estimates of DNA concentration in the positive control samples. IPC Cq values for samples treated with ≤ 0.5000 mM Al did not exceed the defined negative value of 30, despite undetermined human DNA quantification results; however, the IPC assay present in samples treated with ≥ 3.1250 mM Al did detect the presence of the inhibitor (**Fig. 6**). All Ca-treated samples produced DNA quantification estimates that were consistent with the positive control samples and no sample yielded an IPC Cq value sufficient to indicate the presence of a PCR inhibitor. Similarly, samples treated with ≤ 14.750 mM Cu had DNA concentration estimates that were consistent with the positive control samples and those samples also did not have sufficient IPC Cq values to indicate the presence of a PCR inhibitor. DNA samples treated with ≤ 0.5000 mM Fe were consistent with DNA quantification results obtained from the positive control samples and none of the IPC Cq values indicated the presence of PCR inhibitor, despite undetermined human DNA quantification values for samples treated with ≥ 14.750 mM Fe. Ni-treated samples produced DNA quantification results that were consistent with the positive control samples ≤ 1.8250 mM Ni. Samples treated with 3.1250 yielded reduced quantification estimates with IPC Cq values of 28.48 and 30.02, which indicate the potential for Ni inhibitor to be detected in some samples with this concentration of Ni. Samples treated with 18.750 mM Ni had undetermined results for both detectors, demonstrating the ability of the IPC detector to detect the inhibitor at that concentration of Ni. Samples treated with Pb had DNA quantification results that were consistent with the positive control samples ≤ 7.0000 mM Pb. The IPC Cq values did not indicate the presence of a PCR inhibitor until the Pb concentration reached 14.750 mM. Among the metals examined, with the exception of Al, the

Quantifiler kit accurately quantifies human DNA in the presence of ≤ 0.5000 mM Ca, Cu, Fe, Ni, and Pb and does not indicate the presence of a PCR inhibitor. The Quantifiler kit is more sensitive to the presence of Al compared to the other metals, as quantification estimates are accurate only at ≤ 0.0750 mM Al and its presence is not detected by the IPC at that concentration. This kit is most resistant to the presence of Ca, which does not affect either the estimated concentration of DNA in a sample or the amplification of the IPC ≤ 18.750 mM, the maximum concentration tested in these experiments. Results have been successfully obtained from skeletal remains samples using the Quantifiler kit, therefore obtaining results from Ca-inhibited samples was consistent with expectations that the presence of Ca would be well-tolerated by the system. Studies aimed at investigating the mechanism of PCR inhibition caused by Ca have suggested that it is primarily a *Taq* polymerase inhibitor, using a different assay, due to a reduction in amplification efficiency that was marginally improved by adding more polymerase to the reaction [54]. Our results show a slight decrease in the amplification efficiency of the human DNA target at 3.1250 and 18.750 mM, evidenced by minor increases in Cq values; however, the IPC assay exhibited a minor decrease in Cq values at the same concentrations, suggesting an increase in the IPC amplification efficiency. These results are inconsistent with the suggestion that Ca is a *Taq* polymerase inhibitor, which would decrease the efficiency of both assays.

An unanticipated result was observed during the analysis of samples treated with 18.750 mM Cu. Despite preparation at 0.1 ng/µL of 2800M DNA, the estimated mean concentration of human DNA in this sample pair was 10.93 ng/µL. The IPC Cq values were 31.32 and 32.14 for these paired samples, which initially appeared to reflect suppressed amplification of the IPC detector that can occur in samples with a higher quantity of human DNA. Examination of the amplification plots for these samples did not reveal anything suspicious; they visually appeared

consistent with the quantification results obtained. This concentration of Cu-treatment was repeated in two additional quantification runs and the results were consistently higher than expected, although the estimate of DNA quantity ranged from 1.05 to 13.97 ng/μL. In addition, negative template control (NTC) samples treated with 18.750 mM Cu also produced quantification estimates, even though all reagents had separately passed quality control tests to insure they were negative for human DNA content. Fluorescence spectroscopy examination of Cu and the 6-FAM dye used to label the human DNA detector was conducted to determine if the metal was increasing the fluorescent properties of the 6-FAM dye. These experiments failed to elicit an increase in fluorescence intensity when the components were tested in isolation, outside the real-time PCR environment. Subsequent examination of the results using the component view in the SDS Software for the Cu-treated DNA and the Cu-treated NTC samples demonstrate the increase in 6-FAM fluorescence occurs contemporaneously with suppression of the ROX-labeled baseline fluorescent signal, which may account for the increased estimates in DNA concentration. These plots are displayed with those obtained from samples demonstrating inhibitor-induced phenomena, as well as positive and negative control samples in **Fig. 7**. The component view plots illustrate the change in fluorescence (y-axis) over the 40 PCR cycles (x-axis); all plots have been normalized to the scale of the positive control sample and contain an inset plot displaying the default fluorescent scale that was obtained for each sample. The normalized scale is presented to offer a more direct comparison between inhibited and control samples, while the default scale is what is immediately observable to a user when the component view tab is selected in the results section of the SDS Software. **Fig. 7** (upper left) displays the component view of a positive control sample, which illustrates no change in fluorescence from the ROX-labeled baseline component (blue) during the 40 cycles of PCR amplification, and exponential growth of the fluorescence from the VIC-labeled

IPC (green) and 6-FAM-labeled Quantifiler® Human DNA (red) components. Similarly, **Fig. 7** (upper right) is the plot obtained from a no template control (NTC) sample that has exponential growth of only the IPC component with static fluorescence of the baseline and Quantifiler® Human DNA components. Both of these plots illustrate normal results for a positive and negative control sample, respectively. DNA treated with 18.75mM Al in **Fig. 7** (center left) illustrates complete inhibition of amplification for both the Quantifiler® Human DNA and IPC components, although the baseline component is unaffected; whereas DNA treated with 18.750 mM Pb in **Fig. 7** (center right) shows no change in fluorescence of baseline and IPC components, but does show a gradual, sub-threshold increase in fluorescence of the Quantifiler® Human DNA component. Most interesting is the increase in fluorescence in the Quantifiler® Human DNA component in the Cu-treated samples, DNA treated with 18.750 mM Cu and a NTC treated with 18.750 mM Cu, **Fig. 7** (lower left and lower right, respectively). Both plots show static fluorescence from the IPC component with gradual suppression of the ROX-labeled baseline component over the 40 cycles of PCR amplification. Subsequent fluorescence spectroscopy experiments eliminated isolated interactions between the Cu metal and 6-FAM fluorophore dye used to label the Quantifiler® Human DNA detector as the cause of this increased fluorescence. The cause of the variable quantification results obtained from samples treated with 18.750 mM Cu remains unknown; however, it is possible the effect is DNA dependent and/or a consequence of the ROX suppression observed in these component plots.

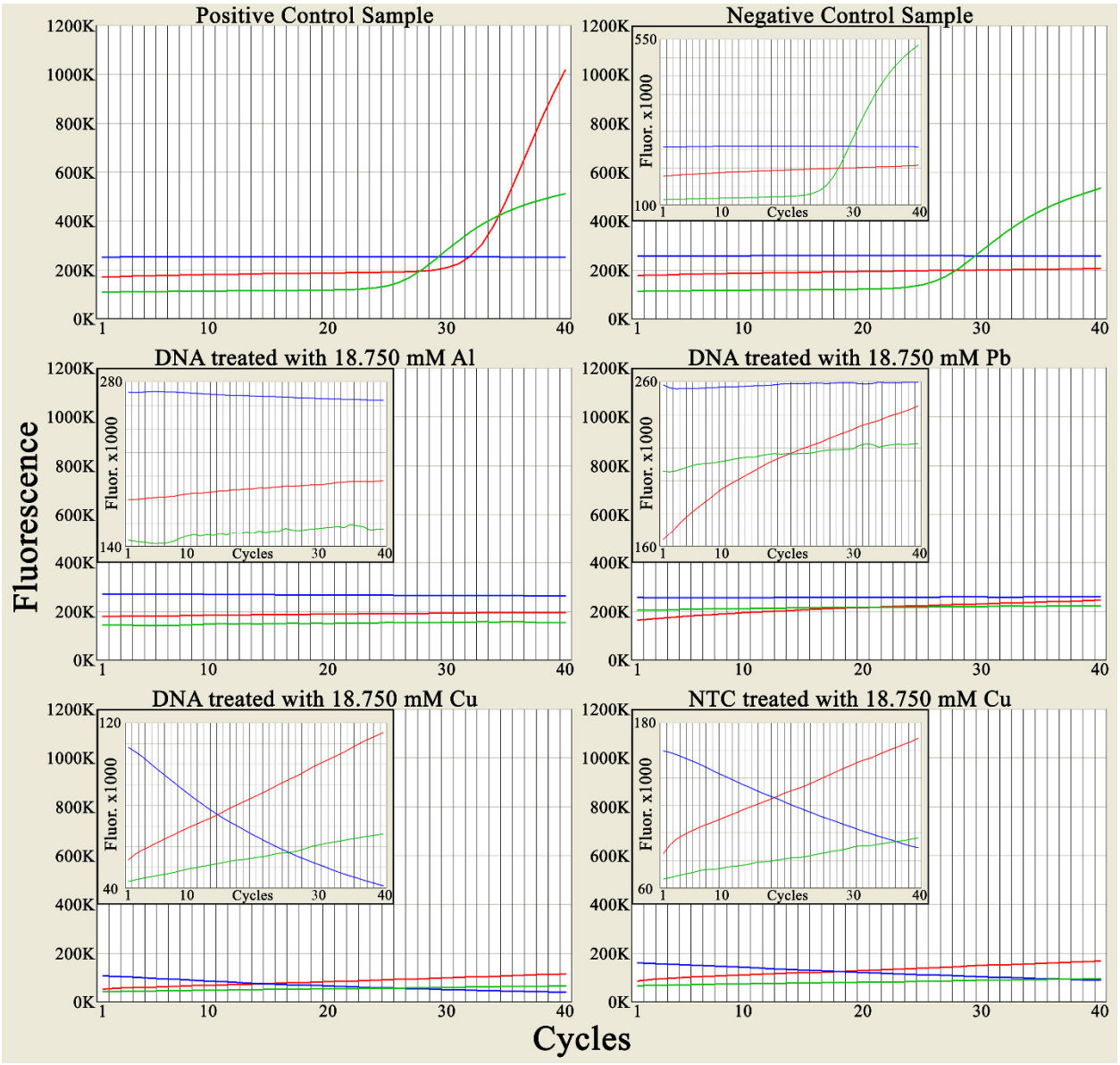

Fig. 7. Component view of selected samples and controls, displaying fluorescence (normalized scale, 0K to 1200K) measured across cycles of PCR (0 to 40). 2800M DNA samples were prepared with metal solutions and quantified in duplicate using the Quantifiler® Human DNA Quantification Kit on a 7500 Real-Time PCR System. Sample plots were obtained using SDS Software v1.2 and includes fluorescence obtained from the ROX-labeled baseline component (blue), VIC-labeled IPC component (green), and 6-FAM-labeled human DNA component (red). Inset plots illustrate fluorescence (default scale; varies by sample) across cycles of PCR (0 to 40). Default fluorescence scale for inset plots times 1000 (Fluor. x1000), are as follows: negative control sample, 100 to 550; DNA treated with 18.750 mM Al, 140 to 280; DNA treated with 18.750 mM Pb, 160 to 260; DNA treated with 18.750 mM Cu, 40 to 120; NTC treated with 18.750 mM Cu, 60 to 180.

In cases where inhibition is less severe and the IPC exhibits exponential growth in the component view, some types of inhibitors may still be present in a sample and affect amplification of the human target, while failing to be detected by the IPC assay. One inhibitory mechanism that may go undetected by IPC is sequestration of DNA in solution, preventing access to the template [33]. Metal ions may inhibit PCR through this mechanism, as they have been implicated in adduct-formation directly with DNA. Metals may also produce substantial crosslinks between DNA and proteins and can even form soluble, high molecular weight complexes with humic substances and DNA [34-37]. If the metal ions are directly interacting with the human DNA template prior to being combined with quantification reagents, it is plausible that there is insufficient unbound metal inhibitor in solution to prevent amplification of the IPC template. This possibility is supported by the detection of most metals by the IPC at higher concentrations. Metal inhibitors have demonstrated the potential to negatively affect the Quantifiler® Human DNA detector at a lower concentration than the IPC detector. The apparent mechanism of metal ion inhibition differs from that of the hematin model used in the developmental validation of the Quantifiler kit and humic acid inhibition described in recent studies; both demonstrated identification of inhibited samples by the IPC detector at a lower concentration than the human DNA detector [65,78]. Samples with negative human DNA results and normal IPC Cq values may still produce full STR profiles without additional purification of the sample [79]. Another study reported 27% of low-quantity samples with “undetermined” quantification values tested produced at least partial STR profiles [80]. These observations are important to relate to samples that may contain PCR inhibitors, as inhibition effectively decreases the amplifiable concentration of a DNA sample. Caution must be exercised when ascertaining whether to discontinue DNA testing of a sample, based solely on a negative result for the human DNA detector and failure of the IPC to indicate the presence of a

PCR inhibitor. Based on these results, laboratory personnel are encouraged to conduct forensic DNA testing of negative samples in cases with limited evidence that may contain inhibitors (*e.g.*, bone, aged blood, environmentally-challenged samples, samples that are contaminated with soil, etc.). DNA extracted from bone samples recovered from burial environments are inherently susceptible to producing false negative quantification results due to the presence of endogenous Ca and soil-derived metals in the samples and should be tested, regardless of the quantification result.

Due to functional similarities between commercially-available qPCR DNA quantification systems, it would be advantageous for the forensic DNA community to directly assess the accuracy of quantification estimates and detection capability of their internal control assays using metal-treated DNA samples. Additional directions for future research should include examination of the effect of PCR inhibitors with different mechanisms of inhibition to determine the accuracy of DNA concentration estimates and the ability of internal controls to detect their presence. It would also be beneficial to determine the performance of various PCR-based testing systems to these inhibitors using a range of DNA concentrations, across the effective concentration of inhibitor, as the performance of a PCR-based system in the presence of a particular inhibitor may be increased when additional template DNA is included in the reaction.

CHAPTER IV.

METAL IONS AS FORENSICALLY-RELEVANT INHIBITORS OF POLYMERASE CHAIN REACTION-BASED DNA TESTING

IV.1. Introduction

The developmental validation studies for Identifiler® Plus and the PowerPlex® 16 HS System included only the hematin and humic acid models of inhibition [13,39]. These kits contain advanced buffering systems that are designed to provide improved results with challenged sample types, such as bone; however, neither Identifiler® Plus or PowerPlex® 16 HS have established performance in the presence of calcium or other bone-associated inhibitors. Calcium is the primary mineral component of skeletal connective tissue, but other metals are present in endogenous samples and may be introduced through exposure to burial environments [1,19]. Aluminum (Al), calcium (Ca), copper (Cu), iron (Fe), nickel (Ni), and lead (Pb) were selected because there is existing documentation that: 1) they are naturally present at high levels in the environment, 2) they have been previously detected in human remains and/or fossilized bone (**Table 15)**, and 3) there is existing evidence they may inhibit PCR [2-5,29,31].

Table 15
Reported levels of metals (ng/g) measured in contemporary (cadaver, autopsy and surgical) specimens versus buried and archaeological bone samples.

Analytes	Cadaver, Autopsy and Surgical Specimens	Buried and Archaeological Bone Samples
^{27}Al	180 – 1,600 [81]	24 – 10,000,000 [4,82]
^{63}Cu	150 – 14,000 [81,83]	1 – 86,000 [4,84]
^{57}Fe	450,000 – 530,000 [83,84]	3 – 6,500,000 [4,82]
^{60}Ni	7,300 – 11,000 [84]	1 – 21,000 [82,84]
^{208}Pb	340 – 57,000 [85,86]	1 – 7,400 [82,84]

The purpose of this study was to determine whether metal ions function as inhibitors of the Identifiler® Plus PCR Amplification Kit and the PowerPlex® 16 HS System, and to determine the levels of the selected metal ions in human skeletal remains from adjudicated unidentified remains cases. Inhibition was assessed by treating control DNA with purified metal solutions, prior to amplification. The advantage of treating control DNA, versus directly spiking PCR reactions, is that it allows for possible interactions between the metal and purified DNA that may not occur in the presence of some of the PCR reagents. This approach maximizes the detection of PCR inhibition; however, control DNA is purified and lacks organic materials, which are likely co-isolated with DNA during extraction of routine bone samples. Some organic materials known to complex with metals include partially-digested collagen fragments and humic substances; potential interactions between the metals and these sample constituents cannot be detected using this approach [6,87].

There are no published works that report quantitative values for co-isolated metals in DNA extracts obtained from bone samples. During the development of DNA extraction procedures, qualitative tests have been performed to detect the presence of calcium using ammonium oxalate

precipitation [27]. DNA extraction procedures from skeletal remains demonstrate improved results when demineralization procedures are incorporated into the methodology [28,88].

IV.2. Materials and Methods

IV.2.1 DNA sample preparation

Samples were prepared using solutions of purified Al, Ca, Cu, Fe, Ni, and Pb obtained in the form of certified analytical standards (High-Purity Standards, Charleston, SC). An aliquot of each standard was diluted to 21 mM and the pH adjusted (between 3.8 and 5.8) using 3 M NH_4OH and 1 M HCl (**Table 16**). Dilutions of the metal stock solutions were created using UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA). Experimentally-treated DNA samples were prepared in duplicate by combining 9 µL of metal solution and 1 µL of 1 ng/µL 2800M DNA (Promega). The naming convention used to describe the inhibited samples reflects the concentration of metal based on the initial units provided and not actual measurements after the addition of DNA; this convention does not reflect the final concentration in the quantification or STR amplifications (**Table 17**). Duplicate positive and negative control samples were also tested. The final concentration of DNA in the inhibitor-treated and positive control samples was 0.1 ng/µL, chosen to reflect the optimal concentration of DNA to add to an STR amplification.

Table 16
Stock metal solutions were prepared at a 21 mM concentration for Al, Ca, Cu, Fe, Ni, and Pb. The measured pH and visual description for each solution are provided.

Characteristics of 21 mM Metal Stock Solutions					
Metal	pH	Description	Metal	pH	Description
Al	4.0	Clear, colorless	Fe	3.8	Orange with red precipitate
Ca	4.2	Clear, colorless	Ni	4.3	Clear, light green color
Cu	4.9	Clear with light blue precipitate	Pb	5.8	Clear with white precipitate

Table 17
The DNA samples are labeled with the naming convention used to describe the approximate metal concentration mixed with 1 ng DNA. The approximate concentrations of metal in the PCR reactions, after samples were combined with reagents required for each assay, are included.

Final Concentration of Metals (mM) in:		
DNA Sample	qPCR Reaction	STR Reaction
0.0025	0.0002	0.001
0.0150	0.0012	0.006
0.0750	0.0060	0.030
0.5000	0.0400	0.200
1.8250	0.1460	0.730
3.1250	0.2500	1.250
7.0000	0.5600	2.800
11.000	0.8800	4.400
14.750	1.1800	5.900
18.750	1.5000	7.500

IV.2.2 DNA amplification

All DNA amplifications were performed in duplicate using AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Applied Biosystems, Foster City, CA) and PowerPlex 16 HS® System (Promega Corporation), according to the manufacturer's instructions for 25 µL PCR reaction setup and thermocycling parameters [43,44].

Experimental amplifications included a separate set of positive and negative control samples for each metal tested. The master mix used for the AmpFLSTR® Identifiler® Plus amplification setup included 10.0 µL of AmpFLSTR® Identifiler® Plus Master Mix and 5.0 µL of AmpFLSTR® Identifiler® Plus Primer Set (Applied Biosystems) per sample. The master mix used for the PowerPlex® 16 HS System amplification setup included 7.5 µL of amplification grade water, 5.0 µL of PowerPlex® HS 5X Master Mix, and 2.5 µL of PowerPlex® 16 HS 10X Primer Pair Mix (Promega) per sample. A 15 µL aliquot of the respective master mix was added to each PCR amplification tube, before adding 10.0 µL of metal-treated DNA sample.

Amplification was performed using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems), in 9600 emulation mode, for both kits. The Identifiler® Plus amplification parameters were: 95°C hold for 11 minutes; 28 cycles of 94°C for 20 seconds and 59°C for 3 minutes; 60°C hold for 10 minutes, and 4°C hold until samples removed from the thermocycler and stored refrigerated. The parameters for the PowerPlex® 16 HS System were an initial 96°C hold for 2 minutes that was followed by two cycling groups, the first consisted of 10 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 70°C for 45 seconds; and the second consisted of 22 cycles of 90°C for 30 seconds, 60°C for 30 seconds, and 70°C for 45 seconds. These cycles were followed by a final 60°C hold for 30 minutes, and 4°C hold until samples removed from the thermocycler and stored refrigerated.

IV.2.3 Analysis and genotyping

Genetic analysis of Identifiler® Plus-amplified samples was performed after combining 1 µL of product or allelic ladder with 9 µL master mix that included 8.75 µL Hi-Di™ Formamide and 0.25 µL of GeneScan™ 500 LIZ® Size Standard (Applied Biosystems). Genetic analysis of PowerPlex® 16 HS-amplified samples was performed after combining 1 µL of product or allelic ladder with 10 µL master mix that included 9 µL Hi-Di™ Formamide and 1 µL of Internal Lane Standard 600 size standard (Promega). Capillary electrophoresis of all samples was conducted using a 3 kV, 10 second injection and POP-6™ Polymer on a 3130*xl* Genetic Analyzer (Applied Biosystems). STR profiles, including individual peak heights and areas, were obtained using a 50 RFU allele detection threshold and GeneMapper® ID Software v.3.2 (Applied Biosystems).

IV.2.4 Statistical analyses

Average allele counts between pairs, expressed as a percent of the expected number of alleles in the 2800M STR profile, were used to compare results between the two systems.

Regression analysis was used to assess allele peak heights as a function of inhibitor concentration; logarithmic transformation was employed to improve the uniformity of the distribution of data points. The linear dependence of allele peak heights and peak areas to the metal ion concentration was examined through Pearson's correlation coefficients. Tests were conducted with a significance level (α) of 5%, where applicable. All statistical analyses were conducted using Microsoft® Excel 2003 (Microsoft Corporation, Redmond, WA).

IV.2.5 Preparation of skeletal remains[2]

Six pulverized bone samples from adjudicated unidentified skeletal remains cases were obtained from the University of North Texas Center for Human Identification. These samples were anonymized and transferred for research testing due to poor quality autosomal STR typing results. The samples were labeled R&D#1 through R&D#6.

Exterior surfaces of skeletal remains were decontaminated using a Dremel® rotary tool (Dremel®, Racine, WI) and a 10% bleach solution. A Dremel® sanding cone was used to remove the surface (periosteum and outer cortical layer) of a 5 by 8 cm area of the bone and sanded area was wiped using the bleach solution, as needed. A Stryker saw (Stryker Surgical Company, Kalamazoo, MI) was used to cut the sanded area into thin sections; each sample was placed into a 50 mL conical tube. A 50% bleach solution was used to immerse the cuttings that were gently agitated and soaked for 1-5 minutes, then the solution was poured off. The bone cuttings were rinsed and agitated with sufficient volumes of distilled water until the waste was clear and no longer retained the smell of bleach. A final rinse using 100% ethanol was performed before allowing the bone cuttings to dry.

