DNA ISOLATION AND ANALYSIS FROM SKELETAL REMAINS: EVALUATING THE UTILITY OF SOIL DNA EXTRACTION KITS

By

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ABSTRACT

DNA ISOLATION AND ANALYSIS FROM SKELETAL REMAINS: EVALUATING THE UTILITY OF SOIL DNA EXTRACTION KITS

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DNA identification of human remains is often necessary in missing person cases or when decedents are unidentified owing to severe decomposition and skeletonization. Although current DNA extraction and analysis practices are often successful, instances of polymerase chain reaction (PCR) inhibition are frequently encountered, especially from buried skeletal remains. In the research presented, the utility of soil DNA isolation kits in skeletal DNA analysis was evaluated and compared to standard skeletal DNA extraction techniques. Mitochondrial (mtDNA) and nuclear DNA yields from buried bovine femora were compared among extraction methods and across lengths of burial. The ability of each technique to remove PCR inhibitors associated with buried skeletal remains (i.e., calcium chloride, collagen, humic acid) was also evaluated. Finally, the extraction methods were tested on ancient and modern human skeletal remains, and mtDNA haplogroup markers and a portion of the control region were sequenced. Soil DNA isolation kits were successfully used to extract skeletal DNA at quantities similar to standard extraction methods, and calcium chloride and humic acid did not result in PCR inhibition when using the soil DNA isolation kits, whereas collagen sometimes did. Concordant control region sequences were obtained from modern skeletal remains among soil kits and standard extraction methods, although extracts of ancient skeletal remains did not consistently produce concordant haplotypes. Based on the above comparisons, soil DNA isolation kits were determined to be a viable extraction technique for skeletal remains that resulted in positive identification of a decedent while quickening the extraction process.

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INTRODUCTION

Identification of the deceased is a major goal for law enforcement, medical examiners, and others in the field of forensic science. In the United States, medical examiner and coroner offices document approximately 4,400 unidentified decedents annually, 1,000 of which remain unidentified after one year (Hickman et al. 2007). From the view of law enforcement, timely identification is important because knowing the identity of the decedent helps to ascertain if a crime occurred and to create a case against a suspect. Furthermore, it is necessary for the decedent's family to be notified as soon as possible so that they can begin the grieving process and make postmortem plans.

Various techniques are used to identify human remains. Conventional methods include visual identification by relatives of the decedent or fingerprint comparison. However, if the fingerprints of the decedent are not on file with law enforcement or if the individual is unrecognizable, such as when the postmortem interval is long or when the remains are burned, another manner of identification is needed. Developing a biological profile of a decedent anthropologically is one identification method used when remains are skeletonized. Biological profiles, generated by forensic anthropologists, include estimates of age, sex, ancestry, and stature (SWGANTH 2010). These characteristics can be compared with missing person reports from the surrounding area to aid in a tentative identification. If an identity is presumed, positive identification can be made by comparing postmortem skeletal or dental radiographs to antemortem records (Mann and Fatteh 1968, Pretty and Sweet 2001). Similarly, evidence of trauma or orthopedic implants can assist identification by comparing the forensic anthropologist's analysis to antemortem medical records (Simpson et al. 2007). The above

identification methods, while useful, are not always successful, especially when dental and medical records are unavailable.

DNA Identification

DNA analysis is often performed if other identification methods render indeterminate or unsuccessful results. Cells contain two types of DNA: nuclear and mitochondrial (mtDNA). Each type has a different cellular location, copy number, and physical structure. The nucleus contains two copies of linear DNA, one received from each parent. Because nuclear DNA consists of a unique nucleotide sequence for each individual, excluding identical twins, identifications can be made with confidence. Mitochondria contain circular DNA that is inherited maternally (Giles et al. 1980); therefore, the same mtDNA sequence is shared among all maternal relatives of an individual (e.g. siblings, mother, grandmother). Human cells can have hundreds of mitochondria (Robin and Wong 1988), each containing an average of 4 – 5 mtDNA copies (Satoh and Kuroiwa 1991), resulting in a greater mtDNA copy number per cell than the single copy regions of nuclear DNA that are analyzed for forensic identification. Despite the difference in copy number, both types of DNA can be used to make positive identifications.

The regions of nuclear DNA examined today in forensic identifications are termed short tandem repeats (STRs). Identifications by STR analysis can be made by comparing a reference DNA sample from the person of interest (e.g. cheek swab or blood) to the STR profile obtained from the evidentiary item. When making an identification of a decedent, a DNA sample from a parent or child is most useful as a reference sample because they share half of their genetic material with the decedent. Although mtDNA is not unique to an individual, it is useful for

identification if nuclear DNA analysis is not possible. DNA degrades after death, which is accelerated when human remains are exposed to harsh conditions, such as damp environments (Graw et al. 2000) or the heat of a fire (Cattaneo et al. 1999). Nuclear DNA is particularly susceptible to degradation, which is a reason why its analysis from skeletal remains often fails; however, mtDNA analysis is often more successful for several reasons. First, as detailed above, mtDNA has a higher copy number per cell, which increases the amount of target material present. Second, mtDNA may be less susceptible to degradation by exonucleases—enzymes that cleave DNA at the end of the strand—due to its circular structure. Finally, the cellular location of mtDNA inside the mitochondrion seems to protect it from degradation (Foran 2006).

Likewise, mtDNA analysis can be useful when it is not possible to obtain a DNA reference sample of the decedent or a close relative, a situation that makes positive identification using nuclear DNA difficult or impossible. When mtDNA analysis is performed, a sample from any member of the decedent's maternal lineage will support or refute a presumed identification. Figure 1 illustrates a map of the human mtDNA genome, including the control region, which has high sequence variability among individuals (Aquadro and Greenberg 1983). Areas with the greatest variability within the control region are termed hypervariable regions and are analyzed to make an identification. The nucleotide sequences (i.e. order of A, C, G, and Ts) of the hypervariable regions are determined for an evidentiary DNA sample and are compared to the revised Cambridge reference sequence (rCRS): a revised version of the first mtDNA genome sequenced, derived from a European individual (Andrews et al. 1999). Polymorphisms are noted when a nucleotide from evidentiary DNA differs from the rCRS, as shown in Figure 2. The combination of polymorphisms from a DNA sample defines an individual's haplotype, which is

the same for maternal relatives. Therefore, haplotypes from a decedent and a potential maternal relative can be compared to support a presumed identification.