[2] Preparation of skeletal remains samples was conducted by the University of North Texas Center for Human Identification.

Dried bone cuttings were enclosed in a small grinding vial with an impactor rod, immersed in liquid nitrogen, and pulverized to fine powder using a SPEX 6750 Freezer/Mill® (SPEX SamplePrep, Metuchen, NJ). The pulverized sample was weighed and placed into a labeled 15 mL conical tube. One gram of the initial sample was consumed for testing for casework analysis and the remainder transferred for subsequent testing after the case was adjudicated. Aliquots weighing 100 mg for each of the six samples were transferred for elemental analysis.

IV.2.6 Elemental Analysis of skeletal remains[3]

Elemental content was determined by inductively coupled plasma-mass spectrometry (ICP-MS) using a Bruker Aurora M90™ ICP-MS System with Autosampler (Bremen, Germany). Elemental analysis was performed in solution mode ICP-MS. Acid digestion was used for sample preparation; approximately 100 mg of each sample was dissolved in ultra-pure concentrated nitric acid (HNO_3), and then diluted with 1% HNO_3. For major and trace metal analysis, dilution factors vary between 20 and 100. All samples were measured in triplicate.

IV.3. Results

IV.3.1 Average allele counts, as a percent of the 2800M DNA profile

The average number of alleles obtained for each pair of metal-treated samples was expressed as a percent of the total number of expected alleles for the 2800M STR profile (Identifiler® Plus, **Fig. 8**; PowerPlex® 16 HS System, **Fig. 9**). Samples treated with 0.0025 mM Al produced full profiles; however, samples treated with 0.0150-0.5000 mM yielded between 9-12% of the expected STR profile. Samples treated with Ca, Cu, Fe, and Ni yielded full STR profiles up to 0.5000 mM, and, respectively, 95%, 9%, 97%, and 81% of the expected profiles were

[3] Elemental analysis of skeletal remains samples was conducted by the Department of Chemistry at the University of North Texas.

obtained from samples containing 3.1250 mM metal. Full 2800M STR profiles were obtained from samples containing up to 3.1250 mM Pb. No alleles were obtained from any samples treated with 18.750 mM of any metal and amplified with Identifiler® Plus.

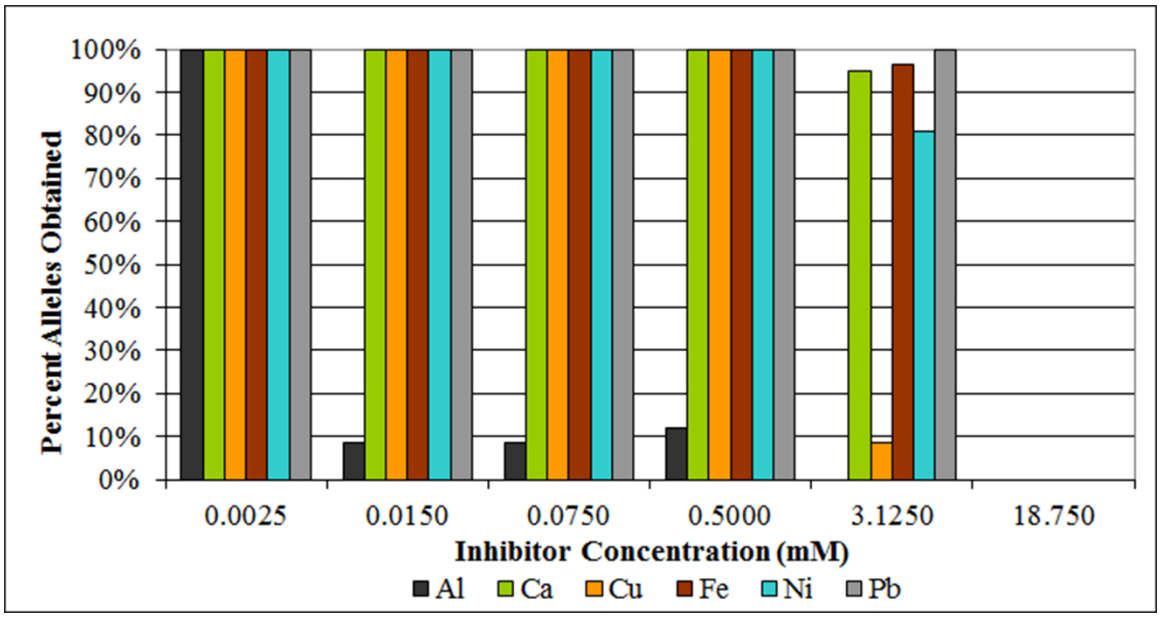

Fig. 8. Duplicate, metal-treated 2800M DNA samples amplified with the Identifiler® Plus PCR Amplification Kit were analyzed and the total alleles obtained were counted. The number of alleles for each duplicate pair were averaged and are expressed as a percent of the expected number of alleles for the complete 2800M STR profile (100% is equal to 29 alleles).

Samples amplified with the PowerPlex® 16 HS and treated with up to 0.0150 mM Al produced full profiles; however, samples treated with 0.0750-3.1250 mM yielded between 0-3% of the expected STR profile. Samples treated with Ca, Fe, Ni and Pb yielded full STR profiles up to 0.5000 mM, and, respectively, 0%, 5%, 40%, and 45% of the expected profiles were obtained from samples containing 3.1250 mM metal. Full 2800M STR profiles were obtained from samples containing up to 3.1250 mM Cu. No alleles were obtained from any samples treated with 18.750 mM of any metal and amplified with the PowerPlex® 16 HS System.

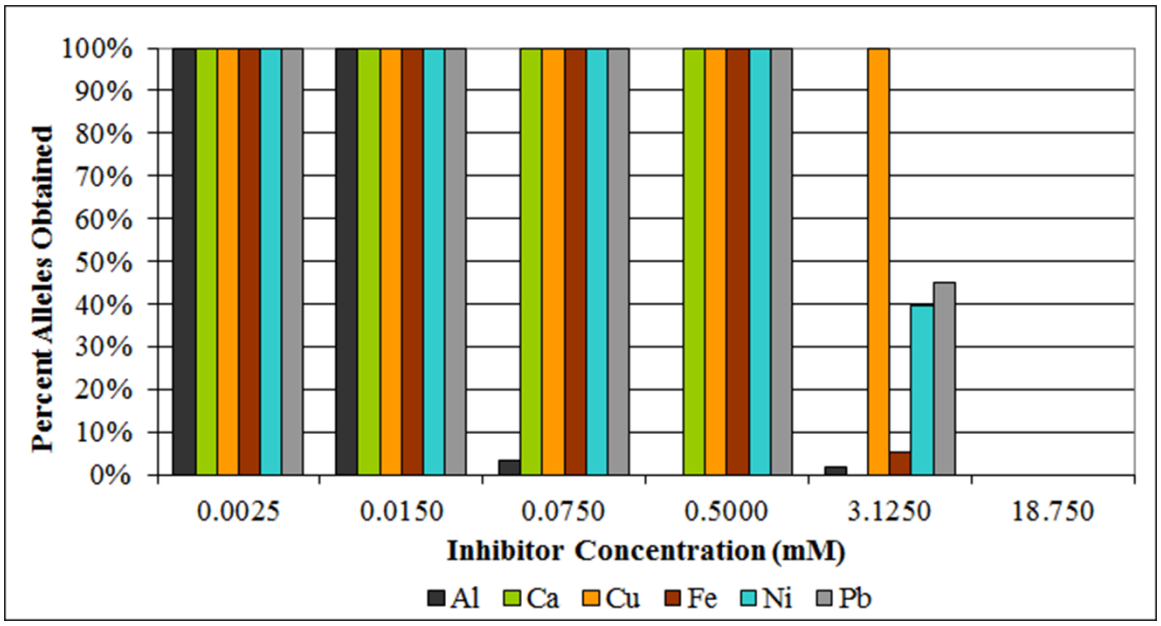

Fig. 9. Duplicate, metal-treated 2800M DNA samples amplified with the PowerPlex® 16 HS System were analyzed and the total alleles obtained were counted. The number of alleles for each duplicate pair were averaged and are expressed as a percent of the expected number of alleles for the complete 2800M STR profile (100% is equal to 29 alleles).

IV.3.2 Test of linear dependence between variables (Pearson's Correlation Coefficients)

Pearson's coefficients indicated a significant negative correlation between the concentration of metal and peak heights (**Table 18**, *lower diagonal*) and peak areas (**Table 18**, *upper diagonal*) obtained with Identifiler® Plus. Fragment size (allele length) was found to have a significant positive correlation to peak areas obtained from samples treated with Fe, Ni, and Pb. No significant correlation was found between allele length and peak heights. There were significant positive correlations found when comparing the results obtained from each set of metal-treated samples, indicating consistent results. Peak heights and areas were equally affected by the presence of metal; only Ca-treated samples yielded a difference in correlation coefficients, the correlation between Ca concentration and peak heights was -0.63 versus -0.65 for peak areas.

Table 18
Allele peak heights (*lower diagonal*) and peak areas (*upper diagonal*) were independently analyzed with respect to the length of the allele in base pairs (bp), the concentration of metal used to treat the DNA samples, and data obtained from each set of metal-treated samples amplified with the Identifiler® Plus PCR Amplification Kit. Significance at the 0.05 level for negative correlation is between -1.0 and -0.0973, and positive correlation is between 0.0973 and 1.0 (*n alleles = 406, df = 404*). Significant values are indicated by bold typeface.

Height \ Area	Size (bp)	Metal conc.	Al	Ca	Cu	Fe	Ni	Pb
Size (bp)	•	-7.7×10^{-17}	0.058	0.061	0.011	**0.10**	**0.14**	**0.10**
Metal conc.	-7.7×10^{-17}	•	**-0.29**	**-0.65**	**-0.47**	**-0.60**	**-0.61**	**-0.60**
Al	0.017	**-0.29**	•	**0.36**	**0.57**	**0.60**	**0.37**	**0.58**
Ca	-0.047	**-0.63**	**0.38**	•	**0.58**	**0.72**	**0.76**	**0.69**
Cu	-0.075	**-0.47**	**0.56**	**0.59**	•	**0.73**	**0.72**	**0.61**
Fe	0.001	**-0.60**	**0.61**	**0.71**	**0.72**	•	**0.83**	**0.89**
Ni	0.035	**-0.61**	**0.34**	**0.75**	**0.71**	**0.81**	•	**0.73**
Pb	-0.003	**-0.60**	**0.57**	**0.70**	**0.61**	**0.88**	**0.72**	•

Pearson's coefficients indicated a significant negative correlation between the concentration of metal and peak heights (**Table 19**, *lower diagonal*) and peak areas (**Table 19**, *upper diagonal*) obtained with the PowerPlex® 16 HS System. Fragment size (allele length) was found to have a significant positive correlation to peak areas obtained from all metal-treated samples; however, no significant correlation was found when fragment size was compared to peak heights. There were significant positive correlations found when comparing the results obtained from each set of metal-treated samples, indicating consistent results between metal-treated sample sets. Peak heights were more affected by the presence of metal than peak areas for all metals-treated samples amplified with the PowerPlex® 16 HS System.

Table 19
Allele peak heights (lower diagonal) and peak areas (upper diagonal) were independently analyzed with respect to the length of the allele in base pairs (bp), the concentration of metal used to treat the DNA samples, and data obtained from each set of metal-treated samples amplified with the PowerPlex® 16 HS System. Significance at the 0.05 level for negative correlation is between -1.0 and -0.1010, and positive correlation is between 0.1010 and 1.0 (*n alleles* = 377, *df* = 375). Significant values are indicated by bold typeface.

Height \ Area	Size (bp)	Metal conc.	Al	Ca	Cu	Fe	Ni	Pb
Size (bp)	•	2.5×10^{-17}	**0.11**	**0.26**	**0.24**	**0.22**	**0.27**	**0.24**
Metal conc.	2.5×10^{-17}	•	**-0.26**	**-0.52**	**-0.58**	**-0.51**	**-0.53**	**-0.55**
Al	0.026	**-0.27**	•	**0.26**	**0.52**	**0.53**	**0.49**	**0.51**
Ca	0.064	**-0.54**	**0.24**	•	**0.72**	**0.74**	**0.82**	**0.80**
Cu	0.008	**-0.60**	**0.49**	**0.67**	•	**0.81**	**0.81**	**0.81**
Fe	0.045	**-0.54**	**0.52**	**0.72**	**0.78**	•	**0.88**	**0.93**
Ni	0.085	**-0.57**	**0.48**	**0.80**	**0.79**	**0.87**	•	**0.92**
Pb	0.048	**-0.58**	**0.48**	**0.77**	**0.78**	**0.93**	**0.91**	•

IV.3.3 Metal-treated samples amplified with AmpFLSTR® Identifiler® Plus

The peak height relative fluorescent units (RFU) of the individual alleles obtained for selected Identifiler® Plus duplicate amplifications of metal-treated samples are compared to the respective positive control samples in **Fig. 10**. The particular samples chosen from each metal series for illustrative purposes demonstrate the patterns of reduction in RFU across the fragment size range with increasing concentration of metal. Expected alleles below the 50 RFU detection threshold are plotted with an RFU value of zero, mean and ranges for each sample exclude these manually-assigned values.

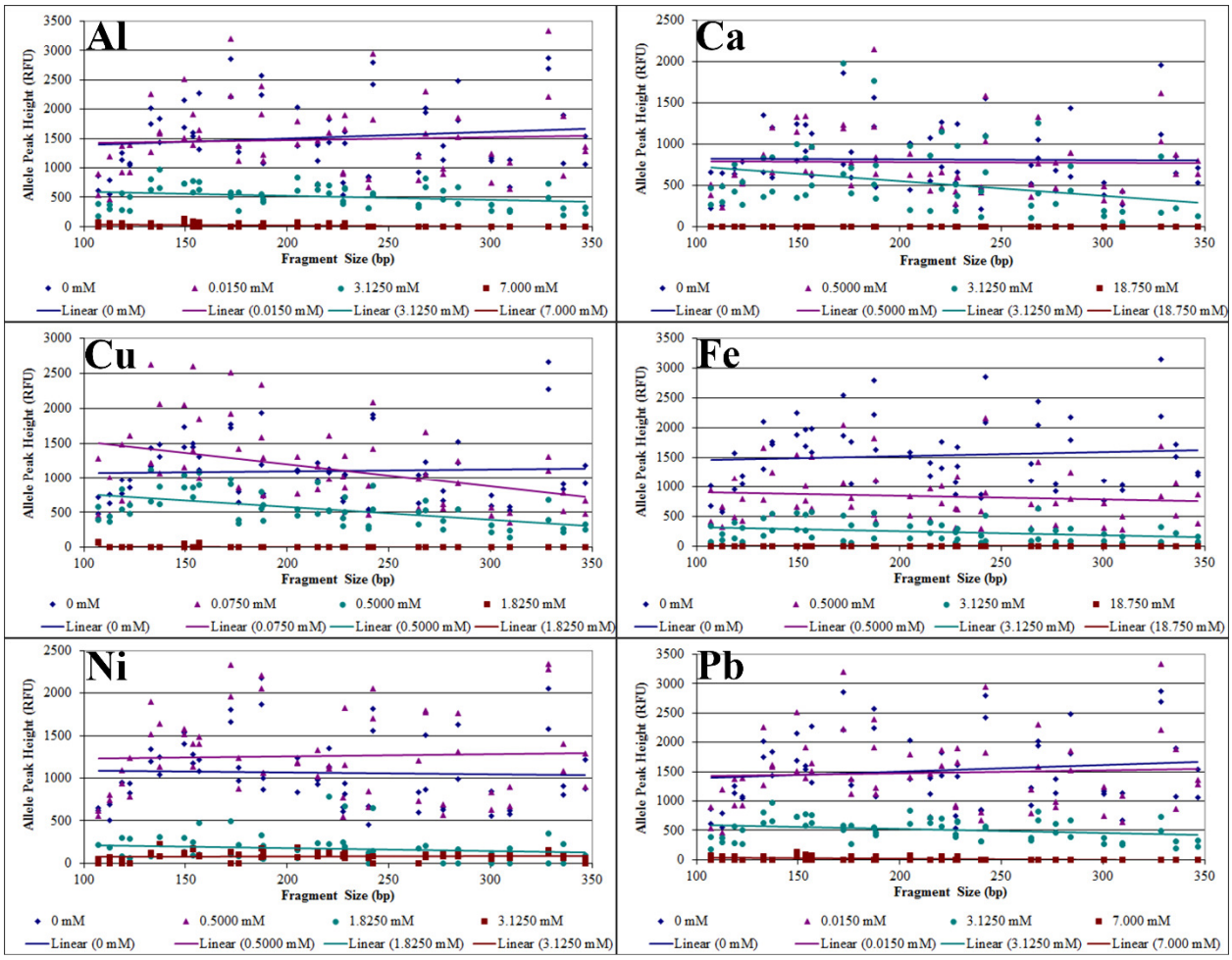

Fig. 10. Duplicate, metal-treated 2800M DNA samples, amplified with the Identifiler® Plus were analyzed and the individual peak heights were plotted in a scatter diagram. Selected samples, illustrating the effect of the metal across a range of concentrations tested, are displayed for each metal. The trend lines are a visual representation of the mean RFU values across the fragment size range for duplicate samples. Allele peak height intensities (RFU) are plotted as a function of fragment size in base pairs (bp); scale of the y-axis varies by metal series, as follows: Al 0 to 2500, Ca 0 to 2500, Cu 0 to 3000, Fe 0 to 3500, Ni 0 to 2500, Pb 0 to 3500.

The Al plot includes the complete 2800M profile alleles obtained for the positive control samples (mean 836, range 257-1658) and samples treated with 0.0025 mM Al (mean 889, range 318-2073). The five alleles obtained between duplicate samples containing 0.0150 mM Al (mean 78, range 53-100) are also displayed. The Ca plot includes the complete profile alleles obtained for the positive control sample (mean 811, range 207-1957) and samples treated with 0.5000 mM Ca (mean 776, range 236-2147). The 55 of 58 alleles obtained from duplicate samples treated with

3.1250 mM Ca (mean 554, range 56-1973) and 18.750 mM Ca (no alleles detected) are included in the plot. Cu-treated samples are displayed in the Cu plot and include the complete profile alleles for the positive control sample (mean 1100, range 474-2658), and samples treated with 0.0750 mM Cu (mean 1150, range 357-2628) and 0.5000 mM Cu (mean 552, range 146-1113). The four alleles obtained between duplicate samples containing 1.8250 mM Cu (mean 65, range 53-81) are also displayed.

The Fe plot includes the complete 2800M profile alleles obtained for the positive control samples (mean 1532, range 574-3147) and samples treated with 0.5000 mM Fe (mean 836, range 282-2153). The 56 of 58 alleles obtained from duplicate samples treated with 3.1250 mM Fe (mean 245, range 55-627) and 18.750 mM Fe (no alleles detected) are included in the plot. The Ni plot includes the complete 2800M profile alleles obtained for the positive control samples (mean 1067, range 452-2197) and samples treated with 0.5000 mM Ni (mean 1259, range 549-2342). The 49 of 58 alleles obtained from duplicate samples treated with 1.8250 mM Ni (mean 205, range 50-784) and 47 of 58 alleles obtained from duplicate samples treated with 3.1250 mM Ni (mean 101, range 50-227) are included in the plot. Pb-treated samples are displayed in the Pb plot and include the complete profile alleles for the positive control sample (mean 1518, range 532-2862), and samples treated with 0.0150 mM Pb (mean 1478, range 466-3337) and 3.1250 mM Pb (mean 512, range 173-965). The 13 of 58 alleles obtained between duplicate samples containing 7.000 mM Pb (mean 69, range 52-136) are also displayed.

IV.3.4 Inhibitory phenomena produced by amplifying metal-treated DNA samples

The analysis of Identifiler® Plus amplification products obtained from metal-treated DNA samples yielded numerous inhibitory phenomena in the resulting genetic data. **Fig. 11** illustrates the reduction in RFU height in the 6-FAM™ channel as the concentration of Fe is increased from

0.0750 to 18.750 mM. The electropherogram obtained from a sample treated with 3.1250 mM Ni in **Fig. 12** illustrates several effects often seen in samples that contain PCR inhibitors, including low signal-to-noise ratio (high baseline), PCR artifacts, and allele dropout. Expected alleles that were below the 50 RFU detection threshold include: D7S820-8; TH01-6; D2S1338-25; D19S433-13,14; TPOX-11; and, AMEL-X, Y. The electropherogram obtained from a sample treated with 0.0750 mM Cu displayed in **Fig. 13** exhibits a characteristic degradation curve, with a "ski-slope" appearance, evidencing the ability of an inhibited sample to appear similar to a low-copy or degraded DNA sample. This demonstrates the need to use caution when assigning cause of PCR failure from challenged sample types, such as bone.

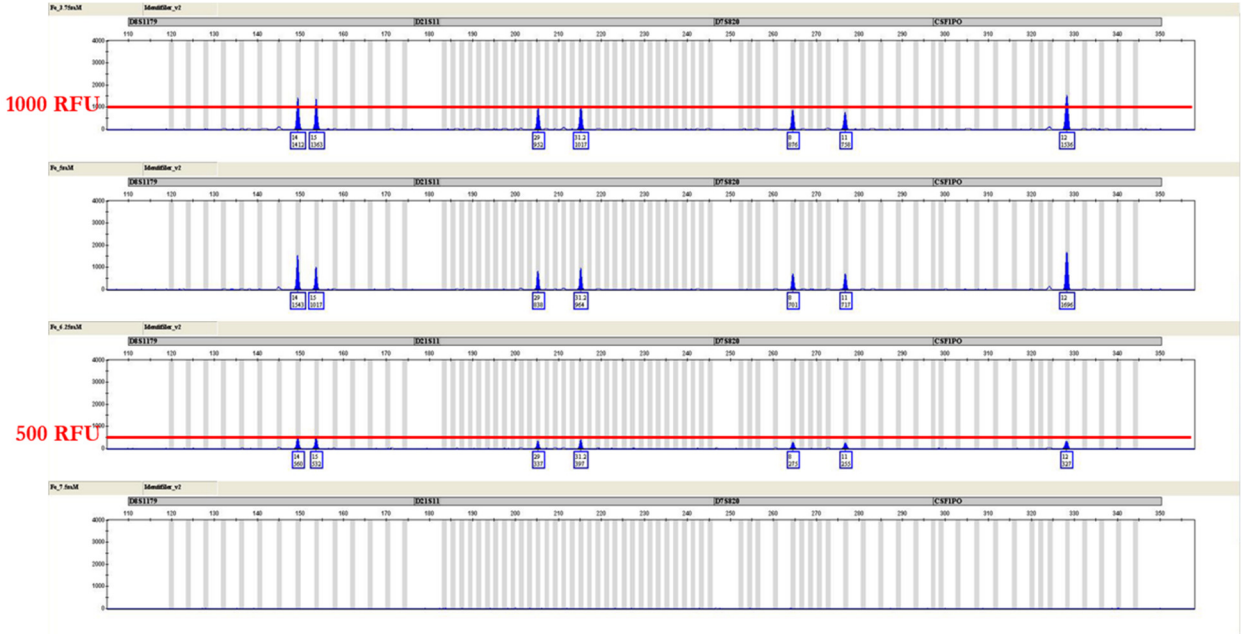

Fig. 11. Duplicate, metal-treated 2800M DNA samples were amplified with Identifiler® Plus and analyzed. The 6-FAM™ channel for samples containing 0.0750, 0.5000, 3.1250, and 18.750 mM Fe (top to bottom), respectively (scale, 0 to 4000 RFU). The red lines have been added as a visual reference, indicating the 1000 RFU and 500 RFU points on the y-axis of the 0.0750 and 3.1250 mM samples, respectively.

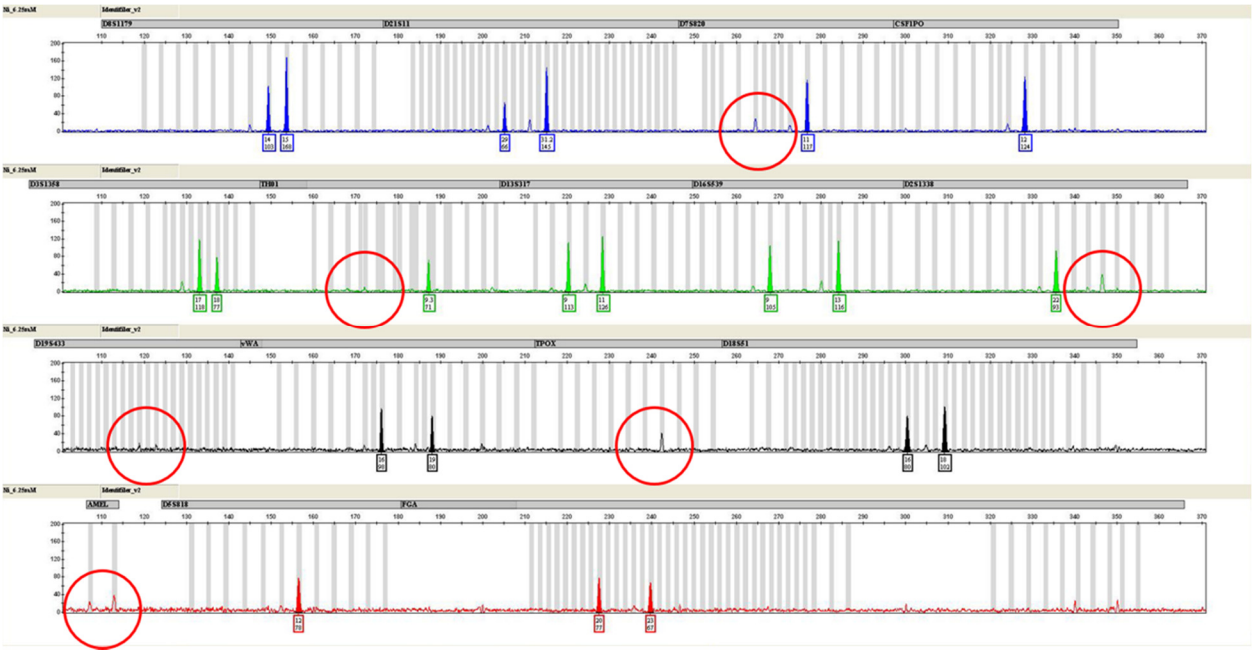

Fig. 12. Duplicate, metal-treated 2800M DNA samples were amplified with Identifiler® Plus and analyzed. The STR profile obtained from a sample containing 3.1250 mM Ni is displayed (scale, 0 to 200 RFU). The red circles identify the locations of the expected alleles that were below the 50 RFU detection threshold.

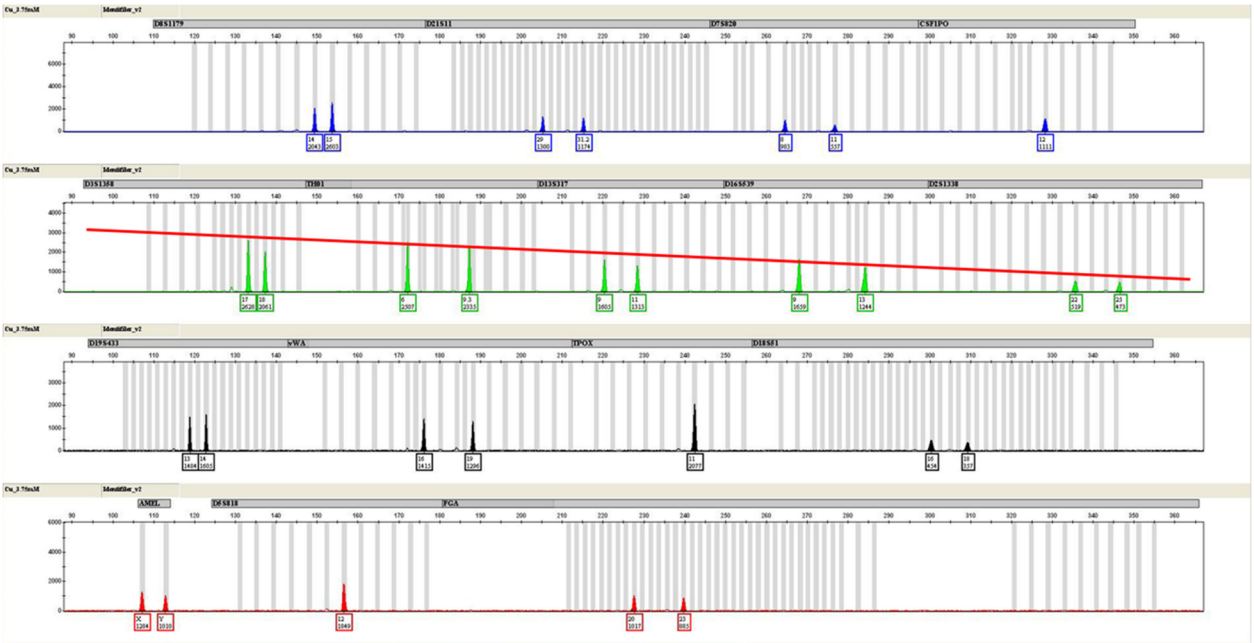

Fig. 13. Duplicate, metal-treated 2800M DNA samples were amplified with Identifiler® Plus and analyzed. The STR profile obtained from a sample containing 0.0750 mM Cu is displayed (scale, 0 to 4250 RFU). Red diagonal line across the VIC® channel has been added for emphasis.

Fig. 14 and **Fig. 15** demonstrate electrophoretic migration issues resulting from the presence of metal ions. **Fig. 14**, an electropherogram obtained from a sample treated with 14.750 mM Pb, illustrates two off-ladder alleles in the PET® channel. The expected heterozygous alleles for that channel are AMEL-X, Y and FGA-20, 23, and there is insufficient data to determine if the migration of PET®-labeled alleles are increased or decreased with respect to the size standard and other dye channels. **Fig. 15** shows the injection failure caused by a sample treated with 18.750 mM Ni. This sample was amplified in duplicate and the samples were prepared for CE separately, with a uniform master mix of Hi-Di™ Formamide and GeneScan™ –500 LIZ® Size Standard. Two injections of each sample were performed and the results of all four injections were the same as the one depicted.

Fig. 14. Duplicate, metal-treated 2800M DNA samples were amplified with Identifiler® Plus and analyzed. The STR profile obtained from a sample containing 14.750 mM Pb is displayed (scale, 0 to 250 RFU). The red circle encompasses two alleles in the PET® channel exhibiting a migratory shift, as a consequence of Pb in the sample.

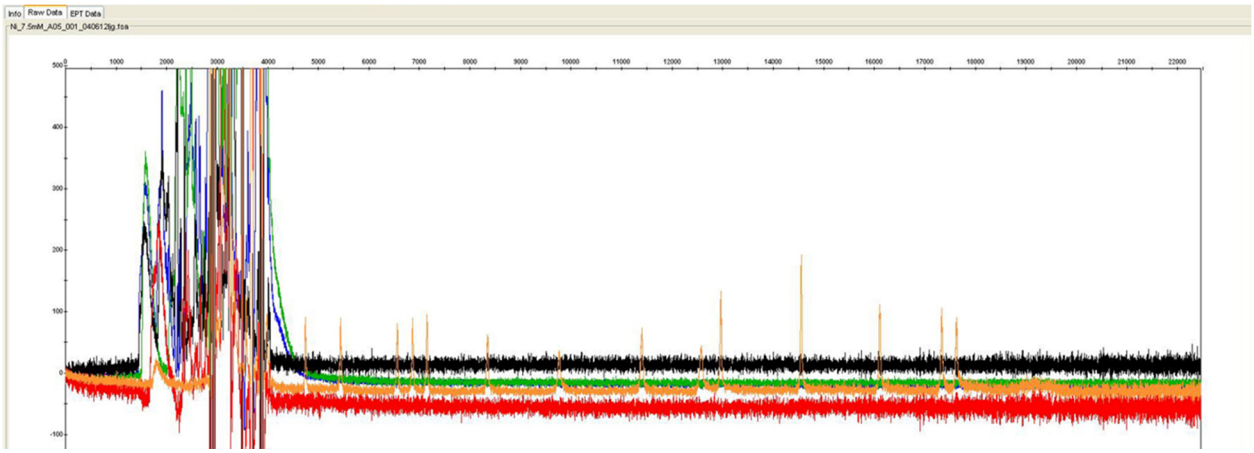

Fig. 15. Duplicate, metal-treated 2800M DNA samples were amplified with Identifiler® Plus and analyzed. The raw data obtained from a sample containing 18.750 mM Ni is displayed (scale, -110 to 500 RFU). GeneScan™ –500 LIZ® Size Standard peaks are visible in the plot; however, insufficient data resulted in sizing failure.

IV.3.5 Metal concentrations present in human skeletal remains

The quantities of six metal isotopes measured in bone powder samples from adjudicated forensic cases are listed in **Table 20**. The minimum and maximum values are provided as a range. The elemental analysis of bone powder samples from adjudicated forensic cases confirmed ^{43}Ca as the most abundant isotope present. ^{57}Fe was determined to be the second most abundant isotope, followed by ^{27}Al, ^{60}Ni, ^{208}Pb, and ^{63}Cu.

Table 20
The quantitative range of ^{27}Al, ^{43}Ca, ^{63}Cu, ^{57}Fe, ^{60}Ni, and ^{208}Pb in bone powder samples from adjudicated forensic cases were measured by ICP-MS, following acid digestion.

Isotope	Quantity in Bone Powder (ng/g)	Isotope	Quantity in Bone Powder (ng/g)
^{27}Al	880 – 512,000	^{57}Fe	1,900,000 – 2,200,000
^{43}Ca	188,000,000 – 207,000,000	^{60}Ni	14,000 – 41,000
^{63}Cu	140 – 440	^{208}Pb	410 – 1,600

IV.4. Conclusions and Discussion

Metal-inhibited DNA samples produce phenomena consistent with characteristics observed in samples with low template or damaged DNA (**Fig. 11**, **Fig. 12**, **Fig. 13**), including

heterozygote peak imbalances and drop out of higher molecular weight alleles [89]. Inhibition of PCR-based DNA testing has been suggested previously; however, the current forensic STR testing systems have not been tested in the presence of calcium or other metal ions [5,31,39-40]. The presented data demonstrate Al, Ca, Cu, Fe, Ni, and Pb to be effective inhibitors of Identifiler® Plus and the PowerPlex® 16 HS System STR testing kits.

One unanticipated finding of this work was the ability of PCR product formed from Ni and Pb-treated DNA to inhibit capillary electrophoresis (**Fig. 14**, **Fig. 15**). A single report from 2005 by Hartzell and McCord, using capillary electrophoresis to examine metal-DNA complex formation, describes covalent binding of Ni to DNA [90]. Amplified STR products treated with 6 mM $NiCl_2$ solution at pH 8.3 resulted in broadening of peaks and peaks with shouldering artifacts, explained as a range of masses, charges, or structures and these effects were observed with the size standard, in addition to the amplified STR products [90]. Complex formation preferable to G/C regions, but were most favorable with poly A/T regions, and mixed purine/pyrimidine regions were the least favored for metal complexation [90]. The smaller molecular weight fragments in the size standard were more affected than the larger ones, and the formation of Ni-DNA complexes was reversible when the sample pH was decreased from 8.3 to 6.6 or when chealation by EDTA was employed [90]. The pH of metal-DNA solutions (3.8-5.8) was below the level that covalent DNA-metal complex formation would be expected (8.3), although the pH of the amplified metal-DNA solution was not measured. It is still likely that the covalent binding of Ni to amplified DNA is the explanation for the observed migration failure at 18.750 mM Ni, despite the advanced buffering of the reaction mixes used in Identifiler® Plus and the PowerPlex® 16 HS System.

Fe-treated DNA was also examined by Hartzell and McCord and no effect was observed when samples were treated with Fe^{3+} and based on their results, it was concluded that Fe^{3+} does

not covalently bind the DNA bases [90]. Experiments with Fe^{2+} did show a reduction in peak height and some quenching of the emitted fluorescence, although it does not appear to be the result of structural changes consistent with Fe-DNA complex formation [90]. While Fe may not covalently bind to DNA, results displayed in **Table 19**, **Fig. 10**, and **Fig. 11** demonstrate the efficacy of Fe as an inhibitor of STR product formation. In addition to complexes formed by covalent binding of the DNA bases, it is possible for metals to form ionic bonds with the backbone of the DNA helix. This was first mentioned in the seminal paper describing the structure of DNA, the potential for ionic interactions between positively-charged metal ions and the negatively-charged phosphate backbone of the double-helix structure was described, "as the phosphates are on the outside, cations have easy access to them" [91].

Cu reportedly has high affinity binding to DNA bases, can intercalate between bases, and has been demonstrated as a PCR inhibitor of non-STR amplifications [5,92]. Al forms at least three DNA crosslinking variants [93]. Pb has also been shown to form complexes with DNA; however, it appears to be a sequence-specific phenomenon that may explain the reduced effect observed as an STR inhibitor [94]. In addition to covalent bonding and ionic interactions directly with the template, metals can also inhibit PCR by competitively inhibiting the enzyme responsible for amplifying the template. Of the metals tested, only Ca has been classified as a *Taq* polymerase inhibitor [54].

Bone samples contain organic inhibitors, such as collagen and humic substances, in addition to inorganic inhibitors demonstrated in this work [6,87]. The levels of Al, Ca, Cu, Fe, Ni, and Pb reported in autopsy and surgical specimens, buried and archaeological specimens, and in forensic skeletal remains samples appear to be sufficient to inhibit STR testing (**Table 15** and **Table 20**). In order to determine the relevance of skeletal-derived metal ions as inhibitors of PCR-

based DNA testing, future experiments should characterize DNA samples from skeletal remains to elucidate if metals co-isolate with DNA during routine extraction.

CHAPTER V.

ELEMENTAL AND DNA ANALYSIS OF CONSTITUENTS OBTAINED FROM EXTRACTING BONE SAMPLES

V.1. Introduction

Aluminum (Al), calcium (Ca), copper (Cu), iron (Fe), nickel (Ni), and lead (Pb) can inhibit STR testing of DNA samples and have been shown to be present in human skeletal remains [76]. It is necessary to ascertain if metals in skeletal remains co-isolate with DNA during routine extraction and purification at a sufficient level to function as PCR inhibitors. Carryover of Ca, during DNA extraction from bone samples, was first established by Fisher *et al.* in 1993 using a qualitative ammonium oxalate precipitation assay [27]. Methods for extracting DNA from bone samples report improvement when combined with demineralization procedures; however, the extracts were not tested for the presence of or change of metal concentration as a consequence of these procedures [28,88].

The purpose of this study was to extract DNA from human skeletal remains from adjudicated forensic unidentified remains cases and to measure the DNA and metal content of the extracts. The extracts will be tested for DNA and metals at several steps during the extraction process to determine efficacy of each step in the procedure. The use of anonymized samples from adjudicated unidentified skeletal remains cases is limited by the loss of knowledge of the sample (skeletal element sampled, age of individual at death, burial conditions, as well as nature and length

of environmental exposure). Conversely, these samples are representative of the real-life challenges encountered daily by forensic scientists who conduct DNA typing of skeletal remains samples and it is imperative to ensure experimental data are reflective of actual sample conditions.

V.2. Materials and Methods

V.2.1 Skeletal remains samples

Six pulverized bone samples from adjudicated unidentified skeletal remains cases were obtained from the University of North Texas Center for Human Identification. These samples were anonymized and transferred for research testing due to poor quality autosomal STR typing results. The samples were labeled #1 through #6.

V.2.2 Sample preparation[4]

Exterior surfaces of skeletal remains were decontaminated using a Dremel® rotary tool (Dremel®, Racine, WI) and a 10% bleach solution. A Dremel® sanding cone was used to remove the surface (periosteum and outer cortical layer) of a 5 by 8 cm area of the bone and sanded area was wiped using the bleach solution, as needed. A Stryker saw (Stryker Surgical Company, Kalamazoo, MI) was used to cut the sanded area into thin sections; each sample was placed into a 50 mL conical tube. A 50% bleach solution was used to immerse the cuttings that were gently agitated and soaked for 1-5 minutes, then the solution was poured off. The bone cuttings were rinsed and agitated with sufficient volumes of distilled water until the waste was clear and no longer retained the smell of bleach. A final rinse using 100% ethanol was performed before allowing the bone cuttings to dry.

[4] Sample preparation of skeletal remains samples was conducted by the University of North Texas Center for Human Identification.

Dried bone cuttings were enclosed in a small grinding vial with an impactor rod, immersed in liquid nitrogen, and pulverized to fine powder using a SPEX 6750 Freezer/Mill® (SPEX SamplePrep, Metuchen, NJ). The pulverized sample was weighed and placed into a labeled 15 mL conical tube. One gram of the initial sample was consumed for testing for casework analysis and the remainder transferred for subsequent testing after the case was adjudicated. Remaining sample weights are provided in **Table 21**.

Table 21
The weights (g) of the pulverized bone samples (numbered 1 through 6), available for additional DNA testing, are provided as a reference. Samples 1 through 5 consisted of white to off-white fine powder, whereas sample 6 presented with incomplete pulverization ("chunky") and tan coloring.

No.	Weight (g)
1	0.486
2	0.791
3	0.323
4	0.819
5	0.367
6	0.802

V.2.3 Demineralization extraction of skeletal remains

DNA extraction was performed using the UNT Center for Human Identification "Demineralization Extraction of Skeletal Remains" protocol, a modified version of the total demineralization extraction [28]. Demineralization buffer was prepared using 0.5004 g of sodium N-lauroyl sarcosinate (SLS) and 50 mL 0.5 M ethylenediaminetetraacetic acid (ETDA) solution, and 4.5 mL of this buffer was added to each sample and a reagent blank. In addition, 0.3 mL of 20 mg/mL Proteinase K (Pro K) enzyme was included, prior to vortexing and incubation for 24 hours at 56°C with agitation. Samples following incubation are depicted in **Fig. 16**.

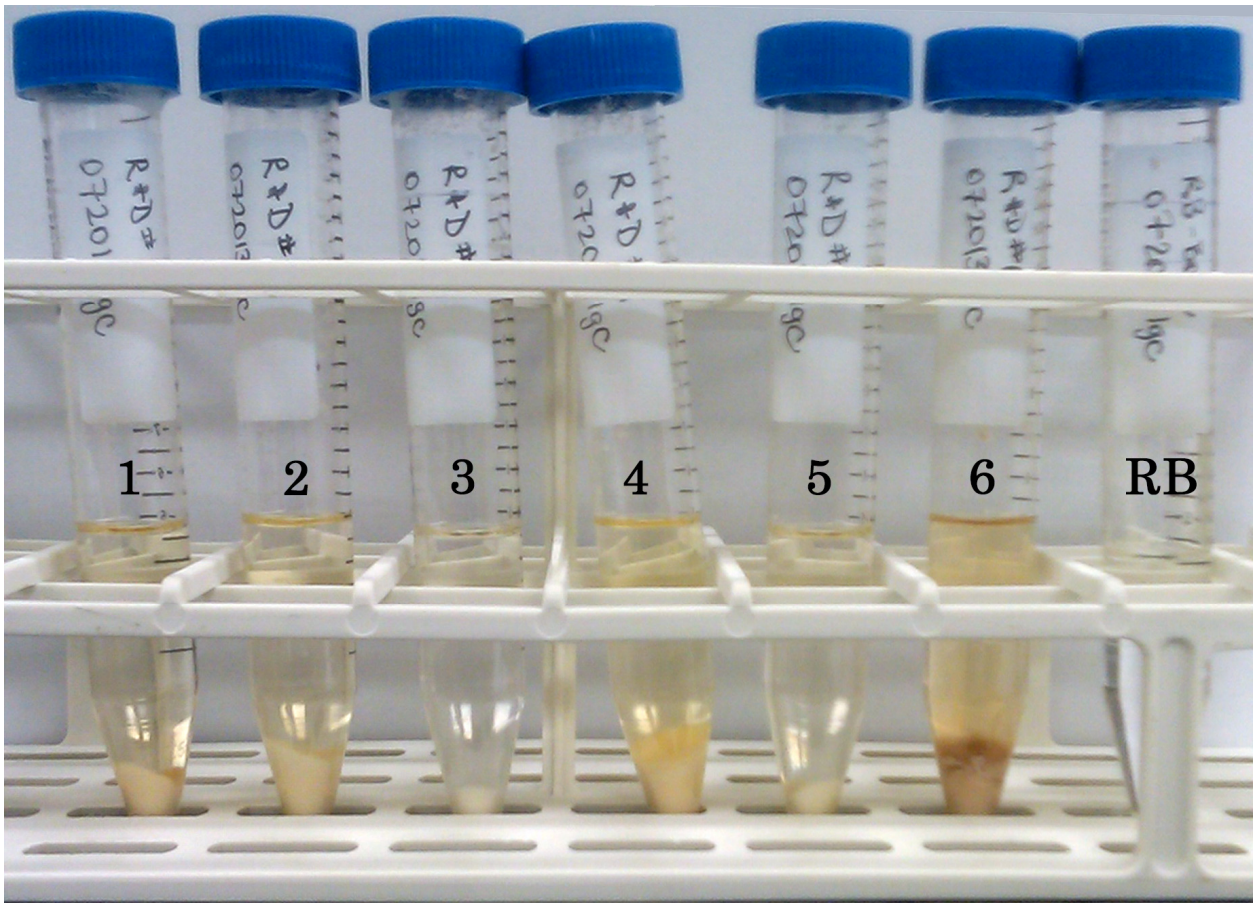

Fig. 16. Samples number 1 through 6 and corresponding reagent blank, following overnight incubation.

Demineralization was followed by protein digestion and DNA purification, by adding 4.8 mL phenol-chloroform-isoamyl alcohol (PCIA) to each sample and reagent blank. The PCIA-treated samples were vortexed and centrifuged at maximum speed for 3 minutes. The 4.8 mL aqueous phase was transferred into an individual Amicon® Ultra-4 PL-50 centrifugal filter device which isolates DNA based on its molecular weight. Two aliquots were removed for subsequent testing: 50 µL for DNA testing and 100 µL for elemental analyses. This fraction was designated fraction "P" for aqueous phase of PCIA purification; the PCIA waste was not retained for additional testing due to the hazardous properties of phenol.