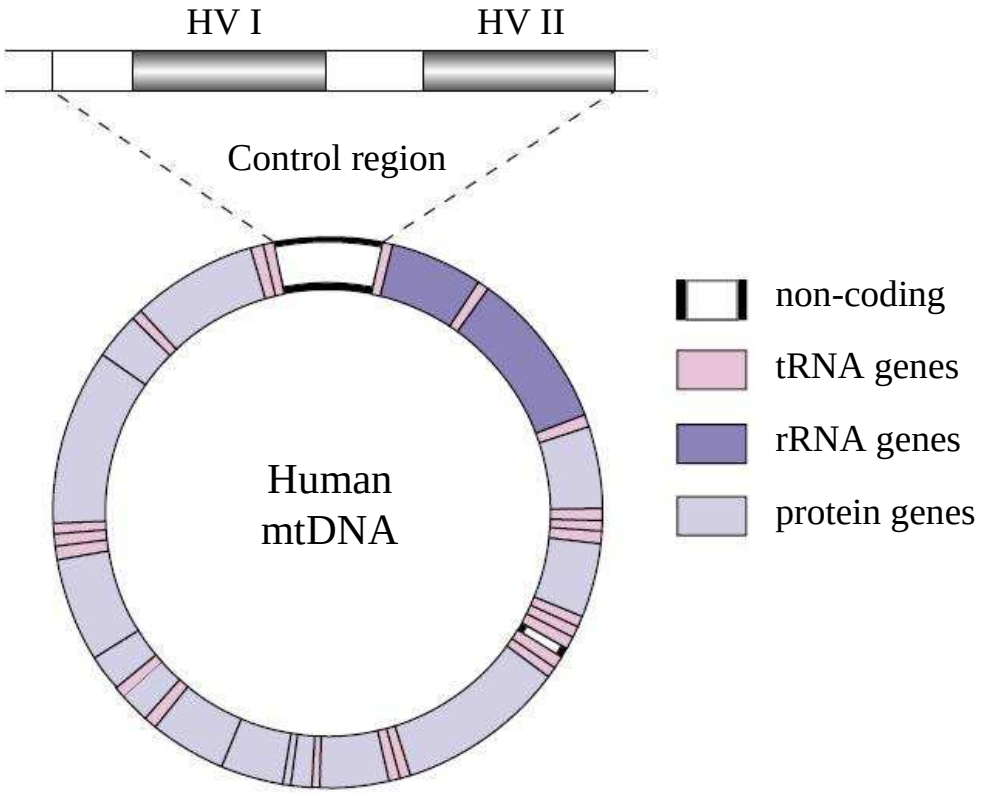


Figure 1. Map of the Human mtDNA Genome
Adapted from Hagelberg (2003). The white color indicates non-coding DNA, while DNA coding for tRNA, rRNA, and proteins is shown in color. Within the control region, hypervariable regions I and II (HV I and HV II) are illustrated in grey. DNA sequences of HV I and HV II are obtained and compared to a reference DNA sample in order to make a forensic identification of a decedent. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

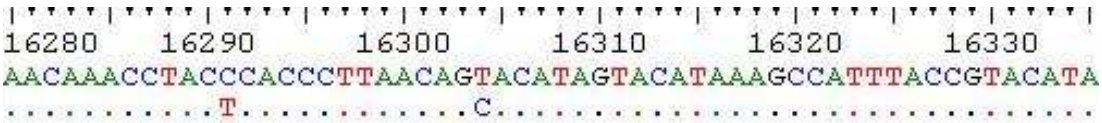


Figure 2. Polymorphisms in the mtDNA Control Region
The DNA sequence shown is a portion of HV I (16280 – 16335 bp). The top sequence is the rCRS, while the bottom is an evidentiary DNA sample. Nucleotides that are the same as the rCRS are indicated by a ".", while any polymorphisms are denoted by the nucleotide listed. The haplotype of this evidentiary DNA sample is 16291 T, 16304 C.

mtDNA Haplogroups

mtDNA haplotypes that are derived from a common mtDNA ancestor, indicated by sharing a characteristic polymorphism, are termed haplogroups (Torroni et al. 2006). Originally, haplogroups were identified by variation in mtDNA restriction enzyme digestion patterns. Single nucleotide polymorphisms (SNPs) have also been documented for haplogroups (i.e. single base mutations specific to each haplogroup). Certain haplogroups correlate with ancestral roots in various geographical regions: Africa (Chen et al. 1995), Asia (Ballinger et al. 1992), and Europe (Torroni et al. 1996). Haplogroups have also been used to document ancient human migration patterns (Torroni et al. 1994a, Bonatto and Salzano 1997, Perego et al. 2009); a map of human migrations is shown in Figure 3. Haplogroups A, B, C, and D are present in Native Americans (Wallace et al. 1985) and Asians (Ballinger et al. 1992), but have not been documented in Africans or Caucasians, supporting the theory that Native Americans were migrants of Asian descent.

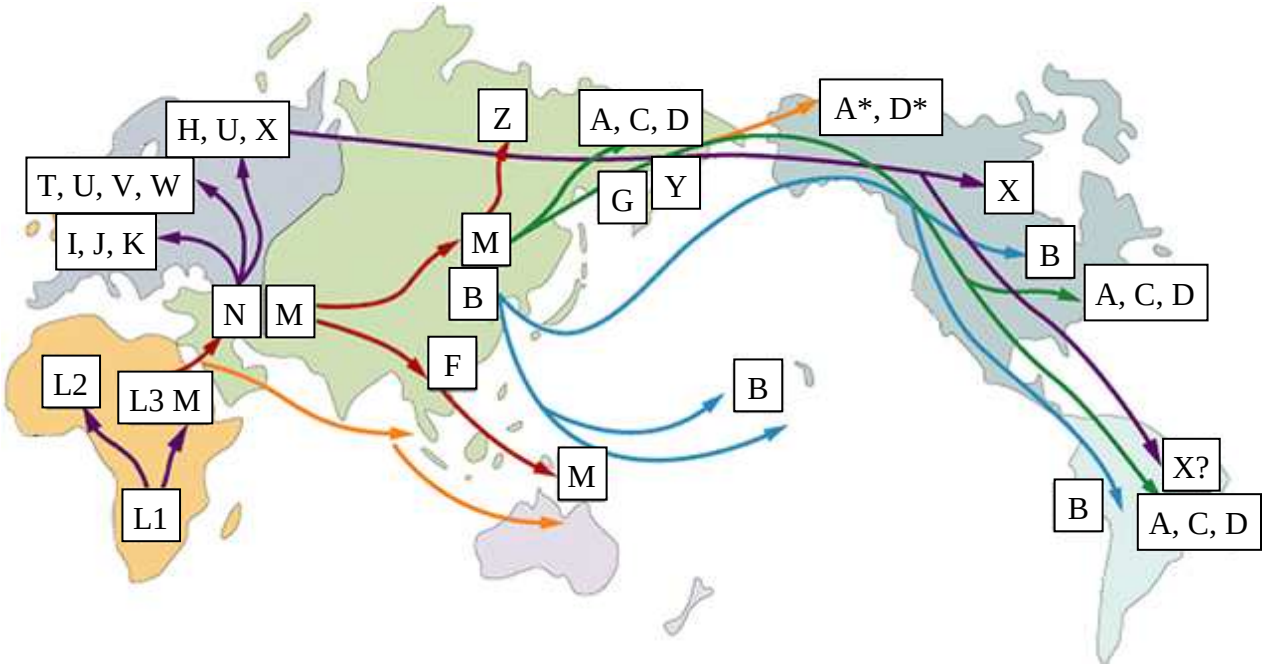


Figure 3. Haplogroup Map of Human Migration
Adapted from Genealogy by Genetics, Ltd., Family Tree DNA (2004). Available at: http://www.worldfamilies.net/reference_mtDNA. Arrows indicate direction of human migration. Note that Native American haplogroups A – D originated from individuals that migrated from Asia, while haplogroup X is present in both Europe and North America.

Torroni et al. (1994b) reported four Caucasian haplogroups (H, I, J, and K) from individuals in the United States and Canada; additional Caucasian haplogroups discovered in Sweden, Finland, and Italy include T, U, V, W, and X (Torroni et al. 1996). However, haplogroup X, characterized by a T at position 6371, is not specific to Caucasians, since it has also been documented in northern Native American populations (e.g. 25% of the Ojibwa, 15% of the Sioux, and 7% of the Navajo; Brown et al. 1998). More recently, SNPs have been identified that can divide haplogroups into subhaplogroups (Pereira et al. 2004, Brandstätter et al. 2006). For instance, the analysis of mtDNA SNPs provided further discrimination of haplogroup X, since the SNPs 16213 A and 200 G were only present in haplogroup X individuals of Native American, not European, descent (Brown et al. 1998). Table 1 lists the restriction enzyme site

polymorphisms and SNPs associated with the Native American/Asian haplogroups studied in this research.

Table 1. Diagnostic Native American Haplogroup Polymorphisms
Adapted from www.mitomap.org. Available at: http://www.mitomap.org/bin/view.pl/MITOMAP/HaplogroupMarkers. Nucleotide positions for restriction enzyme sites represent the first base of the recognition sequence in the top strand, not the actual cut site. "+" indicates a restriction enzyme site gain, while "-" indicates a loss. Nucleotides to the left of the arrows are from the rCRS, while the polymorphic nucleotide is to the right of the arrow. HV I motifs are all transition mutations, unless otherwise indicated. Nucleotides enclosed in brackets, following "del", are part of a deletion.

Haplogroup	Diagnostic Restriction Enzyme Site	Diagnostic SNP	HV I Motif
A	+663 *Hae*III	663 A→G	16223,16290, 16319, 16362
B	8281-8289, 9 bp del	8280:8290 =A[delCCCCCTCTA]G	16183C, 16189, 16217
C	+13262 *Alu*I -13259 *Hinc*II	13263 A→G	16223, 16327, 16298
D	-5176 *Alu*I	5178 C→A	16223, 16362
X	none	6371 C→T	16183C, 16189, 16223, 16278

mtDNA Casework

In forensics, mtDNA haplogroup analysis is useful in determining the maternal ancestry of an individual. For instance, when skeletal remains are encountered by chance, such as when a contractor digs on a plot of land, ancestral information is needed, since investigators must determine if criminal activity may have occurred. A convenient way of doing this is by identifying the remains as Native American—suggesting that the individual was buried long ago—or non-Native American, indicating that it is a modern skeleton and a crime may have been committed. Further, ancestral determination is necessary so that remains are repatriated to the

corresponding tribe if they are of Native American origin. Shunn (2005) used mtDNA haplogroup analysis to classify individuals buried at Fort Michilimackinac (1743 – 1781 AD) as Native American or non-Native American. Although that study was not forensic in nature, it illustrates successful mtDNA haplogroup analysis of skeletal remains.

DNA identifications of victims of war and mass disaster are likely the most challenging forensic cases, because the number of remains that must be efficiently and correctly documented, analyzed, and identified can reach thousands (Leclair et al. 2004, Deng et al. 2005, Edson 2007). Other identification methods are not feasible when remains are commingled or when complete skeletons are not recovered. However, DNA analysis is viable in these cases and has been used to make positive identifications. For example, the Unknown Soldier of the Vietnam War could not be identified by anthropological examination or by "blood typing analysis" of hair from the inside of a flight suit found with the remains (U.S. Department of Defense 1998), but when mtDNA from the remains was compared to reference samples from seven potential families of the decedent, the Unknown Soldier was identified as First Lt. Michael J. Blassie (Holland and Parsons 1999).

The ongoing effort to identify the victims of the World Trade Center terrorist attack on September 11, 2001 provides another example in which DNA analysis has been essential for identification. About 3,000 victims and 20,000 pieces of commingled remains needed identification (Biesecker et al. 2005). Such fragmentation of remains necessitates DNA analysis, since it is the only method that can potentially associate each fragment of bone to an individual and provide families of the victims with the correct remains. Nuclear and mtDNA analyses, or a combination of DNA and other identification methods, were used for the majority of 9/11 victims who have been identified: 879 and 571, respectively (Shaler and Bode 2011). As of

June 2010, 59% of those reported missing—1,626 victims—have been identified (Shaler and Bode 2011); however, Biesecker et al. noted in 2005 that numerous individuals remain unidentified due to DNA degradation or lack of reference samples.

Polymerase Chain Reaction Inhibition

The polymerase chain reaction (PCR), a technique developed by Mullis et al. (1986), is used to amplify specific regions of DNA, resulting in billions of copies of the region of interest. In forensics, PCR is important because it allows DNA profiles to be obtained from minimal starting material, including epithelial cells from briefly handled objects (van Oorschot and Jones 1997) and DNA in ancient bone (Hagelberg et al. 1989). However, DNA amplification may be unsuccessful due to the presence of substances that interfere with PCR, termed PCR inhibitors. Biological fluids and other forensic evidence are sources of PCR inhibitors, which may co-extract during DNA isolation. Some documented PCR inhibitors include hematin (Akane et al. 1994), indigo dye (Larkin and Harbison 1999), urea (Khan et al. 1991), and melanin (Yoshii et al. 1994).

The organic and inorganic portions of bone are also sources of PCR inhibitors. The major components of bone are hydroxyapatite and collagen. Hydroxyapatite, an inorganic complex of calcium phosphate, constitutes 62 – 66% of bone (Bigi et al. 1997), 37% of which is calcium (Field et al. 1974). Calcium ions inhibit DNA amplification by a mechanism that is hypothesized to be competitive inhibition, wherein calcium competes with magnesium, a cofactor necessary for Taq DNA polymerase's enzymatic activity (Bickley et al. 1996, Opel et al. 2010). Furthermore, hydroxyapatite is known to bind DNA (Martinson 1973), which could potentially inhibit PCR. Collagen, another PCR inhibitor, constitutes 90 – 96% of the organic

matrix of bone (Rogers et al. 1952). Figure 4 depicts collagen's highly organized protein structure. Three amino acid chains come together to form a triple helix; the individual helices assemble into microfibrils, which are grouped into fibrils and fibers (Rho et al. 1998). Collagen binds DNA and presumably inhibits PCR by affecting the processivity of Taq DNA polymerase (Scholz et al. 1998, Opel et al. 2010).

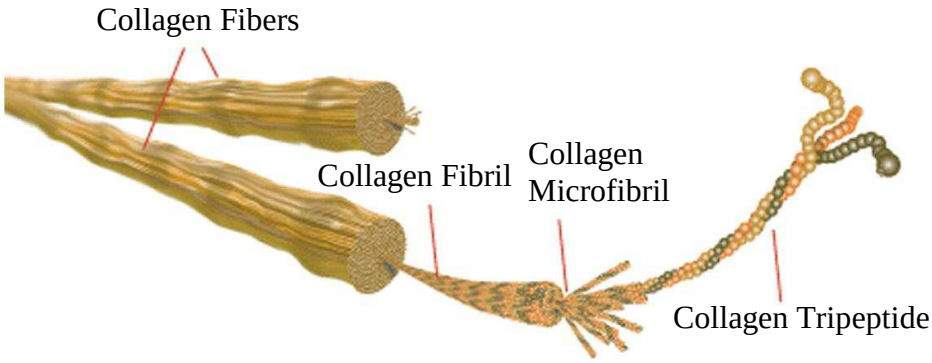


Figure 4. Structural Organization of Collagen
Taken from Sigma-Aldrich Co., LLC. Available at: http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/structural-proteins/collagen.html. Collagen has a highly organized protein structure, consisting of three peptide chains that form a helix, with numerous helices forming microfibrils, which are organized into fibrils and fibers.

In addition to components of bone, skeletal remains are exposed to PCR inhibitors when they come in contact with soil and plant material, particularly when remains are buried. Humic substances—humic acid, fulvic acid, and humin—are complex organic compounds present in soil that originate from the decomposition of plant and animal matter (MacCarthy 2001). Classification of humic substances is based on their pH solubility in aqueous solution. Fulvic acids are soluble at any pH, whereas humic acids are only soluble when the pH is greater than 2, and humin is insoluble in water at any pH (Allard 2006). Since humic and fulvic acids are

soluble in aqueous solutions and have been shown to inhibit PCR (Tebbe and Vahjen 1993, Tuross 1994), they are a concern when extracting DNA from buried skeletal material. The mechanisms of humic and fulvic acid inhibition are unclear. Kreader (1996) suggested that fulvic acid inhibits PCR by binding Taq DNA polymerase. It has been proposed that humic acid may bind DNA and limit its availability for the PCR process (Opel et al. 2010), or bind Taq DNA polymerase's active site (Sutlovic et al. 2008). Humic acid is of concern when analyzing buried skeletal remains because it seeps into collagen (van Klinken and Hedges 1995) and is present in soil that contaminates the powder obtained from drilling or grinding bone. An additional PCR inhibitor associated with soil is tannic acid (Kontanis and Reed 2006), which is prevalent in plant material. Tannic acid has electronegative oxygen atoms that may inhibit Taq DNA polymerase by chelating magnesium ions (Opel et al. 2010).

Assessing PCR Inhibition

If PCR amplification fails, there are two potential causes. First, DNA is degraded or not present in the extract. Alternatively, PCR inhibitors are preventing DNA amplification. An easy way to differentiate the two is by 'spiking' a PCR reaction with high quality DNA that should amplify. If the DNA extract contains PCR inhibitors, the high quality DNA will have little or no amplification, whereas if the spiked DNA successfully amplifies then the initial negative result is due to a lack of DNA. Although useful, spiking PCR reactions is a step in DNA analysis that requires additional time and costs; therefore, it is not an ideal way to assess PCR inhibition in a forensic laboratory.