Once the aliquots were removed, the remaining sample was centrifuged through the filtration unit at 3000 x g until all but 50 µL of sample had passed through the unit. Sample #6 failed to pass through the first unit and was transferred into a new 15 mL conical tube and a second PCIA purification was performed, followed by a second Amicon filtration. Volumes for all retained samples were brought up to 200 µL using UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA). Two aliquots were removed for subsequent testing from both the retentate and filtrate: 50 µL for DNA testing and 100 µL for elemental analyses. These fractions were designated fraction "A" for Amicon retentate and "AW" for Amicon filtrate (waste).

An additional purification was performed using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The 50 µL of each sample remaining in the Amicon filtration unit was transferred into a pre-labeled 1.5 mL microcentrifuge tube, and 250 µL of Buffer PB was added. Samples were vortexed before being transferred into a QIAquick column that was assembled inside a 2.0 mL collection tube, centrifuged at 13,000 rpm for 30-60 seconds, and the waste collected for subsequent testing. Following centrifugation 750 µL of Buffer PE was added to each column, which were centrifuged at 13,000 rpm for 30-60 seconds, and the waste was collected. For elution of the sample, 200 µL of Buffer EB was added and samples were centrifuged for 60 seconds. One 100 µL aliquot was removed for subsequent elemental analyses and the remaining 100 µL of each fraction was retained for DNA testing for both the retentate and filtrate. These fractions were designated fraction "Q" for QIAquick retentate and "QW" for QIAquick filtrate (waste).

V.2.4 Sizing and analysis of DNA fragments

All extracted samples were analyzed in duplicate for DNA fragmentation using the High Sensitivity DNA Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Each DNA extract and reagent blank was prepared by combining 1 µL of sample with 9 µL of High Sensitivity DNA Gel Matrix-Dye Concentrate mixture and 5 µL of High sensitivity DNA Markers 35/10380 bp into individual wells of a primed High Sensitivity DNA chip (Agilent), per manufacturer instructions. Each chip was vortexed for 1 minute at 2400 rpm and analyzed using the corresponding High Sensitivity protocol on the 2100 Bioanalyzer. Gel images were obtained from the 2100 Expert Software v.B.02.08 (Agilent).

V.2.5 DNA quantification

Quantitative PCR (qPCR) was performed using the Quantifiler® Human DNA Quantification Kit. A master mix was prepared according to the manufacturer's instructions, consisting of 12.5 µL Quantifiler® PCR Reaction Mix and 10.5 µL Quantifiler® Human Primer Mix (Applied Biosystems) per sample; 23 µL of master mix was distributed into the requisite wells of a 96-well optical reaction plate [32]. Total reaction volume for qPCR was 25 µL, which included the master mix plus 2 µL of sample. Samples and controls were quantified in duplicate.

Absolute quantification was performed using a 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions [32]. Amplification was performed in 9600 emulation mode using the following parameters: 95°C hold for 10 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data was collected during the 60°C stage for all cycles. The following data were obtained using SDS Software v.1.2 (Applied Biosystems): IPC quantification cycle (Cq), human Cq, human DNA quantity, and the mean (µ) and standard deviation (σ) for

duplicate samples, when available. Samples with "undetermined" Cq values were manually-assigned Cq values of 40.00 and quantities of 0 ng/µL.

V.2.6 DNA amplification

Experimental samples, reagent blanks, and negative control samples were amplified neat, and positive control samples were normalized to 0.1 ng/µL of DNA using TE^{-4} buffer prior to amplification. All DNA amplifications were performed in duplicate using AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions for 25 µL PCR reaction setup and thermocycling parameters [43]. The master mix used for the amplification setup included 10.0 µL of AmpFLSTR® Identifiler® Plus Master Mix and 5.0 µL of AmpFLSTR® Identifiler® Plus Primer Set (Applied Biosystems) per sample. A 15 µL aliquot of this master mix was added to each PCR amplification tube, before adding 10.0 µL of sample. Amplification was performed using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems), in 9600 emulation mode, using the following parameters: 95°C hold for 11 minutes; 28 cycles of 94°C for 20 seconds and 59°C for 3 minutes; 60°C hold for 10 minutes, and 4°C hold until samples removed from the thermocycler and stored refrigerated.

V.2.7 Analysis and genotyping

Genetic analysis of Identifiler® Plus-amplified samples was performed after combining 1 µL of product or allelic ladder with 9 µL master mix that included 8.75 µL Hi-Di™ Formamide and 0.25 µL of GeneScan™ 500 LIZ® Size Standard (Applied Biosystems). Capillary electrophoresis of all samples was conducted using a 3 kV, 10 second injection and POP-6™ Polymer on a 3130*xl* Genetic Analyzer (Applied Biosystems). STR profiles, including individual

peak heights and areas, were obtained using a 50 RFU allele detection threshold and GeneMapper® ID Software v.3.2 (Applied Biosystems).

V.2.8 Elemental Analysis of DNA extracts from skeletal remains [5]

Nitric Acid (HNO_3) preparation of DNA extracts was accomplished by diluting 0.1 mL sample extract into 10 mL of 1% HNO_3; three extract fractions were tested for each sample (P, A, and Q). Elemental content was determined by inductively coupled plasma-mass spectrometry (ICP-MS) using a Bruker Aurora M90™ ICP-MS System with Autosampler (Bremen, Germany). Elemental analysis was performed in solution mode ICP-MS in triplicate, and the following isotopes were measured: ^{27}Al, ^{43}Ca, ^{44}Ca, ^{57}Fe, ^{60}Ni, ^{63}Cu, and ^{208}Pb.

V.3. Results

All between-fraction results for an individual sample should take into consideration that 4.6 mL of fraction P was reduced to 0.2 mL during purification and concentration to create fraction A, and 0.05 mL of fraction A was purified and diluted into 0.2 mL to become fraction Q. This means that fraction A is 23X more concentrated than fraction P, and 4X more than fraction Q; however, it is not possible to quantify the loss of DNA or metal that occurred as a consequence of filtration with the Amicon® Ultra-4 PL-50 centrifugal filter device or the QIAquick PCR Purification Kit.

V.3.1 DNA separation and sizing results

The DNA content of neat purified extracts was separated and sized using the High Sensitivity Kit for the Agilent 2100 Bioanalyzer. Sizing results failed for waste fractions AW and QW for all samples (*data not shown*). The sizing for all P fractions also failed, due to failure of

[5] Preparation for and elemental analysis of DNA extracts obtained from skeletal remains samples was conducted by the Department of Chemistry at the University of North Texas.

the internal migration standards (**Fig. 17**, panel P). Sizing of these fractions was not repeated. DNA content greater than 1000 bp in size was present in fraction A for samples 1, 2, 3, 5, and 6; DNA content less than 1000 bp in size was present in fraction A of sample 4 (**Fig. 17**, panel A). In fraction Q, DNA sizing greater than 1000 bp was visible in samples 1, 2, 3, 5 and 6; although sample 4 appears negative for DNA content (**Fig. 17**, panel Q).

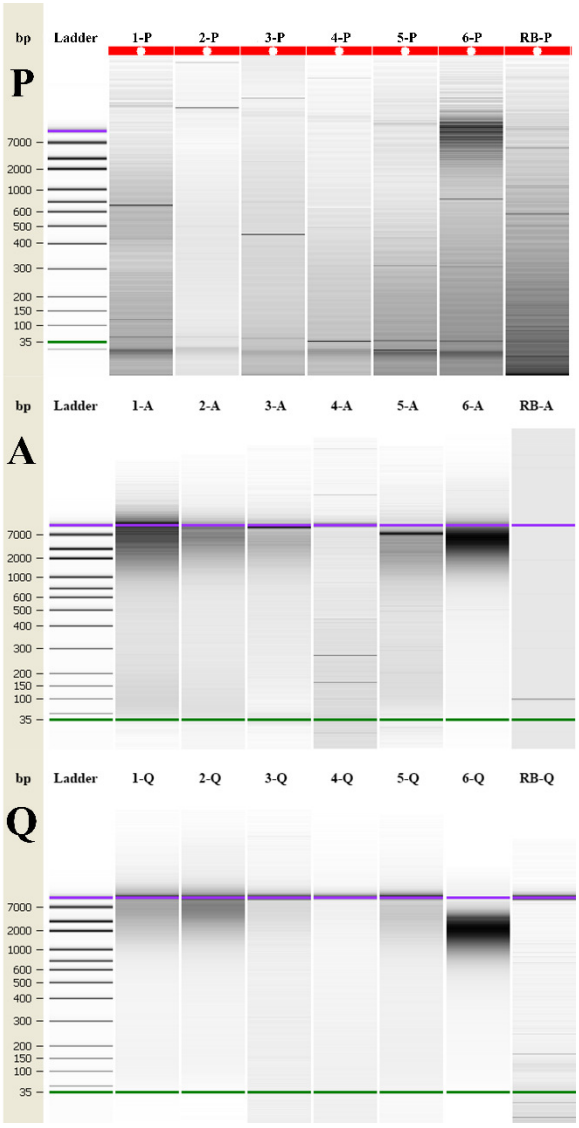

Fig. 17. DNA was extracted from human skeletal remains using a demineralization protocol. Aliquots were obtained following PCIA purification (fraction P), purification and concentration with an Amicon® Ultra-4 PL-50 centrifugal filter device (fraction A), and QIAquick PCR Purification Kit (fraction Q). Gel images obtained from the Agilent 2100 Bioanalyzer are provided

for samples 1 through 6 and the corresponding reagent blank (RB). Sizing markers are 10,380 bp (purple) and 35 bp (green); ladder includes fragments that are 7,000, 3,000, 2,000, 1,000, 700, 600, 500, 400, 300, 200, 150, 100, and 50 bp, in addition to the sizing markers. No sizing data was obtained for the fraction P samples, indicated by the red bar above the gel lane for each sample.

V.3.2 DNA quantification results obtained for skeletal remains samples

The DNA content of neat extracts was quantified using the Quantifiler® Human DNA Quantification Kit. The standard curve parameters were: R^2: 0.995, slope: -3.313, and intercept: 28.65, excluding one of the standard 8 samples. Results for reagent blanks, no template control (NTC) and positive control samples were as expected. Waste fractions AW and QW for all samples yielded undetermined Cq values for the IPC and Quantifiler® Human assays.

The Cq values obtained for the fractions P, A, and Q for all samples are illustrated in **Fig. 18**. All P fractions yielded undetermined Cq values for the Quantifiler® Human and IPC detectors. The Cq values for the IPC assay was undetermined for sample 6, fraction A. All other IPC Cq values were between 0.08 and 0.85 of the positive control mean IPC Cq value (27.13). The average difference between the sample IPC Cq value and the positive control was 0.33 (*sd* = 0.22). The Quantifiler® Human assay was undetermined for one replicate of sample 5, fraction A and fraction Q for samples 3 (both replicates), 4, 5, and 6 (one replicate each).

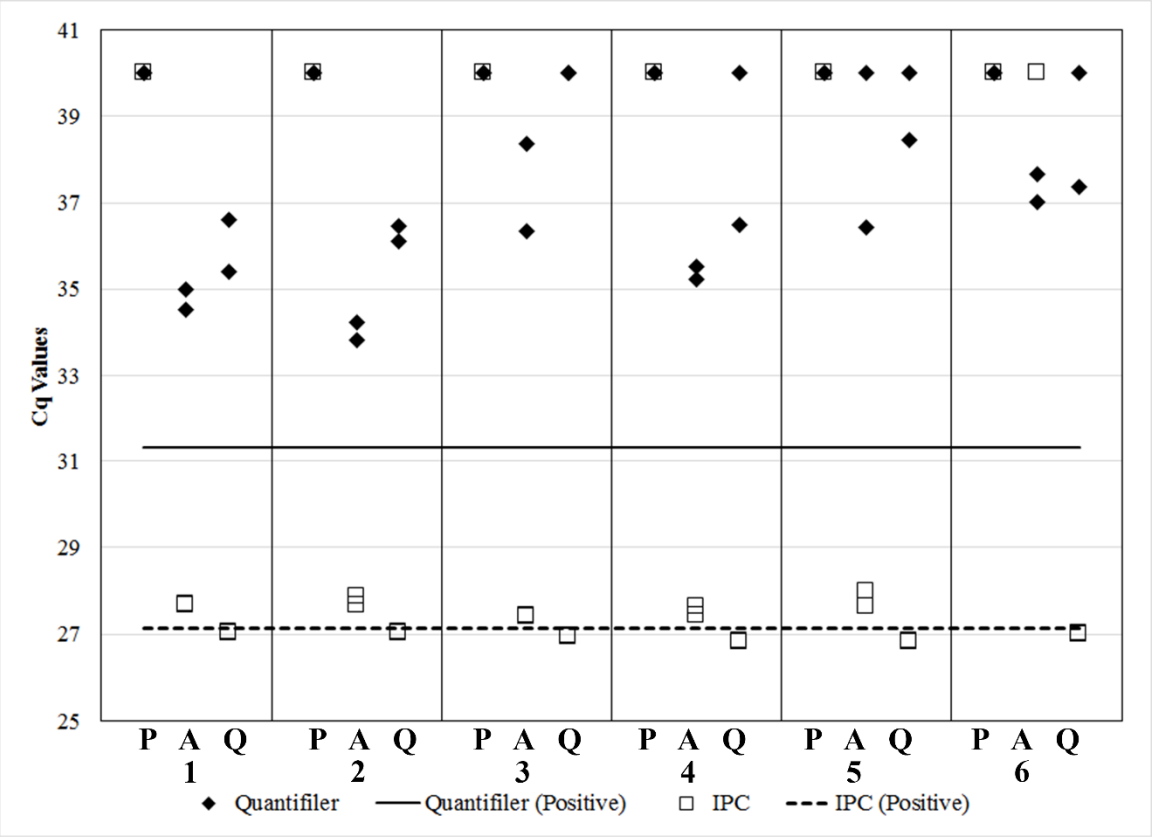

Fig. 18. DNA was extracted from human skeletal remains using a demineralization protocol. Aliquots were obtained following PCIA purification (fraction P), purification and concentration with an Amicon® Ultra-4 PL-50 centrifugal filter device (fraction A), and QIAquick PCR Purification Kit (fraction Q) for six samples, numbered 1 through 6. Neat extracts were quantified in duplicate using the Quantifiler® Human DNA Quantification Kit. Cq values for the Internal PCR Control (IPC) and the human DNA target (Quantifiler) resulting from the neat extracts are illustrated; the solid black line denotes the mean Cq value obtained for the human target (31.31) and the dashed black line indicates the mean Cq value obtained for the IPC target (27.13) in the 0.1 ng/µL control sample, respectively.

The estimated quantity of human DNA from all sample fractions is depicted in **Fig. 19**; samples with undetermined Cq values were manually assigned DNA quantities of zero. This includes all P fractions, and sample 3, fraction Q. The solid green indicates the position of 0.0125 ng and the dashed green line indicates the position of 0.00325 ng in 1 µL; these positions denote concentration thresholds published for full (100% of expected alleles) and partial (50-65% of

expected alleles) STR profiles, respectively, for 10 µL DNA samples amplified with the Identifiler® Plus PCR Amplification Kit [39].

As expected, samples exhibited a loss of estimated DNA quantity between fraction A and fraction Q. Because 25% of fraction A was present in sample Q, it was expected that fraction Q of each sample would reflect that approximate percentage. The calculated percent mean quantity of fraction Q, relative to fraction A for samples 1, 2, 4, 5, and 6 are, respectively, 45%, 21%, 23%, 25%, and 48%. A percent loss could not be calculated for sample 3, as there was no amplifiable DNA detected in fraction Q.

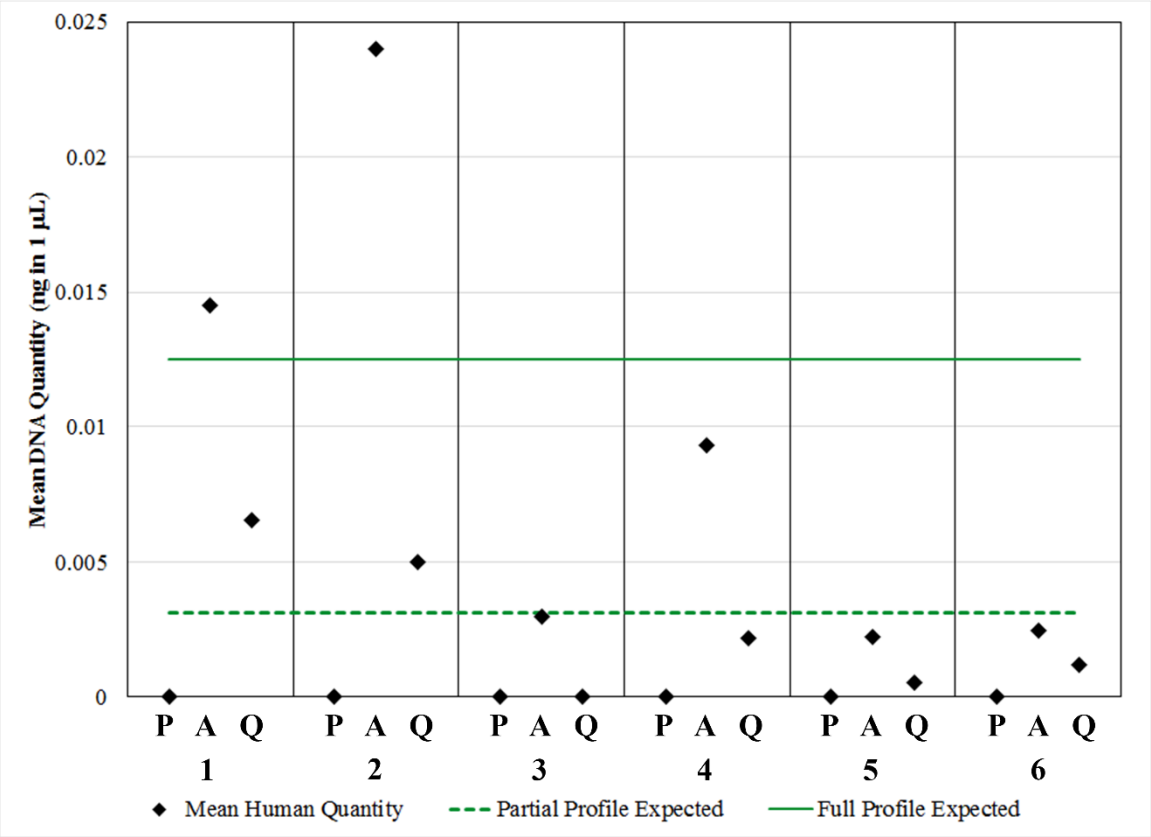

Fig. 19. DNA was extracted from human skeletal remains using a demineralization protocol. Aliquots were obtained following PCIA purification (fraction P), purification and concentration with an Amicon® Ultra-4 PL-50 centrifugal filter device (fraction A), and QIAquick PCR Purification Kit (fraction Q) for six samples, numbered 1 through 6. Neat extracts were quantified in duplicate using the Quantifiler® Human DNA Quantification Kit. Mean human DNA quantities

obtained for the samples are illustrated. The solid green indicates the position of 0.0125 ng and the dashed green line indicates the position of 0.00325 ng.

V.3.3 Skeletal remains samples amplified with AmpFLSTR® Identifiler® Plus

All waste fractions, AW and QW, and working fractions P and A were negative for alleles greater than 50 RFU, the detection threshold used for analysis. Fraction Q for samples 3 through 6 were also negative for alleles greater than 50 RFU. The alleles obtained for fraction Q of samples 1 and 2 are described in detail and compared to the alleles called for the same loci in the positive control sample in **Table 22**. Two alleles were obtained for sample 1, fraction Q and six alleles were obtained from sample 2, fraction Q. The mean peak height for sample 1 alleles was 70 RFU (range 65-75), and the mean for sample 2 alleles was 76 RFU (range 50-150).

Table 22
STR amplification results obtained for sample fractions 1-Q and 2-Q using Identifiler® Plus PCR Amplification kit, compared to the results obtained for the positive control sample at the same loci. Data for each allele includes the allele designation, and peak height and area RFU in parentheses.

Sample	1Q	2Q	Positive Control Sample	
Marker	Allele 1	Allele 1	Allele 1	Allele 2
AMEL	X (65, 1415)		X (3759, 71127)	
D3S1358	16 (75, 1378)	15 (50, 836)	14 (3773, 56770)	15 (3173, 47612)
D19S433		13 (55, 783)	14 (2513, 31690)	15 (2294, 28952)
D8S1179		12 (93, 1301)	13 (7286, 106477)	
D5S818		11 (54, 910)	11 (4552, 78950)	
vWA		14 (53, 771)	17 (2839, 40173)	18 (2548, 36661)
TH01		6 (150, 2198)	8 (3955, 55226)	9.3 (3915, 55476)

V.3.4 Metal concentrations present in human skeletal remains sample extracts

The quantities of seven metal isotopes measured by ICP-MS in fractions P, A, and Q for samples 1 through 6 are listed in **Table 23**. Fraction P contained the highest amounts of all isotopes for all samples. Levels of ^{43}Ca, ^{44}Ca, ^{63}Cu, ^{57}Fe, ^{60}Ni, and ^{208}Pb are higher in fraction A than in fraction Q for all samples. Levels of ^{27}Al are increased in fraction A of sample 1, relative to fraction P of sample 1, and in fractions Q of samples 1, 2, 4, and 5, relative to the respective fraction A.

Table 23
The measurements (mM) of 27Al, 43Ca, 63Cu, 57Fe, 60Ni, and 208Pb in extract fractions P, A, and Q from samples 1 through 6 measured by ICP-MS, following acid digestion. Sample fractions with increased ^{27}Al content, relative to the previous sample fraction, are indicated by bold font.

No.	ID	^{27}Al (x100)	^{43}Ca	^{44}Ca	^{63}Cu (x100)	^{57}Fe	^{60}Ni (x100)	^{208}Pb (x100)
	P	1.66	124	>LOD	7.54	0.575	0.412	0.003
1	A	**2.07**	98.6	12.3	0.161	0.039	<LOD	<LOD
	Q	**2.52**	0.013	0.022	<LOD	<LOD	<LOD	<LOD
	P	2.04	>LOD	>LOD	3.51	1.66	0.409	0.231
2	A	1.28	19.9	20.9	0.329	0.209	0.04	0.026
	Q	**2.42**	0.019	0.004	<LOD	0.002	<LOD	<LOD
	P	<LOD	>LOD	>LOD	2.78	1.06	0.24	0.062
3	A	1.01	3.25	3.29	0.092	0.036	<LOD	<LOD
	Q	<LOD	0.018	<LOD	<LOD	0.002	<LOD	<LOD
	P	1.72	>LOD	>LOD	2.14	1.58	0.369	0.099
4	A	2.05	6.28	6.28	0.118	0.071	0.021	<LOD
	Q	**2.36**	0.033	0.003	0.043	0.004	0.009	<LOD
	P	0.037	>LOD	>LOD	2.2	1.25	0.294	0.041
5	A	<LOD	9.65	9.64	0.21	0.103	0.034	<LOD
	Q	**0.259**	0.024	<LOD	0.0004	0.006	0.011	<LOD
	P	>LOD	>LOD	>LOD	2.87	1.58	6.96	0.046
6	A	22.4	34.983	34.4	0.758	0.394	1.75	0.008
	Q	3.67	0.034	0.03	0.032	0.007	0.014	<LOD

LOD = limit of detection

V.4. Conclusions and Discussion

Aliquots of sample fractions P, A, and Q were obtained from bone samples 1-6. Results are color-coded to indicate the expectation of STR amplification results, based on reported values and previous experimental results in **Table 24** [39,76]. ICP-MS elemental analysis revealed that all samples, except 3 fraction Q and 5 fraction Q, contained sufficient levels of metal ions to inhibit STR testing of a sample with at least 1 ng of DNA in the amplification reaction. DNA separation and sizing on a microfluidic-based platform allowed visualization of DNA in all fractions A and fractions Q. The PCR-based assay used to quantify the amount of amplifiable DNA in each sample

proved to be over-estimating quality of each samples, only indicating the presence of PCR inhibitors in fractions P for all samples and fraction A for sample 6, only. This demonstrates that after routine extraction and purification, current methods used to assess DNA quality and quantity fail to detect inhibitory levels of metal ions in samples extracted from human skeletal remains.