Methods have been developed to simultaneously assess PCR inhibition while quantifying DNA. Quantitative PCR (qPCR), first described by Higuchi et al. (1993), is a technique used to

quantify DNA and is routine in forensic DNA analysis. Amplification of target DNA is measured after each PCR cycle via fluorescently-labeled DNA probes that anneal to target DNA and fluoresce at a specific wavelength. The qPCR computer software sets a fluorescence threshold, indicated by a horizontal line on the amplification chart (Figure 5), and determines when each sample reached this amount of fluorescence. The number of PCR cycles required for a sample to cross the threshold is known as the cycle threshold (C_t) value (Figure 5). C_t values are proportional to the amount of starting DNA in a reaction. As initial DNA concentration increases, fewer cycles are necessary to reach the fluorescence threshold.

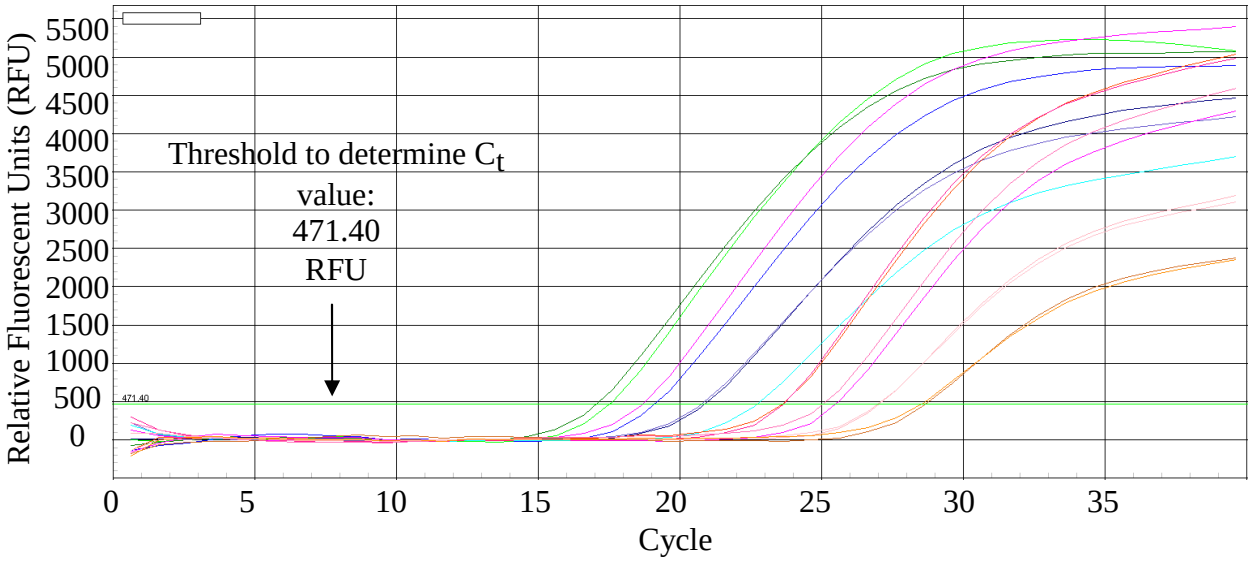


Figure 5. C_t Value Determination
The x-axis is PCR cycle number, and the y-axis is relative fluorescent units (RFU). The fluorescence threshold, determined by the software, is the horizontal green line indicated by the arrow. The C_t values for each of the curves are determined from the cycle at which each crosses the threshold. C_t values of the curves depicted range from approximately 17 to 29.

DNA samples of known concentrations are included in a qPCR run in order to create a standard curve of DNA quantity vs. C_t value, as demonstrated in Figure 6. The C_t values of evidentiary DNA samples are plotted on the standard curve, and DNA concentrations are calculated by reading the corresponding value on the x-axis. A synthetic oligonucleotide termed an internal PCR control (IPC) may also be included in qPCR in order to detect PCR inhibition. The IPC is at the same concentration in every reaction, so C_t values are the same for all reactions (Figure 7). If there is no amplification of target DNA, successful amplification of the IPC indicates that no target DNA exists, however if the IPC does not amplify or amplifies poorly, then PCR is inhibited (Figure 8).

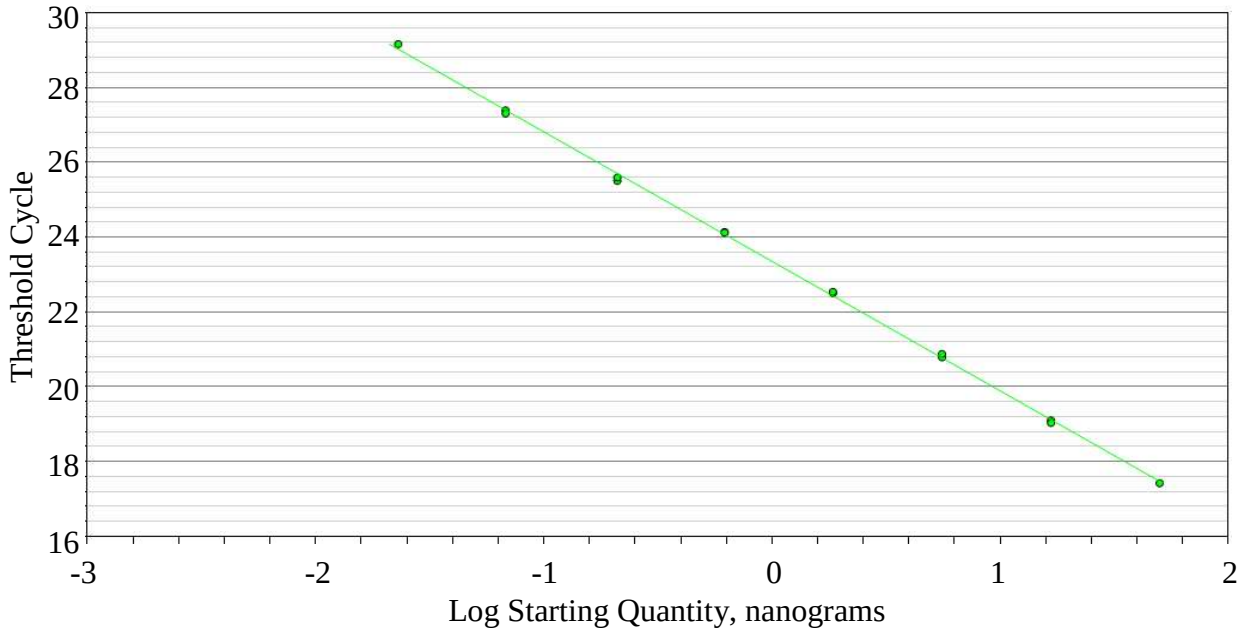


Figure 6. qPCR Standard Curve

The x-axis is a logarithmic scale of DNA quantity in nanograms (ng). The y-axis is the C_t value. A serial dilution of stock DNA of known concentration is made, and duplicates of each dilution are typically included for any qPCR run. As DNA concentration increases, it takes fewer cycles to reach the fluorescence threshold, hence the decrease in C_t value.