Table 24
Summary of elemental and DNA analysis results for samples 1 through 6. Color-coded values indicate expected amplification of 1 ng in 25 µL STR reaction to result in a full STR profile (green), a partial STR profile (black), or complete amplification failure (red).

No.	ID	Elemental Analysis	DNA Separation & Sizing	Quantity of Amplifiable DNA	STR Results
	P	Al and Ca	Sizing failed	IPC undetermined	Negative at 50 RFU
1	A	Al and Ca	DNA >1000 bp	Full STR range	Negative at 50 RFU
	Q	Al	DNA >1000 bp	Partial STR range	2 alleles, low RFU
	P	Al and Ca	Sizing failed	IPC undetermined	Negative at 50 RFU
2	A	Al and Ca	DNA >1000 bp	Full STR range	Negative at 50 RFU
	Q	Al	DNA >1000 bp	Partial STR range	6 alleles, low RFU
	P	Ca	Sizing failed	IPC undetermined	Negative at 50 RFU
3	A	Ca	Some DNA >1000 bp	Partial STR range	Negative at 50 RFU
	Q	< inhibitory level	Some DNA >1000 bp	Human undetermined	Negative at 50 RFU
	P	Al and Ca	Sizing failed	IPC undetermined	Negative at 50 RFU
4	A	Al and Ca	Some DNA >1000 bp	Partial STR range	Negative at 50 RFU
	Q	Al	Some DNA >1000 bp	Partial STR range	Negative at 50 RFU
	P	Ca	Sizing failed	IPC undetermined	Negative at 50 RFU
5	A	Ca	Some DNA >1000 bp	< STR range	Negative at 50 RFU
	Q	< inhibitory level	Some DNA >1000 bp	< STR range	Negative at 50 RFU
	P	Al and Ca	Sizing failed	IPC undetermined	Negative at 50 RFU
6	A	Al and Ca	DNA >1000 bp	IPC undetermined	Negative at 50 RFU
	Q	Al	DNA >1000 bp	< STR range	Negative at 50 RFU

Alleles were only obtained for two of the 15 fractions tested, fraction Q of samples 1 and 2, both of which contained sufficient Al to inhibited 1 ng of DNA template in a 25 µL Identifiler® Plus amplification. While the number of alleles would not be sufficient to identify an individual, the ability to generate any STR data from the sample with an inhibitory level of Al indicates there was likely more DNA than is being measured by the qPCR assay. The results from the DNA separation and sizing assay on the Agilent 2100 Bioanalyzer support this conclusion, as well, showing the presence of DNA in excess of 700 bp.

Separation and sizing failures for fractions P, AW, and QW were likely the result of the high concentration of ions and salts, as the High Sensitivity assay has a limited tolerance to high salt concentrations [95]. An advantage of this analysis is that DNA detection is based on a signal produced from intercalating fluorescent dyes; this type of analysis has been reported to be more accurate and reliable when working with degraded bone samples than PCR-based methods [25]. This improved detection of DNA versus PCR-based was evidenced in the results presented in **Fig. 17**, where DNA is present in fraction A and Q of sample 6 at an apparent increased level compared to samples 1 through 5. It is extremely likely, based on the qPCR results that sample 6 contained sufficient DNA for STR typing, but also PCR inhibitors.

The results of the Quantifiler® Human DNA Quantification Kit assay resulted in IPC Ct values for waste fractions AW, QW, and fraction P of all samples were indicative of inhibition ("undetermined"), as was fraction A of sample 6. The IPC assay results for samples 1 through 5, fraction A, and all samples fraction Q did not indicate the presence of PCR inhibition, despite the measured levels of co-isolated Al and Ca. Previous experimental results, presented in **Chapter III.**, suggest that the IPC result may be unreliable for the detection of metal ion inhibitors.

As described previously, fraction A was 23X more concentrated than fraction P, and 4X more than fraction Q. Comparing the results obtained from the Quantifiler® human assay, fraction Q of samples 1 and 6 had a higher estimated DNA quantity than was expected due to 4X dilution of fraction A, respectively 45% and 48%. This apparent increase reflects a near two-fold increase in amplification efficiency of the sample as a consequence of purification with the QIAquick PCR Purification Kit and/or sample dilution.

Fraction A for samples 1 and 2 were estimated to have DNA concentrations in excess of 0.0125 ng/µL, which is sufficient to expect full Identifiler® Plus STR profiles [39]. Fraction A of sample 4, and fraction Q of samples 1 and 2 were estimated to have DNA concentrations between 0.00325 and 0.125 ng/µL, which are in the range for the development of a partial STR profile after amplification with Identifiler® Plus [39]. Despite these expectations, no STR typing results were obtained from fraction A of samples 1 and 2. Quantifiler® human quantification results did indicate the possibility of partial STR profiles for fraction Q of samples 1 and 2; however, comparing the concentrations to the sensitivity study in the developmental validation the concentrations were suggestive of approximately 50-65% percent of a full profile [39]. No alleles were obtained from sample 4 fraction A that also had an estimated DNA quantity in this range.

There was insufficient 63Cu, 57Fe, 60Ni, or 208Pb to cause inhibition of 1 ng DNA template in any sample fractions. Comparing the level of metal measured in the samples fractions, given the expected concentration and dilution for the various fractions, fraction A for sample 1 had an apparent increase of 25% ^{27}Al, relative to the fraction P; this was much lower than expected because of sample concentration between the two steps, and actually indicates significant loss of ^{27}Al due to purification with the Amicon® Ultra-4 PL-50 centrifugal filter device.

Fraction Q for samples 1, 2, 4, and 5 yielded an increase in ^{27}Al, relative to the respective fraction A. Use of the QIAquick PCR Purification Kit does not typically result in sample dilution; however, for this study, fraction Q was a 25% dilution of fraction A. It was unexpected fraction Q for these four samples yielded higher levels of ^{27}Al, relative to the respective fraction A: sample 1, 22% increase; sample 2, 90% increase; sample 4, 15% increase; and, sample 5, an increase of unknown magnitude (percent could be calculated due to fraction A measurement being < LOD).

In general, the Amicon and QIAquick steps are designed to purify and concentrate DNA samples. The increase in Al levels suggests Al binding to DNA template that is being concentrated with the DNA sample in fraction A. Ca levels decrease between fractions P and A, and fractions A and Q for all samples. This suggests that theoretical DNA-Ca interactions occur in a manner that both Ca and DNA are discarded during both purification steps.

Another factor suggested to improve testing of human skeletal remains is the initial weight of the bone sample. Sample weights between 0.5-1.0 g considered ideal for extraction; however, lower weights (*e.g.* 0.2 g) have been reported to have lower concentrations of inhibitors [28]. The estimated quantity of amplifiable human DNA is plotted against the initial sample weight for fractions P, A, and Q in **Fig. 20**. Quantification results suggest fraction A, retained by a centrifugal filter unit, has more amplifiable DNA content than fraction P, the aqueous phase from PCIA extraction, or fraction Q, retained by the additional purification. The additional purification technique decreases the amplifiable DNA content of each sample; however, increasing initial sample weight may increase amplifiable DNA content.

To determine if initial sample weight affected metal concentration, the metal quantity was compared to the initial sample weight in **Fig. 21**. Metal ions that were co-isolated in the aqueous phase of PCIA-extracted bone samples yielded a similar profile for each isotope, despite samples

being from different individuals, exposed to different burial environments. The initial weight of the bone sample had no effect on the amount of metal ion co-purified with DNA, and all samples contained enough metal to inhibit DNA testing. These results suggest that initial sample weight may not be a significant factor affecting the success of DNA typing of human skeletal remains.

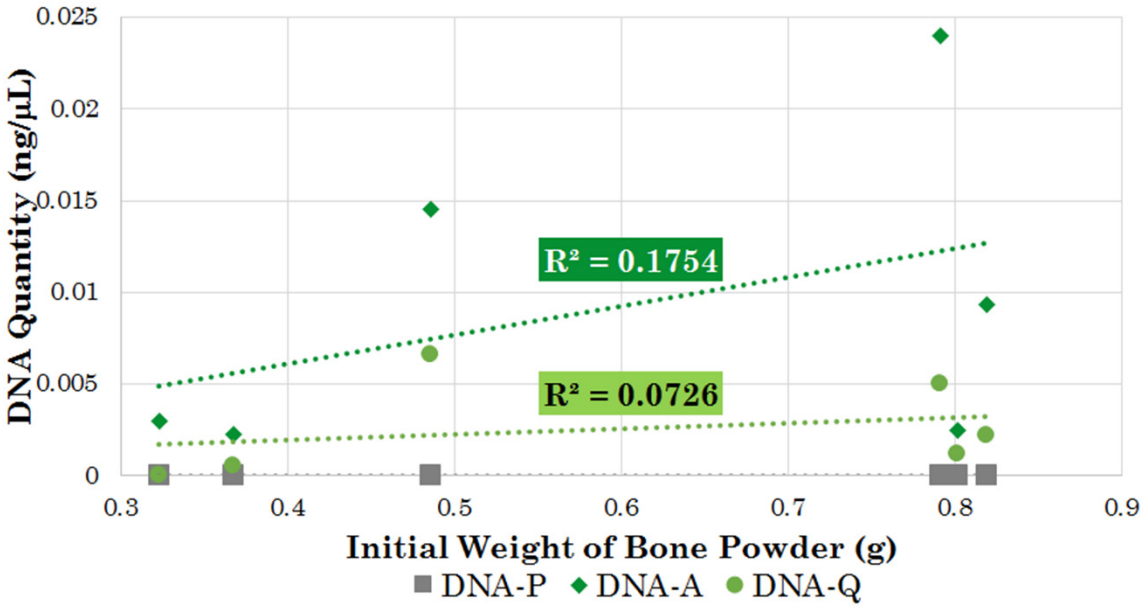

Fig. 20. DNA was extracted from human skeletal remains using a demineralization protocol. Aliquots were obtained following PCIA purification (DNA-P), purification and concentration with an Amicon® Ultra-4 PL-50 centrifugal filter device (DNA-A), and QIAquick PCR Purification Kit (DNA-Q) for six samples. Neat extracts were quantified in duplicate using the Quantifiler® Human DNA Quantification Kit. Sample order (by weight, left to right): 3, 5, 1, 2, 6, 4.

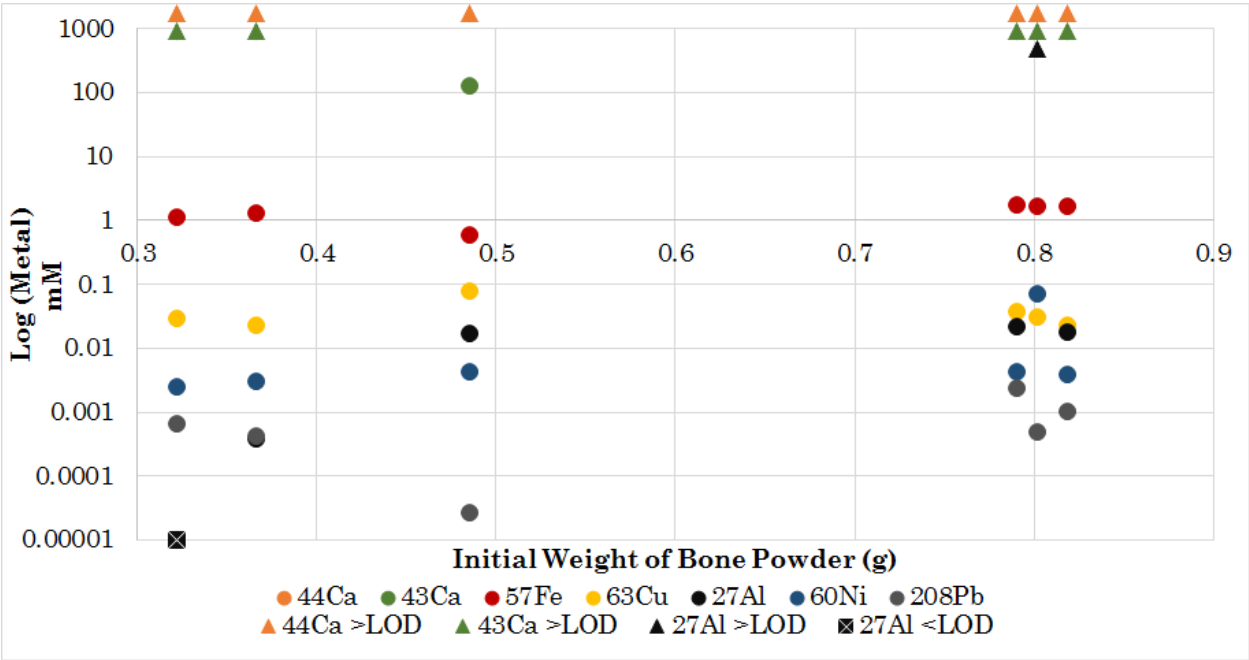

Fig. 21. DNA was extracted from human skeletal remains using a demineralization protocol. Aliquots were obtained following PCIA purification (fraction P) for six samples, numbered 1 through 6. The quantities of seven metal isotopes were measured by ICP-MS. Sample order (by weight, left to right): 3, 5, 1, 2, 6, 4.

CHAPTER VI.

EFFECT OF SKELETAL SAMPLING TECHNIQUE ON DNA ANALYSIS RESULTS

VI.1. Introduction

Apparent skeletal condition (*e.g.* weathering) and initial sample weight are factors that have been evaluated to attempt to improve the DNA typing results obtained from skeletal remains samples [23,28]. Results presented in **Chapter V.** suggest initial starting weight is not a significant factor in the success of DNA typing of human bone samples; however, the possibility that skeletal sampling technique may affect the quality of recovered DNA exists.

Time constraints and limited access to human decomposition facilities make controlled studies to assess damage from decomposition processes and environmental exposure challenging. As a consequence, the scope of this study is limited to DNA damage introduced during sample cleaning and preparation using cadaver bone. The advantage of this study design and sample selection is it allows for isolated examination of the effect of skeletal sampling technique, whether pulverization of the sample contributes to DNA damage observed in profiles obtained from skeletal remains.

VI.2. Materials and Methods

VI.2.1 Cadaver bone samples

A left tibial diaphysis was harvested from a fresh cadaver donated to the Willed Body Program at the University of North Texas Health Science Center. Gross dissection of the lower left limb was performed with a scalpel, forceps, and manual retraction; a Stryker saw (Stryker Surgical Company, Kalamazoo, MI) with a hammer and chisel were used to create transverse cuts and fractures as close to the superior and inferior diaphyseal-metaphyseal junctions as possible, without requiring dissection of the tibial articulations. The diaphysis was packaged in a labeled, re-sealable zipper storage bag and stored frozen, prior to cleaning.

VI.2.2 Sample preparation

Removal of attached soft tissue structures (*e.g.* muscle attachments and periosteum) from the superficial cortical surface and marrow from the medullary cavity was achieved with an assortment of nylon-bristled brushes, a cuticle pusher, and rinsed with water. The cleaned diaphysis, depicted in **Fig. 22**, was allowed to air dry, packaged in a labeled, re-sealable zipper storage bag, and stored frozen.

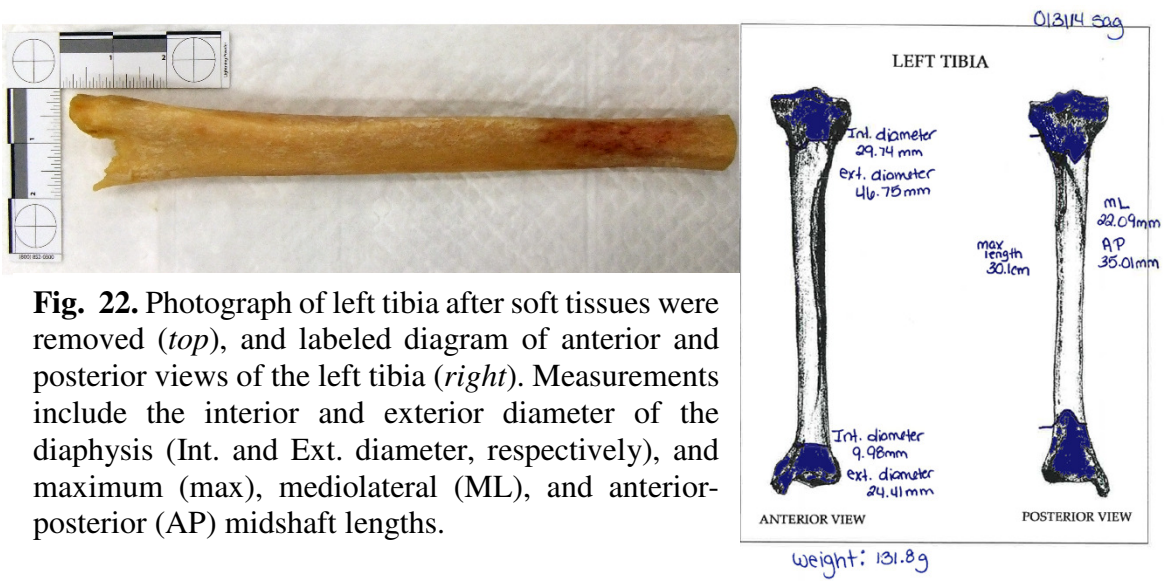

Fig. 22. Photograph of left tibia after soft tissues were removed (*top*), and labeled diagram of anterior and posterior views of the left tibia (*right*). Measurements include the interior and exterior diameter of the diaphysis (Int. and Ext. diameter, respectively), and maximum (max), mediolateral (ML), and anterior-posterior (AP) midshaft lengths.

The tibial diaphysis was allowed to thaw at room temperature before cutting. Samples were obtained by cutting whole pieces of bone (minimally processed, "MIN"), cutting of bone into small pieces along the transverse plane (moderately processed, "MOD"), or pulverized into fine powder (maximally processed, "MAX").

Five whole bone sections were cut from the distal end of the tibial diaphysis using a Dremel® rotary tool with a #545 diamond cutting wheel (Dremel®, Racine, WI), each approximately 1 cm^2 in size, targeting weight between 0.5-1 g. Each piece was weighed and placed into an individual 50 mL conical tube. A 50% bleach solution was used to immerse the bone that was gently agitated and soaked for 1-5 minutes, then the solution was poured off. The bone was rinsed and agitated with sufficient volumes of distilled water until the waste was clear and no longer retained the smell of bleach. A final rinse using 100% ethanol was performed before allowing the bone to dry, and placing them in individual 15 mL conical tubes.

Transverse bone sections were cut from the portion of the distal tibia immediately proximal to the area from which the whole bone sections were obtained. These sections were approximately 0.2 cm in thickness (distal to proximal) and 1 cm in width (medial to lateral). Five groups of cut

bone pieces with total weights similar to the whole bone sections were placed in 50 mL conical tubes and cleaned as described previously before being placed in separate 15 mL conical tubes.

For pulverized bone samples, the cortical surface of the tibial diaphysis was decontaminated using a Dremel® rotary tool and a 10% bleach solution. A Dremel® sanding cone was used to remove the surface (outer cortical layer) of a 5 by 8 cm area of the bone proximal to the portion sampled for the cut bone pieces and the sanded area was wiped using the bleach solution, as needed. A Stryker saw (Stryker Surgical Company, Kalamazoo, MI) was used to cut the sanded area into thin sections; each sample was placed into a 50 mL conical tube and cleaned as described previously. Dried sections were enclosed in a small grinding vial with an impactor rod, immersed in liquid nitrogen, and pulverized to fine powder using a SPEX 6750 Freezer/Mill® (SPEX SamplePrep, Metuchen, NJ). The pulverized bone powder was aliquoted to be consistent with the whole bone sections and placed into five pre-labeled labeled 15 mL conical tubes.

Representative images of the MIN, MOD, and MAX samples are included in **Fig. 23** and individual sample weights of whole bone sections (MIN), cut bone pieces (MOD), and pulverized bone powder (MAX) obtained from the left tibia are provided in **Table 25**.

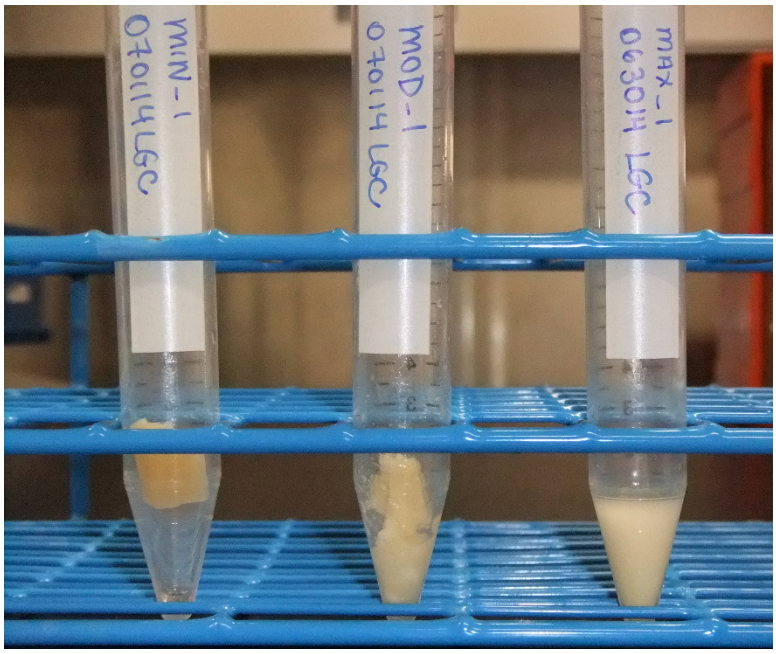

Fig. 23. Samples MIN_1, MOD_1, and MAX_1 samples in 15 mL conical tubes, after incubation and removal of lysate for PCIA purification.

Table 25
The individual weights (g) of whole bone sections (MIN), cut bone pieces (MOD), and pulverized bone powder (MAX) obtained from the left tibia, prior to DNA extraction.

Initial Weight (g) of Cadaver Bone Samples					
MIN_1	0.837	MOD_1	0.907	MAX_1	0.906
MIN_2	0.880	MOD_2	0.891	MAX_2	0.912
MIN_3	0.927	MOD_3	0.915	MAX_3	0.908
MIN_4	0.935	MOD_4	0.899	MAX_4	0.905
MIN_5	0.922	MOD_5	0.887	MAX_5	0.909

VI.2.3 Demineralization extraction of cadaver bone

DNA extraction was performed using the UNT Center for Human Identification "Demineralization Extraction of Skeletal Remains" protocol, a modified version of the total demineralization extraction [28]. Demineralization buffer was prepared using 0.5004 g of sodium N-lauroyl sarcosinate (SLS) and 50 mL 0.5 M ethylenediaminetetraacetic acid (ETDA) solution, and 4.5 mL of this buffer was added to each sample series and a respective reagent blank. In addition, 0.3 mL of 20 mg/mL Proteinase K (Pro K) enzyme was included, prior to vortexing and

incubation for 24 hours at 56°C with agitation. A 1 mL aliquot of the demineralized sample was removed for ethanol (EtOH) purification: 2 mL of ice cold EtOH (99.5%) was added to each sample and reagent blank aliquot, which were vortexed and incubated at -20°C for 30 minutes. Samples were centrifuged at maximum speed for 10 minutes and the supernatant was decanted. The aliquots were washed with 2 mL EtOH (70%) and centrifuged on high for 10 minutes, again the supernatant was decanted. The EtOH was evaporated and samples were dried 45°C and then solubilized at 56°C with 1 mL TE^{-4} (10 mM UltraPure™ Tris-HCL, pH 8.0, Invitrogen Corporation and 0.1 mM UltraPure™ EDTA, pH 8.0, GIBCO Products, Grand Island, NY). The EtOH-purified aliquots were stored refrigerated at 6°C.

Protein digestion and DNA purification of the remaining 3.8 mL demineralized sample was achieved by adding 3.8 mL phenol-chloroform-isoamyl alcohol (PCIA) to each sample and reagent blank. The PCIA-treated samples were vortexed for 30 seconds and centrifuged at maximum speed for 3 minutes. The aqueous phase (3 mL) was transferred into an individual Amicon® Ultra-4 PL-30 centrifugal filter device which isolates DNA based on its molecular weight. The samples were centrifuged through the filtration unit at 3000 x g and 2 mL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA) was added to each filtration unit and samples were again centrifuged until approximately 50 µL remained. Volumes for all retained samples were brought up to 250 µL using TE^{-4} buffer. A final purification was performed using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The sample remaining in the Amicon filtration unit was transferred into a pre-labeled 1.5 mL microcentrifuge tube, and 1250 µL of Buffer PB was added. Samples were vortexed before 750 µL was transferred into a QIAquick column that was assembled inside a 2.0 mL collection tube, centrifuged at 13,000 rpm for 30-60 seconds, and

the waste discarded; this was repeated for the second 750 µL of each sample. Following centrifugation 750 µL of Buffer PE was added to each column, which were centrifuged at 13,000 rpm for 30-60 seconds, and the waste was discarded. For elution of the sample, 200 µL of Buffer EB was added and samples were centrifuged for 60 seconds. Purified samples were stored refrigerated at 6°C.

VI.2.4 Sizing and analysis of DNA fragments

All extracted samples were analyzed in duplicate for DNA fragmentation using the High Sensitivity DNA Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Each DNA extract and reagent blank was prepared by combining 1 µL of sample with 9 µL of High Sensitivity DNA Gel Matrix-Dye Concentrate mixture and 5 µL of High sensitivity DNA Markers 35/10380 bp into individual wells of a primed High Sensitivity DNA chip (Agilent), per manufacturer instructions. Each chip was vortexed for 1 minute at 2400 rpm and analyzed using the corresponding High Sensitivity protocol on the 2100 Bioanalyzer. Gel images were obtained from the 2100 Expert Software v.B.02.08 (Agilent).

VI.2.5 DNA quantification and quality assessment

Quantitative PCR (qPCR) was performed using the Investigator® Quantiplex HYres Kit (QIAGEN, Hilden, Germany) for DNA quantification and the Quantifiler® Trio DNA Quantification Kit (Applied Biosystems) for quality assessment and as a comparative quantification measure. A Quantiplex HYres master mix was prepared according to the manufacturer's instructions, consisting of 9.0 µL Reaction Mix FQ and 9.0 µL Primer Mix IC YQ (QIAGEN) per sample; 18 µL of master mix was distributed into the requisite wells of a 96-well optical reaction plate [96]. Total reaction volume for qPCR was 20 µL, which included the master

mix plus 2 μL of sample. Samples and controls were quantified in triplicate. A "quantitation – standard curve" custom experiment was performed using a 7500 Real-Time PCR System (Applied Biosystems) according to the Quantiplex HYres instructions [96]. Amplification was performed using the following parameters: 95°C hold for 3 minutes; 40 cycles of 95°C for 5 seconds and 60°C for 35 seconds. Data was collected during the 60°C stage for all cycles. The following data were obtained using HID Real-Time PCR Analysis Software v.1.1 (Applied Biosystems): IC quantification cycle (Cq), human Cq, human DNA quantity, male Cq, male DNA quantity, and the mean (μ) and standard deviation (σ) for triplicate samples, when available.

A Quantifiler® Trio master mix was prepared according to the manufacturer's instructions, consisting of 10.0 μL Quantifiler® THP PCR Reaction Mix and 8.0 μL Quantifiler® Trio Primer Mix (Applied Biosystems) per sample; 18 μL of master mix was distributed into the requisite wells of a 96-well optical reaction plate [97]. Total reaction volume for qPCR was 20 μL, which included the master mix plus 2 μL of sample. Samples and controls were quantified in triplicate. A preprogrammed Quantifiler® Trio "quantitation – HID standard curve" experiment was performed using a 7500 Real-Time PCR System (Applied Biosystems) according to the Quantifiler® Trio instructions [97]. Amplification was performed using the default settings for this experiment type, and data was collected for each cycle. The following data were obtained using HID Real-Time PCR Analysis Software v.1.1 (Applied Biosystems): IPC quantification cycle (Cq), small autosomal Cq, small autosomal DNA quantity, large autosomal Cq, large autosomal DNA quantity, male Cq, male DNA quantity, degradation index, and the mean (μ) and standard deviation (σ) for triplicate samples, when available.

VI.2.6 DNA amplification

Experimental and positive control samples were normalized to 0.1 ng/µL of DNA using TE^{-4} buffer prior to amplification, based off the Quantiplex HYres quantification results, whereas reagents blanks and negative control samples were amplified neat. All DNA amplifications were performed in duplicate using AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Applied Biosystems) and the PowerPlex® Fusion System (Promega Corporation, Madison, WI) according to the manufacturer's instructions for 25 µL PCR reaction setup and thermocycling parameters [43,45]. The master mix used for the Identifiler® Plus amplification setup included 10.0 µL of AmpFLSTR® Identifiler® Plus Master Mix and 5.0 µL of AmpFLSTR® Identifiler® Plus Primer Set (Applied Biosystems) per sample. A 15 µL aliquot of this master mix was added to each PCR amplification tube, before adding 10.0 µL of sample. Amplification was performed using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems), in 9600 emulation mode, using the following parameters: 95°C hold for 11 minutes; 28 cycles of 94°C for 20 seconds and 59°C for 3 minutes; 60°C hold for 10 minutes, and 4°C hold until samples removed from the thermocycler and stored refrigerated.

The master mix used for the PowerPlex® Fusion System amplification setup included 10 µL of amplification grade water, 5.0 µL of PowerPlex® Fusion 5X Master Mix and 5.0 µL of PowerPlex® Fusion 5X Primer Pair Mix (Promega) per sample. A 20 µL aliquot of this master mix was added to each PCR amplification tube, before adding 5.0 µL of sample. Amplification was performed using a GeneAmp® PCR System 9700 thermocycler, in Max mode, using the following parameters: 96°C hold for 1 minute; 30 cycles of 94°C for 10 seconds, 59°C for 1

minute, and 72°C for 30 seconds; 60°C hold for 10 minutes, and 4°C hold until samples removed from the thermocycler and stored refrigerated.

VI.2.7 Analysis and genotyping

Genetic analysis of Identifiler® Plus-amplified samples was performed after combining 1 µL of product or allelic ladder with 9 µL master mix that included 8.5 µL Hi-Di™ Formamide and 0.5 µL of GeneScan™ 600 LIZ® Size Standard (Applied Biosystems). Capillary electrophoresis of Identifiler® Plus amplification product was conducted using a 1.2 kV, 24 second injection and POP-4™ Polymer on a 3500xL Genetic Analyzer (Applied Biosystems). STR profiles, including individual peak heights and areas, were obtained using a 50 RFU allele detection threshold and GeneMapper® ID-X Software (Applied Biosystems).

Genetic analysis of PowerPlex® 16 HS-amplified samples was performed after combining 1 µL of product or allelic ladder with 10 µL master mix that included 10 µL Hi-Di™ Formamide and 1 µL of CC5 Internal Lane Standard 500 (Promega). Capillary electrophoresis of PowerPlex® Fusion amplification product was conducted using a 3 kV, 5 second injection and POP-4™ Polymer on a 3130*xl* Genetic Analyzer (Applied Biosystems). STR profiles, including individual peak heights and areas, were obtained using a 50 RFU allele detection threshold and GeneMapper® ID Software v.3.2 (Applied Biosystems).

VI.3. Results

VI.3.1 DNA separation and sizing results

The DNA content of neat purified extracts was separated and sized using the High Sensitivity Kit for the Agilent 2100 Bioanalyzer in duplicate; the normalized 0.1 ng/µL samples for the MAX series was also analyzed. The gel images obtained are in **Fig. 24**, panels include

MIN_neat for whole bone pieces, MOD_neat for cut bone pieces, MAX_neat for the neat extracts from pulverized bone powder, and MAX_0.1 ng/µL for normalized MAX series samples. Samples yielded DNA in the 35 to 300 bp range and DNA >700 bp range. Gauging the visual intensity of the samples, from greatest to least DNA in the 35 to 300 bp range was MAX, MIN, MOD; and from greatest to least DNA in the >700 bp range was MOD, MAX, MIN.

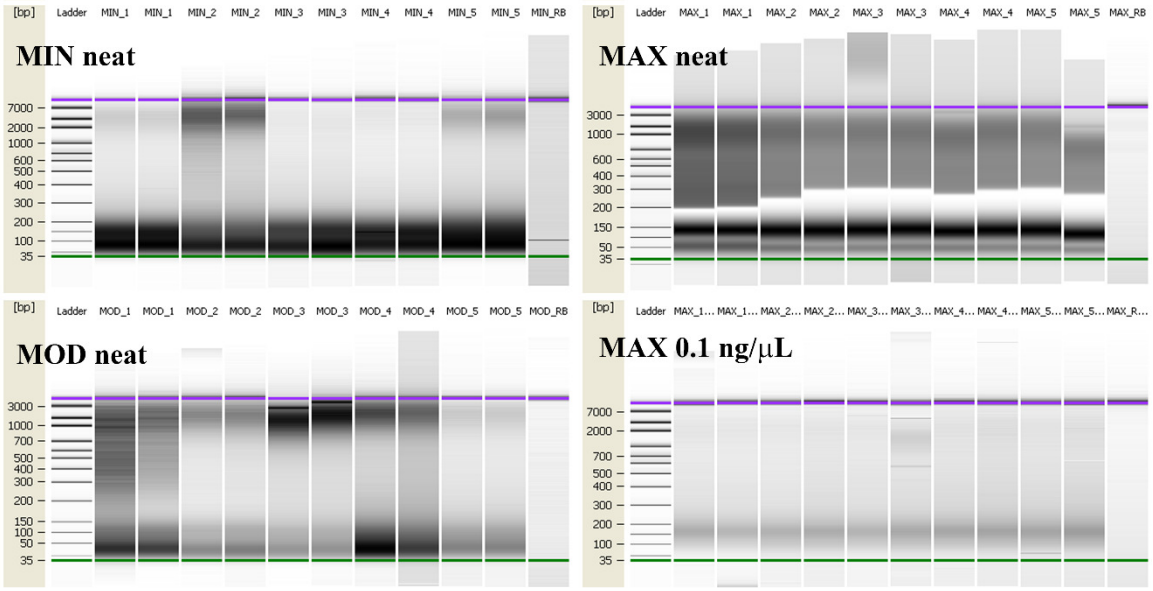

Fig. 24. Bone samples were extracted in quintuplicate using a demineralization protocol. Extracted and purified DNA was analyzed in duplicate on the Agilent 2100 Bioanalyzer using the High Sensitivity Kit; this includes neat extracts for the whole bone sections (MIN_neat), cut bone pieces (MOD_neat), pulverized bone powder (MAX_neat), and normalized extracts for pulverized bone samples (MAX_0.1 ng/µL). Gel images are provided for each series of samples and the corresponding reagent blank (RB). Sizing markers are 10,380 bp (purple) and 35 bp (green); ladder includes fragments that are 7,000, 3,000, 2,000, 1,000, 700, 600, 500, 400, 300, 200, 150, 100, and 50 bp, in addition to the sizing markers.

VI.3.2 DNA quantification results obtained for cadaver bone samples

The DNA content of neat extracts was quantified using the Investigator® Quantiplex HYres Kit. The standard curve parameters for the human DNA target were: R^2: 0.996, slope: -3.633, and intercept: 26.16, inclusive of all data points. The standard curve for the male DNA

target was not analyzed, as the samples were from a female individual. Results for reagent blanks, no template control (NTC) and positive control samples were as expected.

The IC Cq values obtained for all samples are illustrated in **Fig. 25**. The difference between mean IC Cq values for each sample series and the mean IC Cq value for the 0.1 ng/µL positive control samples were 0.09 for the MIN series (range 0.00 to 0.36), 0.11 for the MOD series (range 0.00 to 0.33), and 1.24 for the MAX series (range 0.05 to 4.25). The variation was smallest within the MOD series (*sd* = 0.08) and the MIN series (*sd* = 0.09), and greatest within the MAX series (*sd* = 1.41).

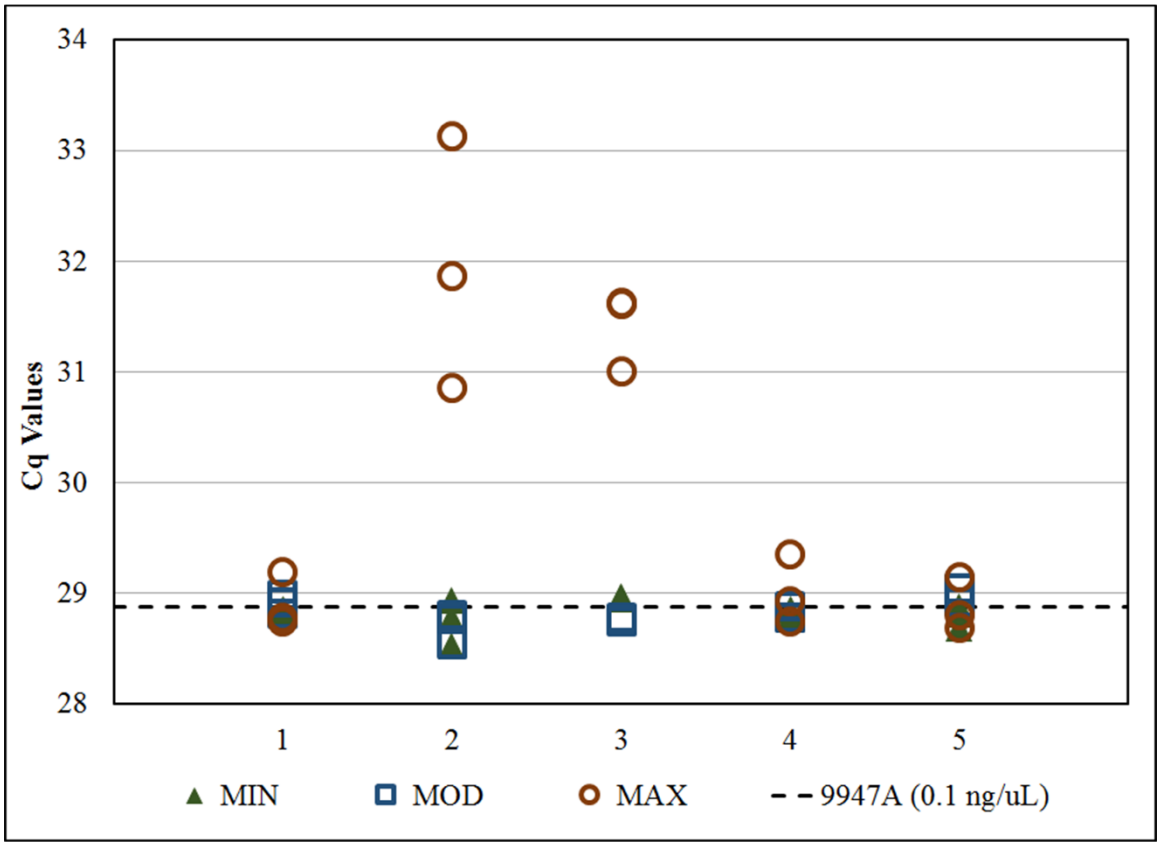

Fig. 25. Whole bone sections (MIN), cut bone pieces (MOD), and pulverized bone powders (MAX) were extracted in quintuplicate using a demineralization protocol. Purified DNA extracts were quantified in triplicate using the Investigator® Quantiplex HYres Kit. Cq values for the Internal Control from the neat extracts are illustrated; x-axis identifies extraction replicate number 1 through 5.

The estimated quantity of human DNA in all samples is depicted in **Fig. 26**, plotted against the initial sample weight (g). The MIN series samples yielded an average of 0.19 ng/µL of DNA (range 0.11 to 0.21); MOD series, average 0.25 ng/µL (range 0.07 to 0.48); MAX series 7.12 ng/µL (range 6.30 to 8.09). The MAX series samples had estimated quantities that were 38 to 58 times greater than the MIN series and 17 to 93 times greater than the quantities of the MOD series.

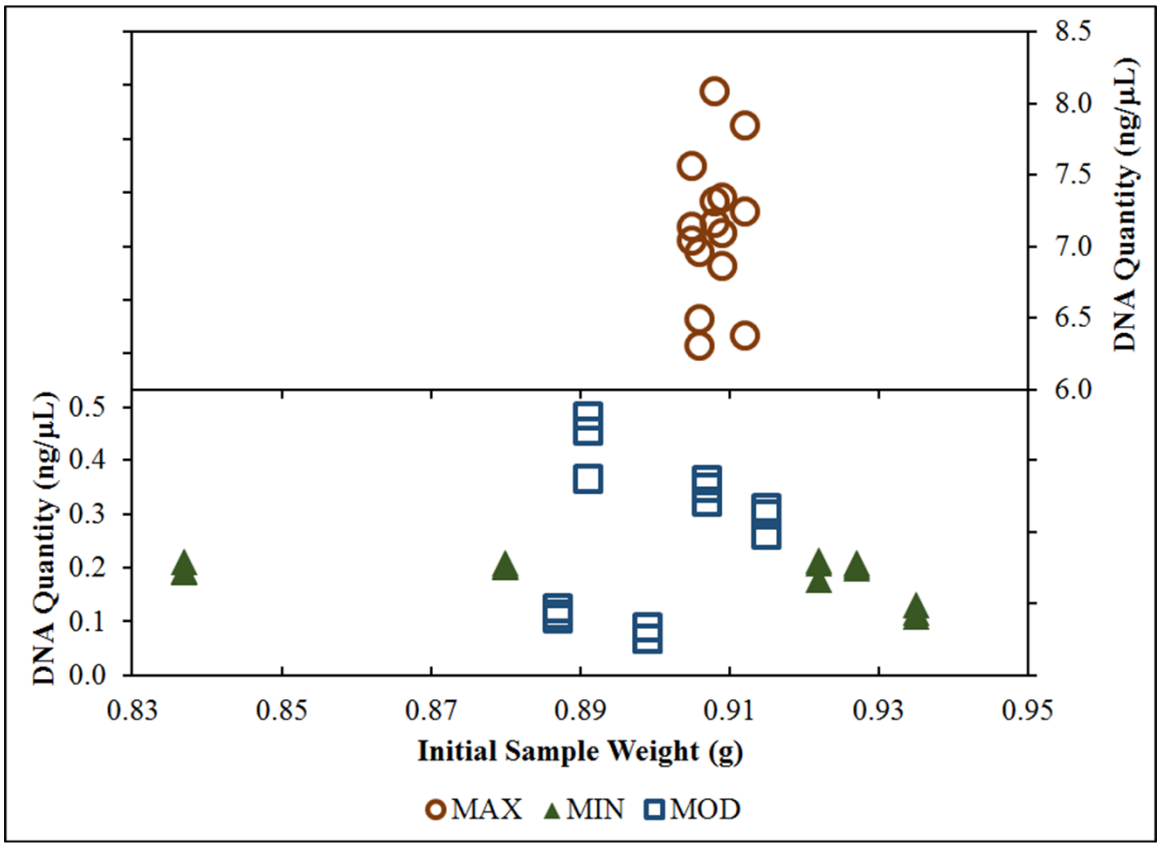

Fig. 26. Whole bone sections (MIN), cut bone pieces (MOD), and pulverized bone powders (MAX) were extracted in quintuplicate using a demineralization protocol. Purified DNA extracts were quantified in triplicate using the Investigator® Quantiplex HYres Kit. The calculated human DNA quantity (ng/mL) is compared to the starting weight (g) of the respective bone sample. MIN and MOD series are plotted against the 0.0 to 0.5 ng/µL scale on the bottom panel and the MAX series values are plotted against the 6.0 to 8.5 ng/µL scale on the top panel.

The DNA content of neat extracts was quantified using the Quantifiler® Trio DNA Quantification Kit. The standard curve parameters, inclusive of all standard data points for all

targets, were for the large autosomal human target: R^2: 0.998, slope: -3.504, and intercept: 25.343; small autosomal human target: R^2: 1, slope: -3.242, and intercept: 27.526; human male target: R^2: 1, slope: -3.242, and intercept: 27.526. Results for reagent blanks, no template control (NTC) and positive control samples were as expected.

The Cq values obtained for the IPC and large autosomal human targets for all samples are illustrated in **Fig. 27**. The difference between mean IPC Cq values for each sample series and the mean IPC Cq value for the 0.1 ng/µL positive control samples were 0.27 for the MIN series (range 0.05 to 0.51), 0.28 for the MOD series (range 0.11 to 0.49), and 0.34 for the MAX series (range 0.09 to 0.50). The variation was greatest within the MOD series (*sd* = 0.18) and the MIN series (*sd* = 0.18), and least within the MAX series (*sd* = 0.15).