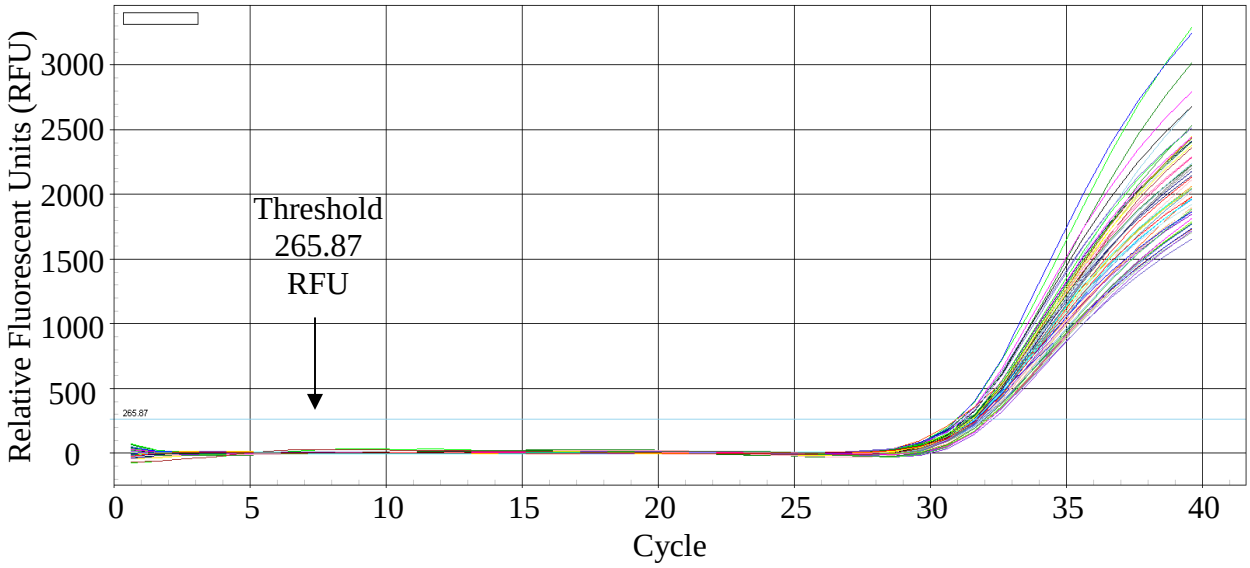


Figure 7. qPCR IPC Curves

The x-axis is cycle number, and the y-axis is RFU. The threshold to determine C_t values is indicated by the arrow. Curves depicted indicate amplification of the IPC in each reaction, and if no inhibition exists, the C_t values are the same for every reaction. In this example, each IPC C_t value falls between approximately cycles 31 and 32, indicating that PCR amplification is occurring with no indication of inhibition.

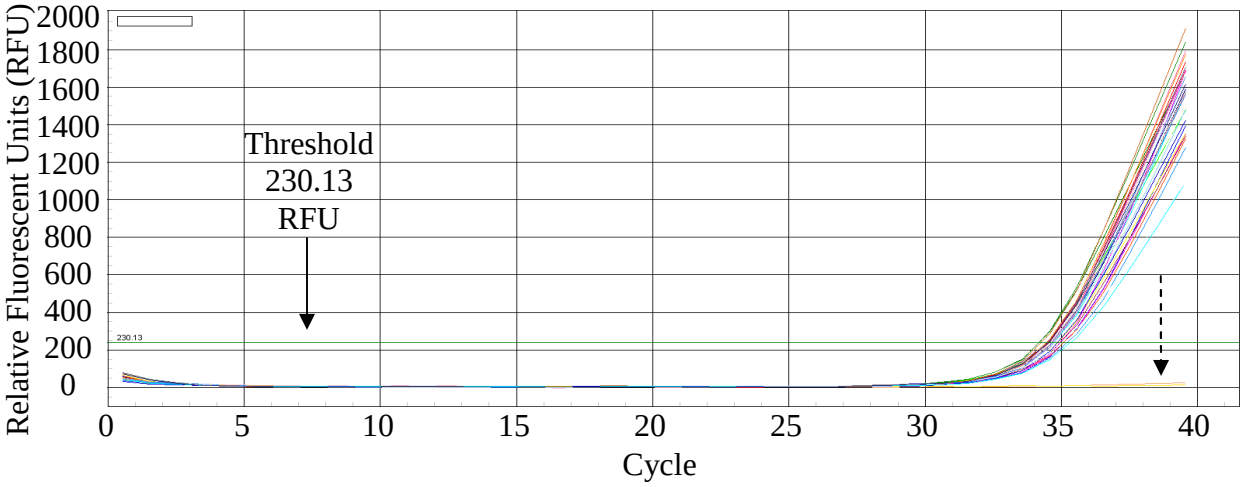


Figure 8. IPC Curve Indicating PCR Inhibition

The x-axis is cycle number, and the y-axis is RFU. The threshold to determine C_t values is indicated by the solid arrow. The IPC curves designated by the dashed arrow demonstrate no amplification because they do not pass the fluorescence threshold. These PCR reactions are inhibited, suggesting that PCR inhibitors are present in the DNA extracts.

DNA Isolation and Purification

DNA extraction protocols from skeletal remains vary widely, but three commonly-used approaches include organic (Kurosaki et al. 1993, Loreille et al. 2007), silica-based (Nelson and Melton 2007, Kim et al. 2008, Lee et al. 2010, Amory et al. 2012), and salting-out (Cattaneo et al. 1995, Coticone et al. 2010) methods. During an organic extraction, a tissue digestion buffer containing a detergent, a buffering agent, and a chelating agent, and proteinase K are added to the sample and incubated at 56˚C to lyse cells (Butler 2012). Next, phenol is added, mixed, and the organic and aqueous phases are separated by centrifugation. Lipids, proteins, and similar cellular components move to the organic phase, while DNA remains in the aqueous phase. The aqueous layer is isolated and combined with chloroform, and the phases are separated again. The aqueous layer may contain water-soluble PCR inhibitors such as humic acid and calcium, which is a limitation of this method.

However, organic extraction is often coupled with further purification techniques, including DNA precipitation (Kurosaki et al. 1993, Kalmár et al. 2000) or the use of centrifugal filter columns (Yang et al. 1998, Loreille et al. 2007, Rohland and Hofreiter 2007, Rucinski et al. 2012). Centrifugal filters purify DNA by retaining it in the filter, while small contaminants pass through. Depending on the molecular weight limit of the filter, PCR inhibitors can also remain in the retentate. For instance, collagen obtained from bone was retained using a 30 kilodalton (kDa) filter (Jørkov et al. 2007), although this may not be of concern since collagen is theoretically removed during organic extraction due to its solubility in the organic phase. On the other hand, humic acid is soluble in the aqueous layer and has been retained using filters with molecular weight limits up to 100 kDa (Lobartini et al. 1997), suggesting that organic extraction may be ineffective at removing humic substances.

An extraction method that removes PCR inhibitors and isolates DNA without using hazardous organic solvents was developed by Boom et al. (1990). This method used a salt solution and silica particles to extract DNA from human serum and urine. Silica binds DNA under high salt conditions, via the mechanism depicted in Figure 9. Positively-charged ions from a chaotropic salt, such as sodium, bind to negatively-charged oxygen atoms of silica, forming an "ionic bridge" that allows negatively-charged DNA to bind to the silica. The silica is washed several times to remove proteins and other components, including PCR inhibitors. Addition of a low salt solution causes water molecules to associate with silica and break the ionic bridge, eluting DNA from the silica (Melzak et al. 1996).

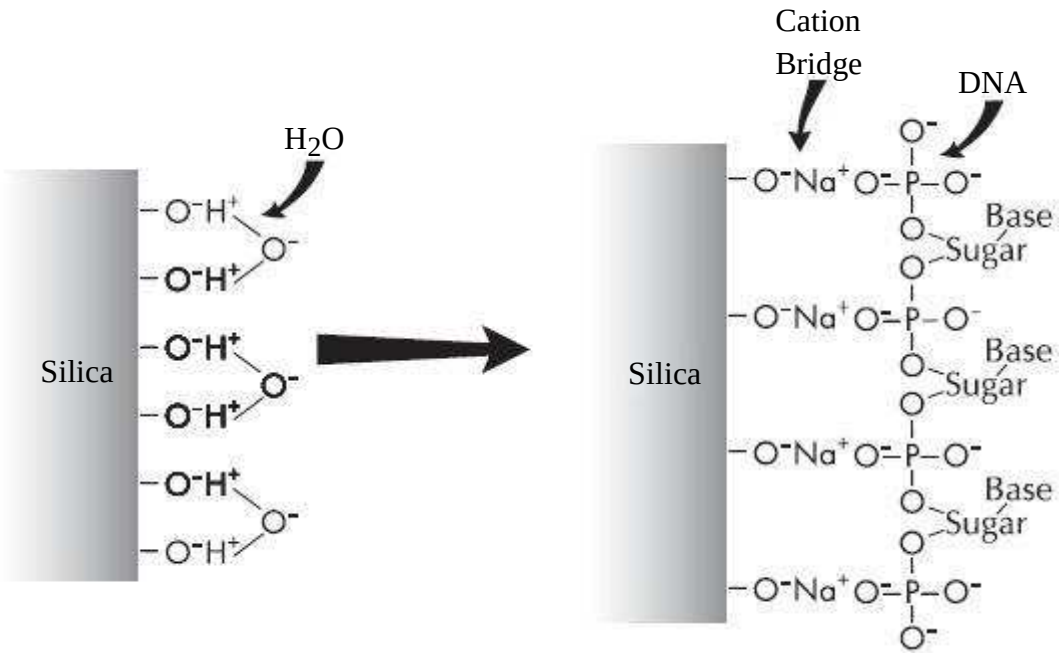


Figure 9. A Proposed Mechanism of Silica Binding DNA
Taken from Qbiogene, Inc. Available at: http://www.qbiogene.com/products/geneclean/geneclean_overview.shtml. Under high salt conditions, cations bind to silica, forming a bridge that allows negatively-charged DNA to bind to negatively-charged silica. After addition of a low salt solution, water molecules reassociate with silica, eluting DNA.

Miller et al. (1988) described an extraction method termed salting-out, which was used to remove proteins and isolate DNA from blood. Salting-out extractions consist of incubating a sample in a tissue digestion buffer, followed by precipitation of proteins via addition of a saturated salt solution. Centrifugation is used to separate the supernatant containing DNA from the protein pellet. Precipitation by salting-out is dependent on the hydrophobicity of proteins (Scopes 1994). In an aqueous solution, proteins are usually folded in a manner that minimizes the exposure of hydrophobic amino acids. Under high salt conditions, the hydrophobic regions become exposed and proteins aggregate, resulting in precipitation (Shih et al. 1992, Scopes 1994).

Numerous variations and modifications of the above extraction techniques have been reported in both the forensic science literature (Cattaneo et al. 1995, Loreille et al. 2007, Nelson and Melton 2007, Coticone et al. 2010, Lee et al. 2010, Amory et al. 2012, Dukes et al. 2012, Rucinski et al. 2012) as well as in the literature on ancient skeletal DNA (Kurosaki et al. 1993, Yang et al. 1998, Kalmár et al. 2000, Rohland and Hofreiter 2007, Kim et al. 2008). The main results of these studies are summarized in Table 2. Note that this is not a comprehensive list of all skeletal DNA extraction procedures; rather, it is meant to demonstrate some of the variation that exists.

Table 2. Summary of Published Skeletal DNA Extraction Methods

Reference	Skeletal Samples	DNA Extraction Protocol(s)	Measures	Results
Kurosaki et al. (1993)	• Human remains from Japan, $1^{st} - 5^{th}$ century	• Demineralization of bone using EDTA, phenol/chloroform/isoamyl alcohol extraction, ethanol precipitation of DNA, glass powder purification	• Short-VNTR amplification	• Short-VNTR genotypes obtained for every individual • When DNA amplification from bone failed, genotypes were obtained from a tooth or additional bone
Cattaneo et al. (1995)	• Fresh human bone • Human remains, 3 and 9 months postmortem	• Sodium acetate precipitation of proteins, isopropanol precipitation of DNA • Phenol/chloroform extraction, ethanol precipitation of DNA	• Nuclear DNA amplification	• No amplification from several bones using phenol/chloroform extraction and ethanol precipitation of DNA • Successful amplification from sodium acetate precipitation of proteins and isopropanol precipitation of DNA
Yang et al. (1998)	• Human remains, 15 – 5,000 years postmortem	• Demineralization of bone using EDTA, phenol/chloroform extraction, column concentration • Demineralization of bone using EDTA, phenol/chloroform extraction, column concentration, silica column purification • Demineralization of bone using EDTA, proteinase K digest applied directly to concentration column and/or silica column	• Nuclear DNA amplification	• Amplification using phenol/chloroform extraction, column concentration, and silica column purification • PCR inhibition from phenol/chloroform extracts when silica column was not used • Amplification when proteinase K digests were added to concentration column and/or silica column

Table 2 (cont'd)

Kalmár et al. (2000)	• Human remains from Hungarian cemetery, 7th – 15th century	• DNA precipitated from supernatant of bone powder digest using sodium acetate and ethanol • Demineralization of bone using EDTA, phenol/chloroform extraction, isopropanol precipitation of DNA	• mtDNA sequencing	• Complete mtDNA sequences obtained using sodium acetate/ethanol precipitation of DNA • No amplification using phenol/chloroform extraction and isopropanol precipitation of DNA
Loreille et al. (2007)	• Human remains, 14 – 100 years postmortem	• Phenol/chloroform extraction, column concentration • Bone powder completely dissolved using EDTA, phenol/chloroform/isoamyl alcohol extraction, column concentration	• qPCR of mtDNA • STR amplification	• Completely dissolving bone powder significantly increased DNA recovery from degraded remains and increased number of STR alleles obtained
Nelson and Melton (2007)	• Human remains (law enforcement identification and historical cases)	• Silica extraction	• mtDNA sequencing	• At least partial mtDNA sequences for ~83% of cases • Multiple attempts to extract DNA from burned bone failed • Historical cases less likely to yield full mtDNA sequences

Table 2 (cont'd)

Rohland and Hofreiter (2007)	• Cave bear remains, Pleistocene age (over 20,000 years old)	• Demineralization of bone using EDTA, phenol/chloroform extraction, column concentration • Silica-based kit extractions • Paramagnetic silica-based kit extraction • Demineralization of bone using EDTA, silica extraction; further optimization of this method	• qPCR of mtDNA	• No significant difference between DNA yields of the method with the highest DNA recovery (paramagnetic silica-based kit) and any other method • Optimization of the demineralization/silica extraction increased DNA yields 2-fold • Addition of detergents did not improve DNA yields • Addition of EDTA and proteinase K increased DNA yields
Kim et al. (2008)	• Human remains that previously failed to yield amplifiable DNA, 500 – 3,300 years postmortem	• Demineralization of bone using EDTA, silica extraction • Demineralization of bone using EDTA, silica extraction, ion exchange columns, column concentration	• mtDNA amplification • Nuclear DNA amplification (Amelogenin)	• The majority of bones yielded amplifiable mtDNA using silica extraction, and 4 of 9 bones had Amelogenin amplification • All bones had mtDNA and Amelogenin amplification using silica extraction and ion exchange columns
Coticone et al. (2010)	• Pig bones • Human bones	• Ultrasonic treatment, sodium chloride precipitation of proteins, ethanol precipitation of DNA, silica bead purification	• STR amplification (human) • Amplification of one STR locus (pig)	• Successful extraction of DNA using acoustic energy • Full STR profiles from human bone

Table 2 (cont'd)