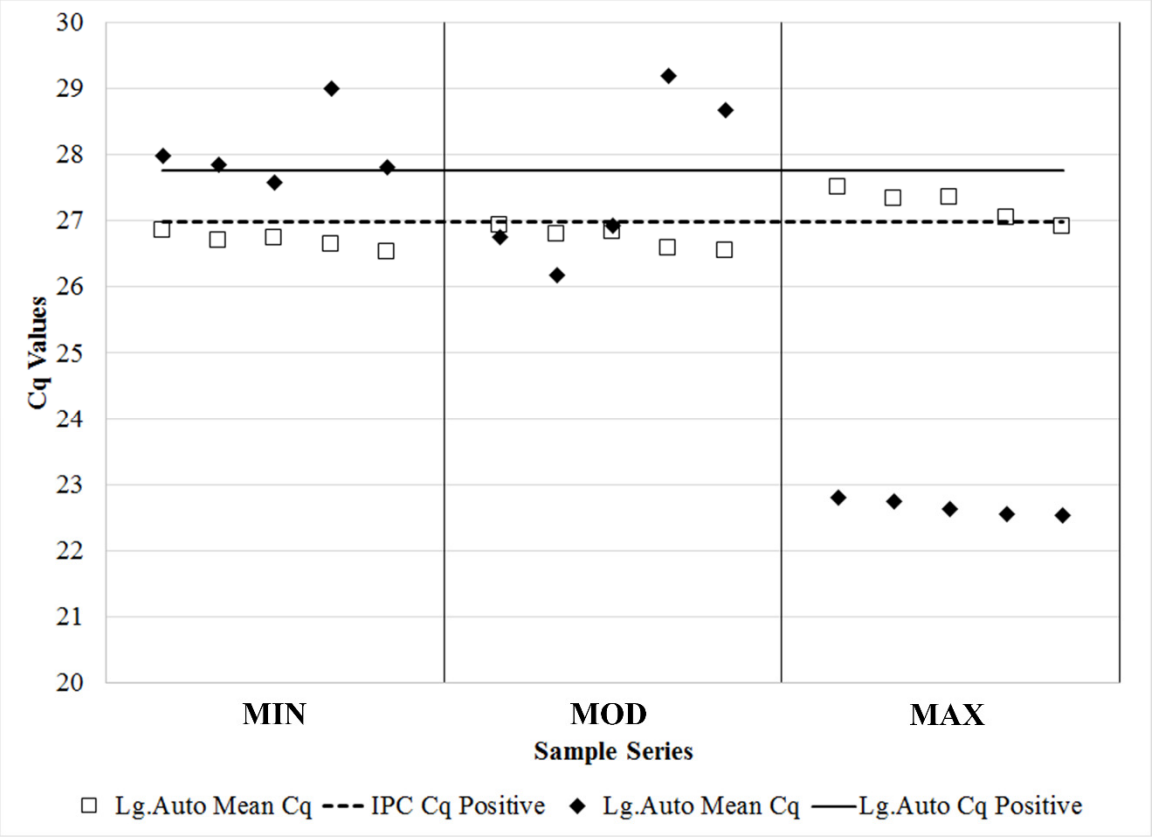

Fig. 27. Whole bone sections (MIN), cut bone pieces (MOD), and pulverized bone powders (MAX) were extracted in quintuplicate using a demineralization protocol. Purified DNA extracts

were quantified in triplicate using the Quantifiler® Trio DNA Quantification Kit. Mean Cq values for the Internal PCR Control (IPC) and the large autosomal human DNA fragment (Lg.Auto) resulting from the neat extracts are illustrated; the solid black line denotes the mean Cq value obtained for the large autosomal fragment and the dashed black line indicates the mean Cq value obtained for the IPC target in the 0.1 ng/µL positive control sample, respectively.

The average quantities and ranges obtained from the Quantifiler® Trio large autosomal assay were as follows: MIN series samples yielded an average of 0.18 ng/µL of DNA (range 0.09 to 0.25); MOD series, average 0.30 ng/µL (range 0.08 to 0.58); MAX series 5.89 ng/µL (range 5.35 to 6.36). The MAX series samples had estimated quantities that were 26 to 59 times greater than the MIN series and 11 to 67 times greater than the quantities of the MOD series.

The estimated mean quantity of human DNA resulting from analysis of the large autosomal target for all samples is compared to the quantification estimate obtained from the Quantiplex HYres Kit for the respective sample replicate in **Fig. 28**. The Quantifiler® Trio estimates were greater than the Quantiplex HYres for the lower quantity samples (**Fig. 28**, bottom panel), and lower for the higher quantity samples (**Fig. 28**, top panel).

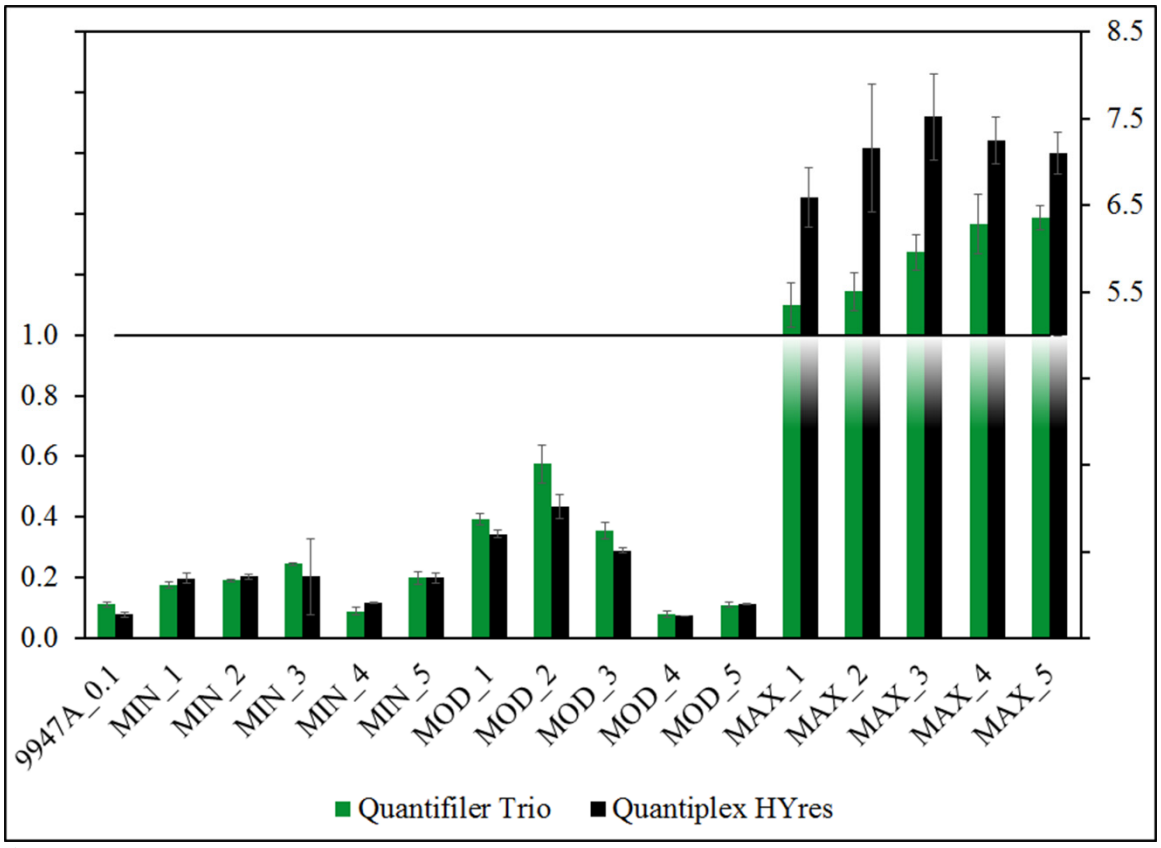

Fig. 28. Whole bone sections (MIN), cut bone pieces (MOD), and pulverize
d bone powders (MAX) were extracted in quintuplicate using a demineralization protocol. Purified DNA extracts from each of the five replicates, numbered 1 through 5, were quantified in triplicate using the Quantifiler® Trio DNA Quantification Kit and the Investigator® Quantiplex HYres Kit. The mean DNA quantities obtained for the large autosomal human target in Quantifiler® Trio is compared to the human DNA target in the Quantiplex kit for the respective replicate; error bars denote standard deviation.

VI.3.3 DNA damage assessment with the Quantifiler® Trio DNA Quantification Kit

The mean Degradation Index (DI) obtained for samples illustrated in **Fig. 29**, with the respective standard deviation. The positive control samples had a mean DI of 0.6 (*sd* = 0.1). The MOD series had the lowest mean DI of 3.9 (range 1.7 to 8.6), followed by the MAX series with a mean of 4.1 (range 3.8 to 4.3), and the MIN series had the highest mean DI at 7.6 (range 3.3 to 15.5). The mean variation was smallest in within the DI values obtained for the MAX series with an *sd* = 0.2, followed by the MOD series at 2.8, and the MIN series at 5.0.

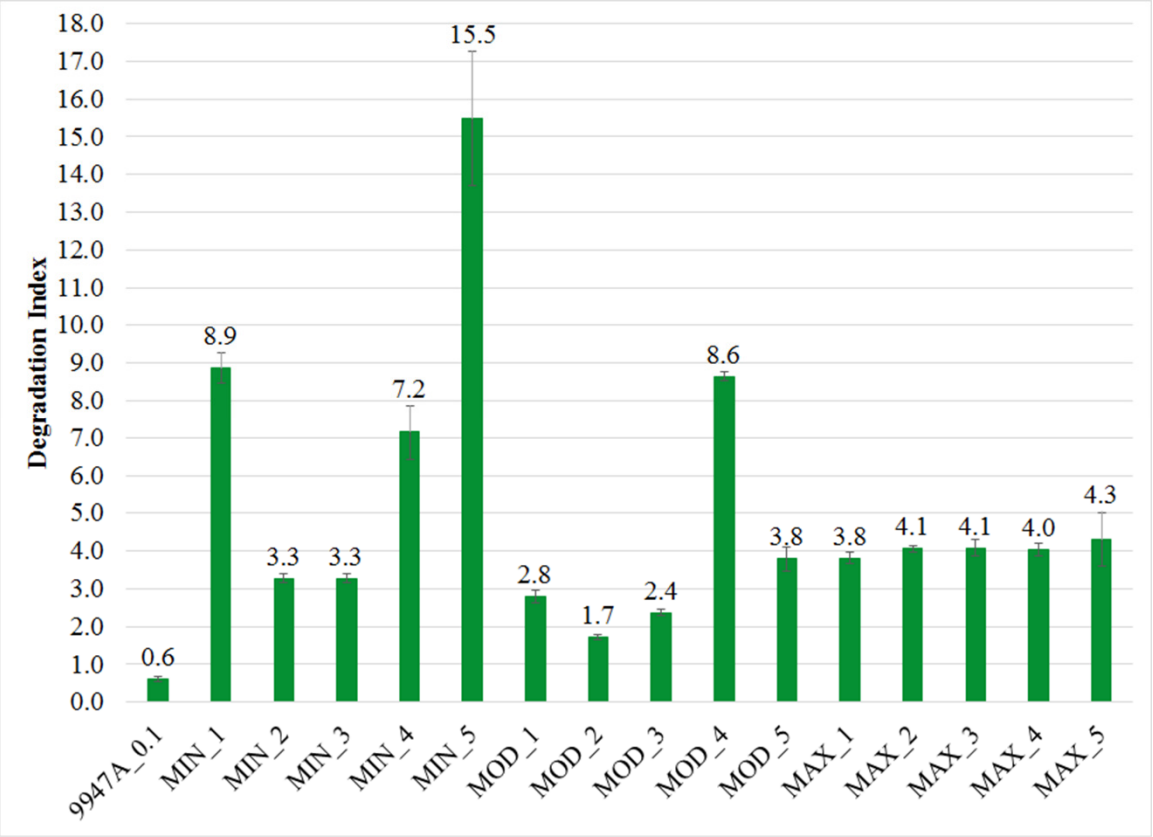

Fig. 29. Whole bone sections (MIN), cut bone pieces (MOD), and pulverized bone powders (MAX) were extracted in quintuplicate using a demineralization protocol. Purified DNA extracts were quantified in triplicate using the Quantifiler® Trio DNA Quantification Kit. The Degradation Index is the ratio of the estimated quantity of the small autosomal DNA fragment compared to that of the large autosomal DNA fragment. The mean obtained for the five replicates in each series is plotted; error bars reflect the standard deviation of the respective mean.

VI.3.4 Cadaver bone samples amplified with AmpFLSTR® Identifiler® Plus

All expected alleles were obtained for normalized samples, amplified in triplicate with the Identifiler® Plus PCR Amplification Kit, and analyzed on a 3500xL Genetic Analyzer. Examples of electropherograms obtained for a MIN, MOD, and MAX sample are illustrated in **Fig. 30**, **Fig. 31**, and **Fig. 32**, respectively, with a normalized scale of 22.5K RFU.

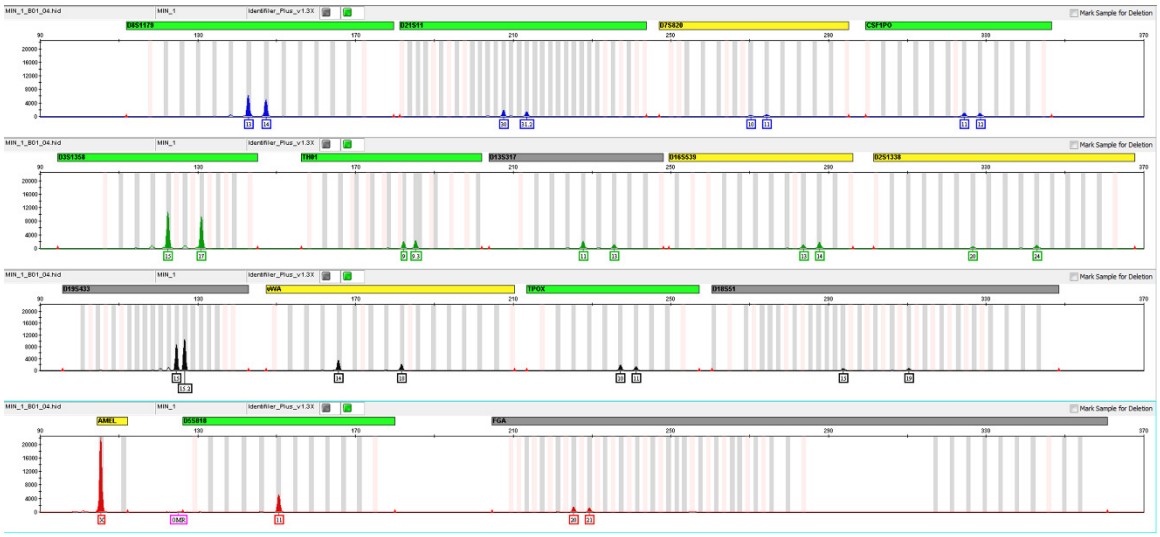

Fig. 30. Extracted and purified DNA obtained from a whole bone section (MIN) was amplified using the Identifiler® Plus PCR Amplification Kit and analyzed on a 3500xL Genetic Analyzer. The resulting data was analyzed using GeneMapper® ID-X Software with a 50 RFU detection threshold; the resulting electropherogram is illustrated at a scale of 22.5K RFU.

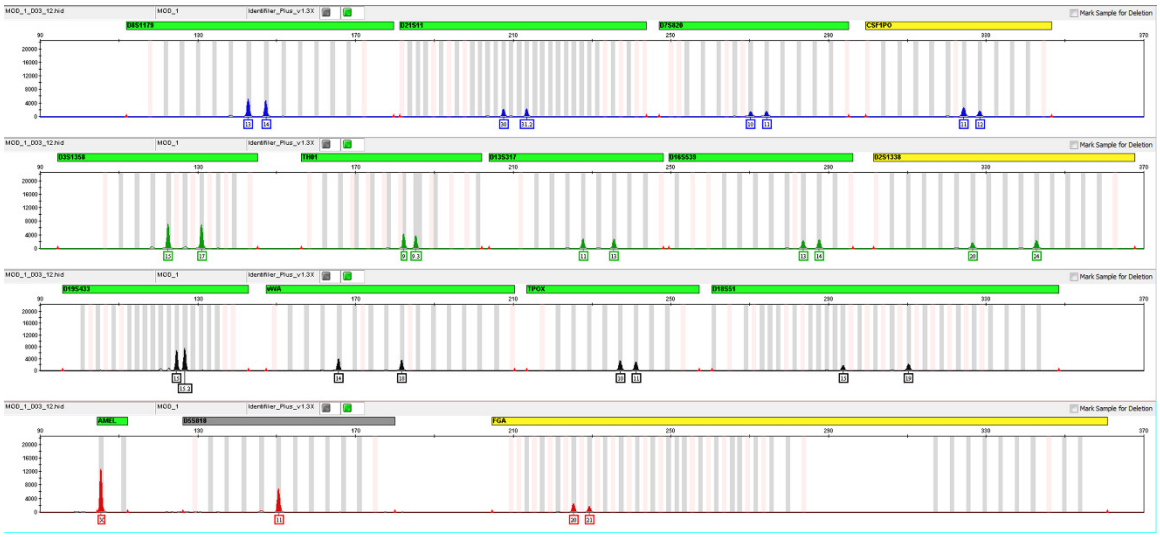

Fig. 31. Extracted and purified DNA obtained from cut bone pieces (MOD) was amplified using the Identifiler® Plus PCR Amplification Kit and analyzed on a 3500xL Genetic Analyzer. The resulting data was analyzed using GeneMapper® ID-X Software with a 50 RFU detection threshold; the resulting electropherogram is illustrated at a scale of 22.5K RFU.

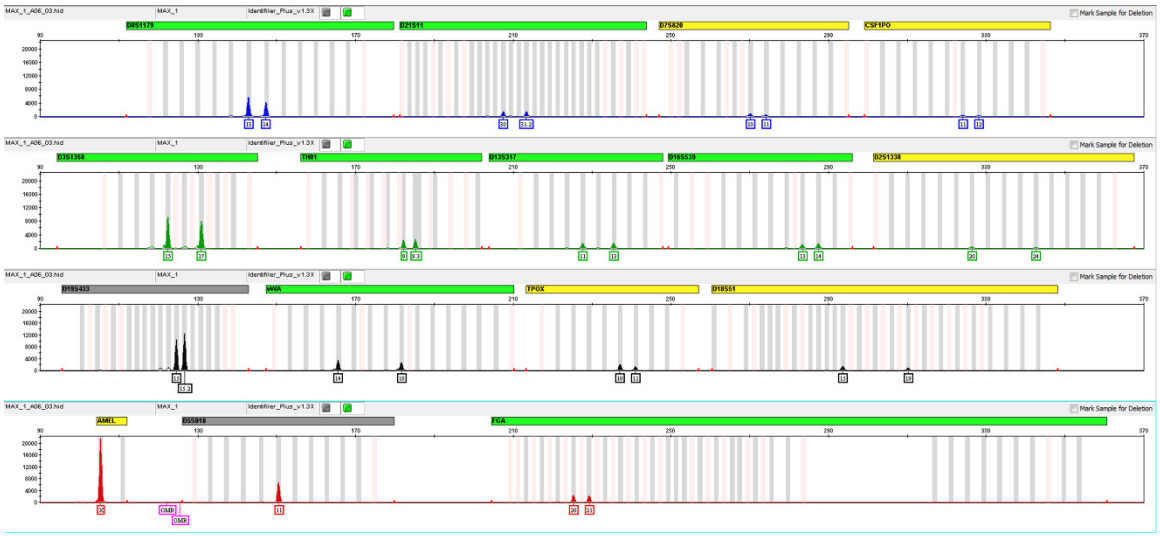

Fig. 32. Extracted and purified DNA obtained from pulverized bone powder (MAX) was amplified using the Identifiler® Plus PCR Amplification Kit and analyzed on a 3500xL Genetic Analyzer. The resulting data was analyzed using GeneMapper® ID-X Software with a 50 RFU detection threshold and the resulting electropherogram is illustrated at a scale of 22.5K RFU.

Fig. 33 and **Fig. 34** illustrate comparisons between representative MIN, MOD, and MAX samples in the VIC® and PET® channels, respectively, normalized at 11K RFU. These comparisons depict preferential amplification of lower molecular weight loci, heterozygote peak imbalances, and differences in peak height patterns observed for MIN, MOD, and MAX samples.

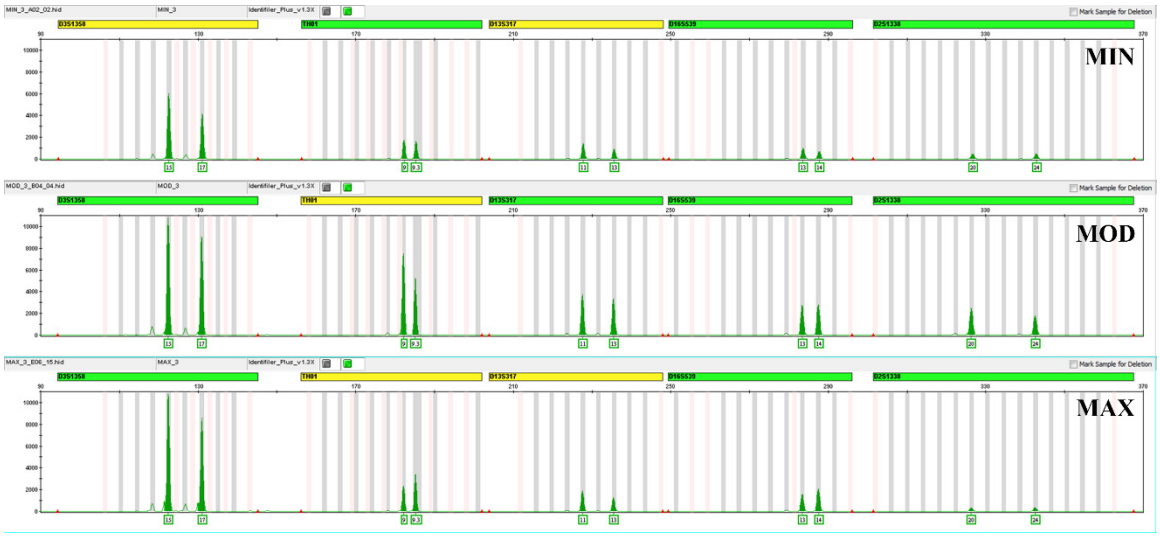

Fig. 33. Whole bone sections (MIN), cut bone pieces (MOD), and pulverized bone powders (MAX) were extracted in quintuplicate using a demineralization protocol. Purified DNA extracts were amplified in triplicate using the Identifiler® Plus PCR Amplification Kit and analyzed on a

3500xL Genetic Analyzer. The resulting data was analyzed using GeneMapper® ID-X Software with a 50 RFU detection threshold. The VIC® channel is compared between representative samples for each sample series is depicted at a normalized scale of 11K RFU.

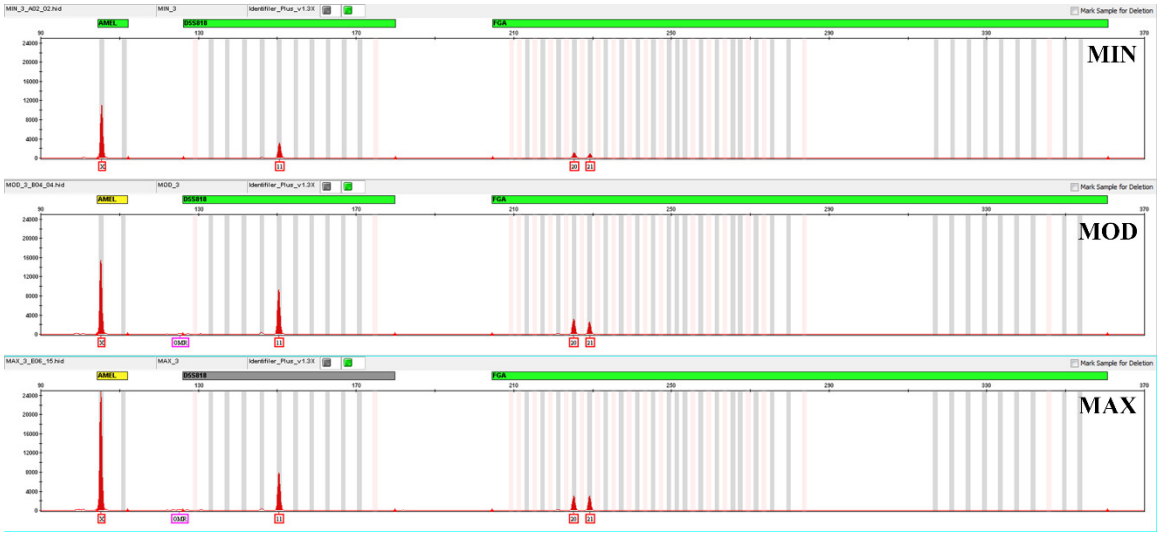

Fig. 34. Whole bone sections (MIN), cut bone pieces (MOD), and pulverized bone powders (MAX) were extracted in quintuplicate using a demineralization protocol. Purified DNA extracts were amplified in triplicate using the Identifiler® Plus PCR Amplification Kit and analyzed on a 3500xL Genetic Analyzer. The resulting data was analyzed using GeneMapper® ID-X Software with a 50 RFU detection threshold. The PET® channel is compared between representative samples for each sample series is depicted at a normalized scale of 11K RFU.