Lee et al. (2010)	• Human remains from the Korean War	• Silica-based kits • Bone powder completely dissolved using EDTA, silica-based kits	• Genomic DNA recovery in presence and absence of PCR inhibitors • STR amplification	• Low genomic DNA recovery (< 51%) • High concentration of humic acid hindered DNA quantification • Completely dissolving bone powder increased number of STR alleles
Amory et al. (2012)	• Excavated human remains of individuals deceased between 1992 and 1999	• Bone powder completely dissolved using EDTA, column concentration, silica column purification • Silica-based kit extraction with some modifications, column concentration	• qPCR of nuclear DNA • STR amplification	• Completely dissolving bone powder improved quality of STR profiles and quantity of DNA recovered • Protocol including dissolving was successfully automated on the QIAcube platform
Dukes et al. (2012)	• Unidentified human remains recovered in 2000, 2006, and unknown dates	• Optimization of reaction volume, digest solutions, and incubation time for an automatable silica-coated paramagnetic bead extraction	• Nuclear DNA quantification • STR amplification	• Complete STR profile recovered from bone extracted with optimized method; partial profile when using a protocol previously published by the authors
Rucinski et al. (2012)	• Exhumed human remains buried 6 – 118 months	• Silica-based kit extraction, column concentration • Demineralization of bone using EDTA, phenol/chloroform/isoamyl alcohol extraction, column concentration	• Nuclear DNA quantification • STR amplification	• Greater DNA recovery with phenol/chloroform extraction • Complete STR profiles using phenol/chloroform extraction; fewer alleles using silica-based kit extraction

As demonstrated by the variations in Table 2, there is no consensus on an optimal DNA isolation method for skeletal remains. Rucinski et al. (2012) reported that a silica-based kit recovered approximately six-fold less DNA from skeletal remains than organic extraction. Silica kits also resulted in low genomic DNA recovery: 20 – 51% of DNA input (Lee et al. 2010). However, other authors have demonstrated that nuclear DNA or STR profiles can be obtained from skeletal DNA extracted using silica-based procedures (Yang et al. 1998, Kim et al. 2008, Lee et al. 2010, Amory et al. 2012, Dukes et al. 2012). Conflicting results were also reported from organic extractions of skeletal remains. Some authors successfully typed DNA from skeletal remains using organic extraction (Loreille et al. 2007, Rucinski et al. 2012), while others demonstrated that organic extraction of bone did not result in nuclear (Cattaneo et al. 1995) or mitochondrial DNA (Kalmár et al. 2000) amplification. PCR inhibition was also found from organic extractions of skeletal remains (Yang et al. 1998, Rucinski et al. 2012). Salting-out has been shown to recover amplifiable nuclear DNA from skeletal remains (Coticone et al. 2010) and to result in greater mtDNA sequencing success than organic extraction (Kalmár et al. 2000). Due to the disagreement on an optimal skeletal DNA extraction technique, there is a need to develop and/or optimize a method that maximizes both DNA recovery and PCR inhibitor removal.

Soil DNA Isolation Kits

Buried skeletal remains come into contact with soil, exposing them to humic substances that inhibit PCR. Numerous manufacturers produce kits designed to remove humic substances and to isolate DNA from soil, since it is performed for various research purposes: measuring microbial biodiversity (Fierer and Jackson 2006), assessing microbial community changes in

response to soil management practices (Crecchio et al. 2004), and discrimination among soil types for forensic purposes (Heath and Saunders 2006, Lenz and Foran 2010). However, published research using soil DNA extraction kits to isolate DNA from skeletal remains does not exist. Given that soil DNA extraction kit protocols contain steps similar to those used in skeletal DNA extraction (e.g. the use of a silica column or protein precipitation followed by DNA precipitation), it seems that soil kits could be adapted to extract skeletal DNA.

Similar to the skeletal DNA extraction methods detailed above, the ability of each soil DNA isolation kit to recover DNA and remove PCR inhibitors varies. Dineen et al. (2010) compared six soil DNA purification kits by spiking three soil types (sand, sandy clay, and sandy loam) with various amounts of *Bacillus cereus* T-strain spores, extracting DNA, and quantifying nuclear DNA via qPCR. A FastDNA® SPIN kit had the highest yield from all soil types; however, this kit and SoilMaster™ DNA kit extracts of loam showed PCR inhibition, as indicated by an increase in C_t values of the IPC, and they required dilution for successful quantification. Conversely, PowerSoil® DNA and E.Z.N.A.® Soil DNA kit extracts from loam contained amplifiable DNA, with little or no PCR inhibition. Although the two kits recovered less nuclear DNA than the FastDNA® SPIN kit, they effectively removed PCR inhibitors, which is an important consideration when selecting an extraction method.

Whitehouse and Hottel (2007) also compared soil DNA isolation kits for the recovery of bacterial DNA and removal of PCR inhibitors. Multiple soil types (clay, silt loam, potting soil) were spiked with various concentrations of a *Francisella tularensis* culture, DNA was extracted using each kit, and *F. tularensis* DNA was quantified by qPCR. PCR inhibitors were removed

effectively using each soil DNA isolation kit. When soils were spiked with the lowest concentration of *F. tularensis*, DNA was recovered from all soil types using an UltraClean™ kit and from silt loam using a SoilMaster™ kit. However, when normalized per gram of soil input, the UltraClean™ and PowerMax™ kits recovered the most *F. tularensis* DNA, indicating that these kits are advantageous for maximizing DNA yield.

Study Aims

A wide variety of DNA extraction methods are used for DNA analysis of skeletal remains, with no agreement on an optimal technique. Furthermore, these methods have not completely eliminated PCR inhibition encountered when processing skeletal remains. Soil DNA isolation kits have been designed to remove PCR inhibitors present in soil, and since buried skeletal remains come in contact with these inhibitors, soil DNA isolation kits have the potential to improve DNA recovery and analysis from buried remains.

In the research presented here, the utility of soil DNA isolation kits for extracting PCR inhibitor-free DNA from soiled bones was examined, and the quantity of DNA recovered was compared to standard DNA extraction methods. The extraction methods used included a PowerSoil® DNA Isolation kit, a SoilMaster™ DNA Extraction kit, a QIAamp DNA Investigator kit, and organic extraction. DNA was isolated from segments of fresh cow femora buried for one week to eight months using each extraction method, followed by quantification of nuclear and mitochondrial DNAs. The extraction methods' efficiency of PCR inhibitor removal was assessed by addition of known inhibitors associated with buried skeletal remains to each

extraction and evaluating the success of mtDNA amplification. Finally, DNA from human skeletal remains was extracted using each technique, and mtDNA was amplified and sequenced. Numerous human bones were tested, including femora from an unidentified decedent, a femur discovered on Beaver Island in Lake Michigan, and ancient skeletal remains recovered from caves in Belize dating from 700 to 900 AD. Overall, the research presented was a methodical analysis of the ability of both standard skeletal DNA extraction techniques and soil DNA isolation kits to recover DNA from skeletal remains and to remove PCR inhibitors.

MATERIALS AND METHODS

Bovine Skeletal Remains

Preparation and Burial

Segments of fresh femora from mature Holstein dairy cows were provided by the Michigan State University (MSU) Meats Laboratory. Excess soft tissue was removed using a scalpel, and the bones were stored at -20˚C until burial. The segments were buried in fertile garden soil in Williamston, MI at a depth of 6 – 12 in for various lengths of time. Burial dates are shown in Table 3, which ranged from August 2012 to April 2013.

Table 3. Burial Dates of Bovine Femora
Segments were buried for various lengths of time and were identified by time (1W, 1M, 2M, 4M, 8M) and replicate number (1, 2, 3, 4).

Bone Identifier	Date Buried	Date Retrieved	Number of Days Buried
1W-1	10/25/2012	11/1/2012	7
1W-2			
1W-3			
1W-4			
1M-1	10/25/2012	11/25/2012	31
1M-2			
1M-3			
1M-4			
2M-1	8/22/2012	10/21/2012	60
2M-2			
2M-3			
2M-4			
4M-1	8/22/2012	12/16/2012	116
4M-2			
4M-3			
4M-4			
8M-1	8/22/2012	4/7/2013	228
8M-2			
8M-3			
8M-4			

Drilling Bones

Bones were drilled in a UV hood, which was cleaned with 70% ethanol and UV irradiated for 5 min prior to and between drillings. Removable drill bit components were soaked in 10% bleach for 10 min, rinsed with water, and UV irradiated in a Spectrolinker™ XL-1500 UV Crosslinker (Spectronics Corporation, Westbury, NY) on each side for 5 min (~2.5 J/cm^2) prior to use and between drillings. Other supplies for drilling bones, including a Dremel 395 MultiPro® rotary tool (Robert Bosch Tool Corporation, Mount Prospect, IL), weighing paper (VWR International, LLC, Radnor, PA), and 1.5 mL microcentrifuge tubes were UV irradiated on each side for 5 min.