The peak height relative fluorescent units (RFU) for the individual alleles obtained from the triplicate Identifiler® Plus amplifications are depicted in **Fig. 35**, plotted against the average fragment size for each allele. MIN, MOD, and MAX series include all alleles for all samples and are compared to a positive control sample. All experimental series exhibited approximately three-fold decrease in RFU for alleles greater than 150 bp in length, compared to those less than 150 bp in length. Compared to the allele heights of the positive control sample, this observed phenomena in the experimental series appears to be a reduction of peak height intensity in the higher molecular weight loci.

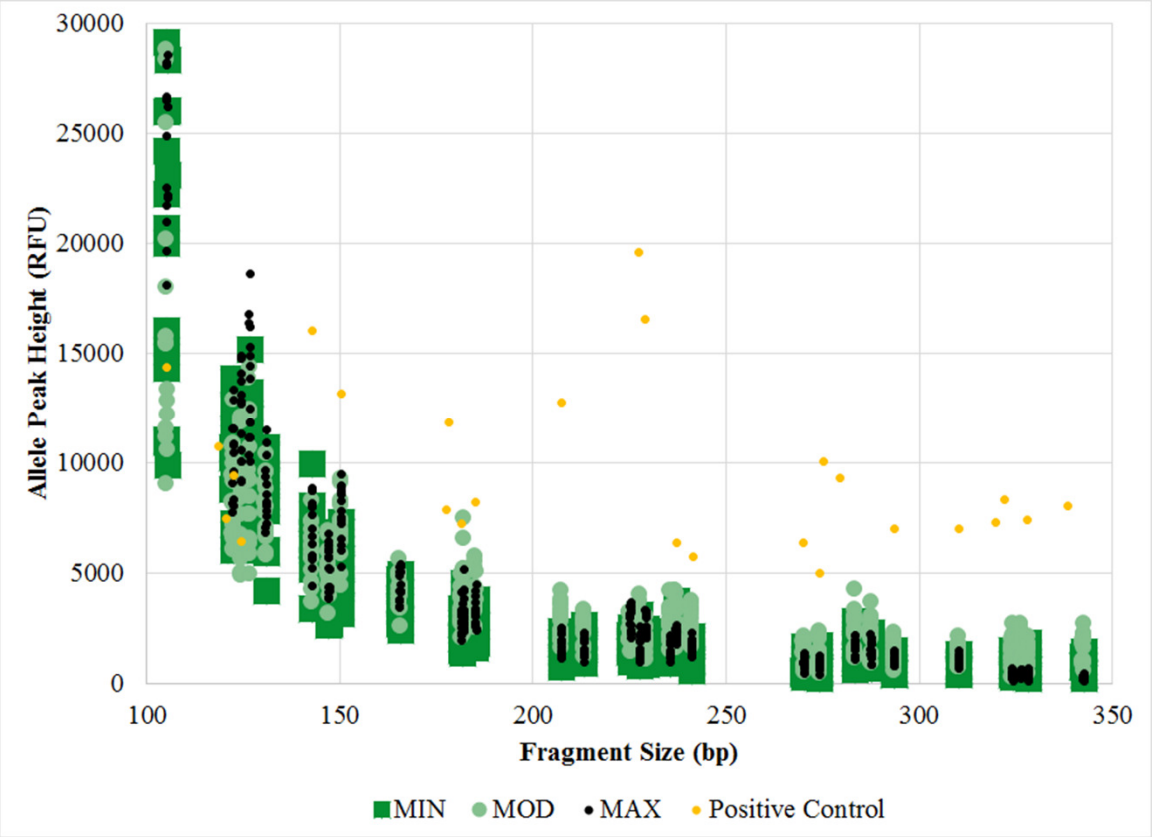

Fig. 35. Whole bone sections (MIN), cut bone pieces (MOD), and pulverized bone powders (MAX) were extracted in quintuplicate using a demineralization protocol. Purified DNA extracts were amplified in triplicate using the Identifiler® Plus PCR Amplification Kit and analyzed on a 3500xL Genetic Analyzer. Allele calls were made using GeneMapper® ID-X Software with a 50 RFU detection threshold. Allele peak heights (RFU) were compared to the average fragment size (bp) for the respective alleles in bone samples, and a positive control sample.

The allele peak height intensities (RFU) for Identifiler® Plus amplified loci were averaged and the standard deviation was calculated for each of the four dyes (6-FAM™, VIC®, NED™, and PET®). The means and standard deviations (SD) are listed in **Table 26** for the MIN, MOD, MAX series samples and the 9947A positive control samples. All experimental series samples had lower means than the positive control samples, and lower SD values for all channels except PET®. The MIN series had the lowest mean peak heights for all channels, except PET®, and the SD was between that obtained for the MOD and MAX samples. The MOD series had the highest mean

peak intensities for 6-FAM™ and VIC® channels, whereas the MAX series had the highest means for NED™ and PET® channels. The MOD series had the lowest SD values for all channels.

Table 26
Mean and standard deviation (SD) values for allele peak heights for experimental (MIN, MOD, MAX) samples and control (9947A) samples amplified with Identifiler® Plus. Data obtained from individual dye channels were analyzed separately.

	6-FAM™ (blue)		VIC® (green)		NED™ (yellow)		PET® (red)	
Series	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MIN	2193	2115	3170	3339	3783	3559	7148	7939
MOD	2768	1859	3831	2528	4217	2936	7023	6272
MAX	2294	2417	3239	3370	4953	4991	9381	9268
9947A	9160	4288	9569	4770	8528	3569	9914	4487

Table 27 displays the minimum and maximum allele peak height (RFU) obtained for each data series in the four channels and the ratio of the minimum to maximum heights, calculated to quantitatively express the decrease in height values across the size range. The MOD series had the highest percent for all channels; the 6-FAM™ ratio was two to four fold higher in the MOD versus the MIN and MAX samples, respectively; the VIC® ratio, three and seven times higher; the NED™ ratio, two times higher; and, the PET® ratio, twice that of the MIN series and 3% higher than the MAX series. The positive control sample ratios were higher than the MOD series by two, four, five, and three-fold, respectively, for 6-FAM™, VIC®, NED™, and PET® channels.

Table 27

Average minimum (Min) and maximum (Max) values for allele peak heights and the calculated percent of minimum to maximum values for experimental (MIN, MOD, MAX) and control (9947A) samples amplified with the Identifiler® Plus PCR Amplification Kit. Data obtained from individual dye channels (6-FAM™, VIC®, NED™, PET®) were analyzed separately.

	6-FAM™ (blue)			VIC® (green)			NED™ (yellow)			PET® (red)		
Series	Min	Max	%	Min	Max	%	Min	Max	%	Min	Max	%
MIN	805	6322	13%	729	10264	7%	779	8846	9%	1459	19797	8%
MOD	1303	6038	22%	1614	8639	20%	1378	8219	19%	2045	16548	14%
MAX	380	7012	5%	301	10189	3%	1045	12405	8%	2667	24186	11%
9947A	7439	16056	46%	8060	10748	75%	7034	7501	94%	5738	14388	40%

VI.3.5 Cadaver bone samples amplified with the PowerPlex® Fusion System

The expected alleles obtained for normalized samples, amplified in duplicate with the PowerPlex® Fusion System, and analyzed on a 3130*xl* Genetic Analyzer are summarized in **Table 28**. The expected allele count for a single sample was 42, and the total allele count expected for the complete series (duplicate amplification of quintuplicate extractions) was 420.

Table 28
The total number of alleles (allele count) and average percent of the expected profile for the cadaver bone samples are provided for each sample series. The expected profile obtained with the PowerPlex® Fusion System for the cadaver bone consisted of 42 alleles; thus, duplicate amplification of the quintuplicate extracts was equal to 420 total expected alleles.

Series	Allele Count	Percent of Expected Profile
MIN	387	92%
MOD	410	98%
MAX	366	87%

The peak height relative fluorescent units (RFU) for the individual alleles obtained from the duplicate PowerPlex® Fusion System amplifications are depicted in **Fig. 36**, plotted against the average fragment size for each allele. MIN, MOD, and MAX series include alleles for all samples, expected alleles below the 50 RFU detection threshold were manually-assigned values of zero. The plot includes a buccal reference sample, extracted using Chelex® 100 resin, and a positive control sample, both amplified in duplicate. The MIN, MOD, and MAX series exhibited an approximately two to three-fold reduction in allele peak height intensities for loci greater than 200 bp, compared to those less than 200 bp. Compared to the buccal and positive control sample alleles, this phenomena appears as an increase in peak height intensity of the lower molecular weight loci.

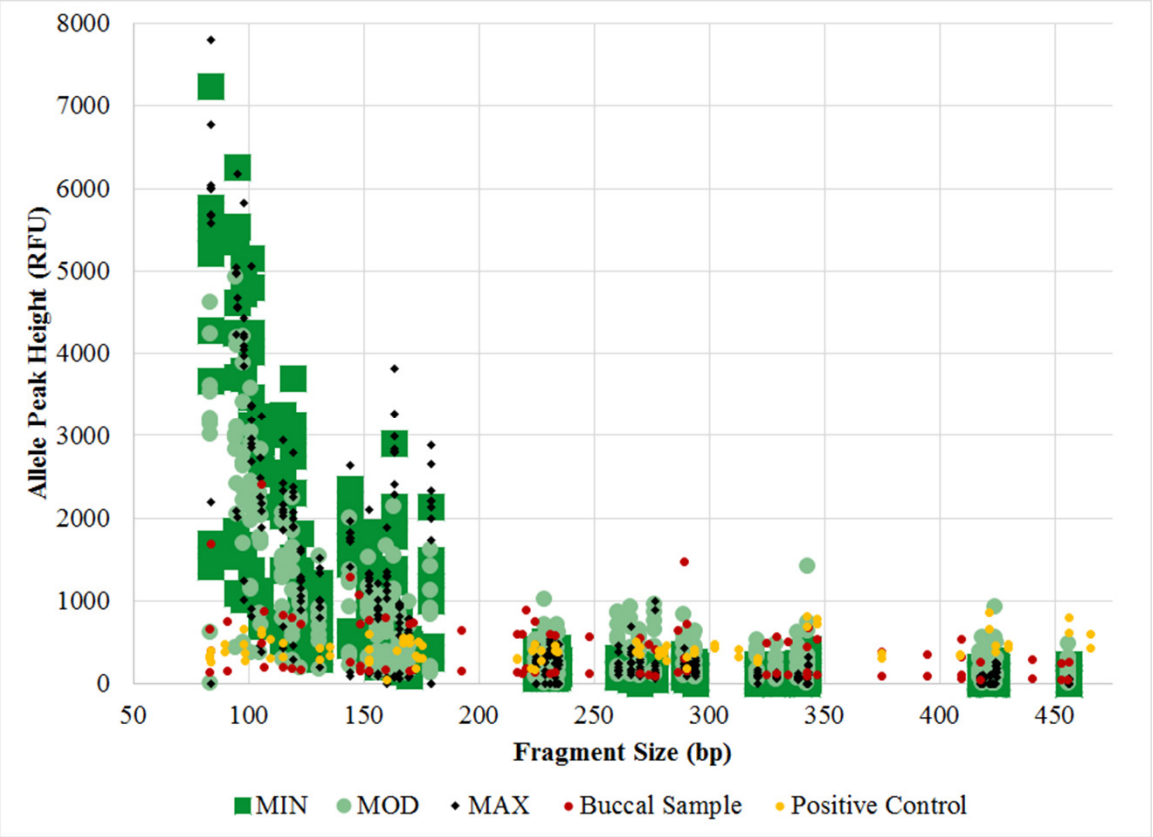

Fig. 36. Whole bone sections (MIN), cut bone pieces (MOD), and pulverized bone powders (MAX) were extracted in quintuplicate using a demineralization protocol. Purified DNA extracts were amplified in duplicate using the PowerPlex® Fusion System and analyzed on a 3130xl Genetic Analyzer. Allele calls were made using GeneMapper® ID Software v.3.2 with a 50 RFU detection threshold. Allele peak heights (RFU) were compared to the average fragment size (bp) for the respective alleles in bone samples, as well as for buccal and positive control samples.

The allele peak height intensities (RFU) for PowerPlex® Fusion System amplified loci were averaged and the standard deviation was calculated for each of the four dyes (Fluorescein, JOE, TMR-ET, CXR-ET). The means and standard deviations (SD) are listed in **Table 29** for the MIN, MOD, MAX series samples, the buccal reference samples, and the 9947A positive control samples. All experimental series samples had higher means than the buccal and positive control samples, and lower SD values for all channels except JOE when compared to the buccal sample. The positive control samples had the lowest SD values for all channels. The MOD series had the lowest mean peak heights for all channels, except TMR-ET where MAX was the lowest, and the

SD was between that obtained for the MOD and MAX samples, except for CXR-ET where it was the lowest. The MIN series had the mean peak intensities that were between those obtained for the MOD and MAX samples for all channels and the highest SD values among the three series. The MAX series had the highest means for all channels and the lowest SD values for all channels, except CXR-ET.

Table 29
Mean and standard deviation (SD) values for allele peak heights for experimental (MIN, MOD, MAX) and control (Buccal and 9947A) samples amplified with the PowerPlex® Fusion System. Computation included values for callable alleles (>50 RFU) only; data obtained from individual dye channels (Fluorescein, JOE, TMR-ET, CXR-ET) were analyzed separately.

	Fluorescein (blue)		JOE (green)		TMR-ET (yellow)		CXR-ET (red)	
Series	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MIN	665	286	847	451	1031	413	910	397
MOD	605	217	719	278	914	359	770	288
MAX	930	216	1162	266	1264	274	1029	326
Buccal	409	358	370	330	479	446	471	435
9947A	380	1	459	24	467	8	404	34

Table 30 displays the minimum and maximum allele peak height (RFU) obtained for each data series in the four channels and the ratio of the minimum to maximum heights, calculated to quantitatively express the decrease in height values across the size range. The MOD series had the highest percent for all channels; the Fluorescein ratio was two to three fold higher in the MOD versus the MIN and MAX samples, respectively; the JOE and TMR-ET ratios, two and four times higher;; and, the CXR-ET ratio, three times that of the MIN series and two times higher than the MAX series. The buccal sample ratios were higher than the MOD series by four, two, and two-fold, respectively, for Fluorescein, JOE, and TMR-ET channels. The buccal sample was equivalent to the ratio obtained for the MOD series in the CXR-ET channel. The positive control sample ratios

were higher than the MOD series by eleven, three, two, and three-fold, respectively, for Fluorescein, JOE, TMR-ET, and CXR-ET channels.

Table 30
Average minimum (Min) and maximum (Max) values for allele peak heights and the calculated percent of minimum to maximum values for experimental (MIN, MOD, MAX) and control (Buccal and 9947A) samples amplified with the PowerPlex® Fusion System. Computation included values for callable alleles (>50 RFU) only; data obtained from individual dye channels (Fluorescein, JOE, TMR-ET, CXR-ET) were analyzed separately.

	Fluorescein (blue)			JOE (green)			TMR-ET (yellow)			CXR-ET (red)		
Series	Min	Max	%	Min	Max	%	Min	Max	%	Min	Max	%
MIN	74	4193	2%	75	1881	5%	91	3973	3%	86	3134	3%
MOD	141	3233	5%	163	1414	13%	183	3065	7%	179	2333	9%
MAX	75	5746	2%	72	2689	3%	79	4589	2%	137	3070	4%
Buccal	226	1041	22%	184	661	25%	155	949	15%	152	1454	10%
9947A	280	527	53%	303	758	40%	115	823	14%	183	726	26%

VI.4. Conclusions and Discussion

Skeletal sampling technique was evaluated by extracting DNA from whole bone section (MIN), cut bone pieces (MOD), and pulverized bone powder (MAX) to determine whether pulverization of the sample contributes to DNA damage observed in profiles obtained from skeletal remains. DNA separation and sizing results obtained with the Agilent 2100 Bioanalyzer revealed DNA in all bone sample extracts, with a significant amount of low molecular weight DNA in the MAX series, compared to the MIN and MOD samples series. Visual comparison of the higher molecular weight DNA region in **Fig. 24** reveals an increase in intensity in the higher molecular weight DNA in the MOD series extracts. This finding suggests only superficial DNA is being recovered from the skeletal connective tissue.

Internal control Cq values for both the Investigator® Quantiplex HYres and Quantifiler® Trio Quantification Kit (**Fig. 25** and **Fig. 27**) did not indicate the presence of PCR inhibition in most samples. Five of the MAX series replicates did have elevated internal control Cq values,

which may indicate PCR inhibitors present in those samples. Those samples did have a higher quantity of DNA (>6.0 ng/μL); however, all MAX series samples had quantities in that range but did not all have elevated Cq values. Estimated quantity of human DNA was compared to initial sample weights in **Fig. 26**, and similar to the analysis in **Chapter V.**, no relationship between initial weight and recovered DNA quantity was found. The Quantiplex HYres and Quantifiler Trio results were compared in **Fig. 28** and the DNA quantities were not significantly different, both kits revealing substantially more amplifiable DNA in the MAX series compared to both the MIN and MOD series samples.

Because none of the Cq values obtained from the Quantifiler® Trio IPC assay were indicative of inhibition, the DI interpretation suggests that it is primarily a consequence of DNA degradation [97]. The extracts for this experimental data set were not evaluated for the co-isolation of metals; based on prior studies (**Chapter III.**), some amount of inhibition is likely a factor and the effect of it on Quantifiler® Trio assay and the resulting DI is unknown. A DI < 1 indicates no degradation, this was only obtained for the positive control DNA sample. Most other samples had DI values between 1-10, indicating they were "slightly to moderately degraded" [97]. Only one MIN sample replicate had a mean DI >10, which is interpreted as a "significantly degraded" sample [97]. The highest DI values were obtained for the MIN series samples, which was unexpected as those samples were subjected to the least amount of mechanical force prior to DNA extraction. The lowest DI values were obtained for the MOD series samples, which did correspond to the highest quality STR profiles. This is the intended purpose of the DI value for a sample and it appears consistent with STR profile quality obtained for both the Identifiler® Plus and PowerPlex® Fusions System STR typing chemistries.

The improvement in the quality of the Identifiler® Plus and PowerPlex® Fusion System STR profiles obtained from the MOD series samples, compared to both the MIN and MAX series, suggests DNA recovery from bone samples is limited to the superficial DNA and that current methodologies do not recover DNA from deep within the skeletal connective tissue. Additional studies should be conducted using human skeletal remains to determine if this observed pattern is reproducible in bone samples exposed to natural burial environments.

SUMMARY AND DIRECTIONS FOR FUTURE RESEARCH

These studies have contributed data to advance efforts employing DNA testing of challenged samples, especially human identity testing of skeletal remains using STR markers. The specific aims of this study were to: 1. demonstrate comprehensive characterization of established PCR inhibitors, hematin and humic acid, and establish the inhibitory potential of metal ions; 2. elucidate the inhibitory effect of endogenous and environmentally-introduced metals, present in human skeletal remains, on PCR-based DNA testing; and, 3. evaluate the effect of pre-extraction processing on DNA extracted from bone samples.

The results from hematin and humic acid-treated control DNA samples, contextualized through comparison to available concentrations co-isolated with DNA during extraction, strongly suggest that hematin inhibition is not relevant to extracted and purified forensic samples. Humic acid appears to function as an inhibitor at concentrations expected to co-isolate with DNA. Because the predominant source of PCR inhibition data is generated during developmental validation stability studies, more value would be provided to the forensic DNA community if these studies employed inhibitory models that reflect materials that co-isolate with DNA at inhibitory levels and are examined at concentrations that are at or above those relevant to expected sample types. Comprehensive characterization should include the ability to detect the inhibitor using qPCR assays as well as concentrations that reduce the intensity of allele peak heights, in addition to the

number of callable alleles or percent of the expected profile. Currently, there are no contemporary studies that examine DNA extracts for the presence of potentially inhibitory substances. Changing the design of these studies would be aided by revising validation guidelines, such as those published by the Scientific Working Group on DNA Analysis Methods (SWGDAM) [98].

Metal ions were demonstrated to function as inhibitors of PCR-based quantification and STR typing assays, and often elude detection by internal control assays included in qPCR kits. Internal PCR controls in quantification systems are less susceptible to metal inhibition than sample template, making metal ion inhibitors challenging to detect; this is inherent to the purported mechanism of metal inhibition of PCR, primarily DNA sequestration. This creates the potential for obtaining false negative human DNA quantification results, and probative samples that may contain metal ions should be tested for STR profiles, regardless of the quantification results. Metal ions were also demonstrated to be present in human skeletal remains and co-isolate with DNA, following routine extraction and additional purification, at levels sufficient to inhibit both quantitative and STR PCR assays. Al and Ca models of inhibition are strongly encouraged for all PCR-based testing kits before use with DNA derived from human skeletal remains. Examples of STR profiles generated from metal-treated control DNA samples exhibit profile characteristics often associated with damaged or degraded DNA; these findings encouraged the evaluation of damage as a confounding factor.

The results of experiments designed to determine if pulverization was a significant factor contributing to the recovery of damaged DNA from skeletal remains were inconclusive, as both whole bone pieces and pulverized bone powder samples yielded a significant amount of damaged DNA template. These observations suggest that DNA recovery from bone samples is limited to superficial DNA only. While degradation assessment via qPCR was valuable for predicting the

quality of an STR profile from the same extract, the results are not exclusive to degradation and PCR inhibition should always be considered, especially if the DNA was obtained from skeletal remains. There is sufficient support to conduct future studies, on a larger scale with recovered skeletal remains, to determine if thin, transverse sectioning of bone provides better STR profiles than pulverization of the sample into a fine powder.

The forensic DNA community would also benefit from development of DNA extraction methods that are more effective at the removal of Al, Ca, and other metal ions, in advance of PCR-based testing. Ideally, this chemistry will be amenable to automated processing to reduce the labor investment and hazardous chemical exposure associated with current techniques. The DNA extracts produced by any novel methodology should be assessed for the presence of co-isolated materials, and if found, comprehensive characterization of the inhibitor is encouraged.

Future research should include examination of all variables used for extraction of DNA from human skeletal remains. These variables include pre-processing techniques, including chemicals used for cleaning and sampling techniques, and selection of reagents, such as enzymes used for chemical digestion of bone tissue and chelation chemicals used to remove metals from solution. Parameters, such as incubation times and temperatures, should be optimized and the efficiencies of purification techniques should be well-documented. As new DNA typing systems become validated for routine use, experiments should be included to assess the performance in the presence of comprehensively characterized inhibitors that are relevant to the targeted sample types.

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