Upon unearthing the bovine bones, loose soil was removed by tapping them on a hard surface or using a scalpel. The top layer of a region on the outside of the bone was sanded down with a drill bit to remove surface contaminants. A 7/64 in cobalt drill bit (RIGID, Elyria, OH) was used to drill small holes into the bone. Drilling continued until approximately 400 milligrams (mg) of bone powder was collected on a piece of weighing paper. The powder was subsequently homogenized via gentle agitation. A 1.5 mL microcentrifuge tube was weighed on a PB153-S precision balance (Mettler-Toledo, LLC, Columbus, OH), and approximately 100 mg of the homogenized powder was added to the tube. This was performed for each of the four extraction methods. Tubes with bone powder were stored at -20˚C until extraction.

DNA Extraction

Four methods were used to extract DNA from bone powder: a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA), a SoilMaster™ DNA Extraction Kit (Epicentre® Biotechnologies, Madison, WI), a QIAamp DNA Investigator Kit (QIAGEN, Germantown, MD), and an organic extraction. Reagent blanks were created for each extraction. DNA extracts were stored at -20˚C.

PowerSoil® Extraction

The PowerSoil® kit contains patented Inhibitor Removal Technology® to remove humic substances and uses a silica spin filter to isolate DNA. All tubes and columns were UV irradiated on each side for 5 min, while solutions were not. The manufacturer's protocol was used with the following modifications. Approximately 100 mg of bone powder was added to the PowerBead tubes for digestion, which were incubated at 70˚C for 1 h instead of vortexing for 10 min. The entire supernatant was transferred to a new 2 mL collection tube following the incubation with Solution C2. DNA was eluted by adding 75 µL of TE (10 mM Tris—pH 7.5; 1 mM EDTA) warmed to 55˚C to the center of the filter membranes and centrifuging for 30 sec at 10,000 x g. The elution was repeated a second time. The extracts were concentrated using 30 kDa Amicon® Ultra-0.5 mL Centrifugal Filters (Millipore Corporation, Billerica, MA). Prior to use, 5 µL of salmonid DNA (1 µg/µL) and 495 µL of TE were added to Amicon® filters, which were centrifuged at 14,000 x g for 10 min. Flow-through was discarded, and the PowerSoil®

extracts were added to the Amicon® filters and centrifuged for 5 min at 14,000 x g. The filters were inverted into new Amicon® tubes and centrifuged for 3 min at 1,000 x g to collect retentates.

SoilMaster™ Extraction

The SoilMaster™ kit protocol involves a hot detergent lysis, salting-out protein precipitation, resin-filled column chromatography, and DNA precipitation using spermidine. All tubes and columns were UV irradiated on each side for 5 min, while the reagents were not. The manufacturer's protocol was used with 100 mg of bone powder input, with slight modifications. Tubes containing bone powder and solution for cell lysis were incubated at 70˚C. Entire supernatants were transferred to new 1.5 mL microcentrifuge tubes following incubation. Pellets were vacuum-dried for 15 min using a Maxima® C Plus Vacuum Pump (Thermo Fisher Scientific Inc., Waltham, MA) and resuspended in 25 µL of TE (provided in kit; 10 mM Tris—pH 7.5, 1 mM EDTA).

QIAamp DNA Investigator Extraction

The DNA Investigator kit protocol incorporates a hot detergent lysis and isolates DNA using silica spin filters. Tubes, columns, and solutions were UV irradiated on each side for 5 min. Extractions were performed according to the manufacturer's protocol for isolation of total DNA from bones and teeth, including the use of carrier RNA, with the following elution modification. Buffer ATE (20 µL) was added to the center of the membranes, which were

incubated at room temperature for 5 min prior to centrifugation. The elution was repeated two times, for a total of three elutions.

Organic Extraction

All tubes, columns, and solutions, except for proteinase K and organic solvents, were UV irradiated on each side for 5 min. Five hundred microliters of digestion buffer (20 mM Tris—pH 7.5; 50 mM EDTA; 0.1% SDS) and 5 µL of proteinase K (20 µg/µL) were added to the 1.5 mL microcentrifuge tubes with bone powder, which were vortexed and incubated overnight at 56˚C. An equal volume (500 µL) of phenol was added to the tubes, followed by vortexing for 15 sec and centrifuging for 5 min at maximum speed. The aqueous layers were transferred to new 1.5 mL microcentrifuge tubes, to which 500 µL of chloroform was added. Tubes were vortexed for 15 sec and centrifuged for 5 min at maximum speed. The aqueous layers were transferred to 30 kDa Amicon® filters (pre-treated with salmonid DNA as described above) and centrifuged for 10 min at 14,000 x g. Flow-through was discarded, and the columns were washed with 300 µL of TE and centrifuged for 10 min at 14,000 x g. Flow-through was discarded, and the TE wash was repeated. Flow-through was discarded, and 300 µL of low TE (10 mM Tris—pH 7.5; 0.1 mM EDTA) was added to the columns, which were centrifuged for 10 min at 14,000 x g. Columns were inverted into new Amicon® tubes and centrifuged for 3 min at 1,000 x g to collect retentates.

Quantitative PCR

Extract volumes were measured immediately prior to DNA quantification. Amplification was performed on an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) and fluorescence detected with an iQ5 multi-color real-time PCR detection system (Bio-Rad Laboratories). Sequences of primers and probes are listed in Table 4. Bovine primers and the probe targeting the mitochondrial *ATPase 8* gene were designed using Primer3 software (Rozen and Skaletsky 2000), based on the *Bos taurus* complete mitochondrial DNA sequence obtained from the National Center for Biotechnology Information (2005, BLAST Accession NC_006853). Primers and the probe targeting the bovine nuclear *Melanocortin-1-Receptor* (MC1R) gene, as well as IPC primers, probe, and template, were designed by Lindquist et al. (2011). Primers and probes were ordered from Sigma-Aldrich (St. Louis, MO) or Integrated DNA Technologies (Coralville, IA). Bovine DNA standards were created via serial dilution of stock DNA from cow muscle that was quantified with a DU-520 UV-Visible Spectrophotometer (Beckman Coulter Inc., Brea, CA). A serial dilution in low TE with 20 µg/mL glycogen yielded eight DNA standards with concentrations of 50, 16.67, 5.56, 1.85, 0.62, 0.21, 0.069, and 0.023 ng/µL.

Table 4. Primer and Probe Sequences for qPCR

Primer Name	Sequence	Amplicon Length
F *ATPase 8*	5'-CAA AAC ACC CCT TGA GAA ACA-3'	88 bp
R *ATPase 8*	5'-AGG GTT ACG AGA GGG AGA CC-3'	
ATPase 8 probe	5'-6FAM-CCT CTT TTA TTA CCC CTG TAA TTT T-BHQ1-3'	
F *MC1R*	5'-AAT AAA TCA TAA ACC AGC CTG CTC TTC ATC AC-3'	77 bp
R *MC1R*	5'-AAT AAA TCA TAA AGC TAT GAA GAG GCC AAC GA-3'	
MC1R probe	5'-6FAM-CAC AAG GTC ATC CTG CTG TGC C-MGBNFQ-3'	
F IPC	5'-AAG CGT GAT ATT GCT CTT TCG TAT AG-3'	77 bp
R IPC	5'-ACA TAG CGA CAG ATT ACA ACA TTA GTA TTG-3'	
IPC probe	5'-VIC-TAC CAT GGC AAT GCT-MGBNFQ-3'	
IPC template	5'-AAG CGT GAT ATT GCT CTT TCG TAT AGT TAC CAT GGC AAT GCT TAG AAC AAT ACT AAT GTT GTA ATC TGT CGC TAT GT-3'	

qPCR reactions were set up in 0.2 mL optically clear flat-capped PCR strips (USA Scientific®, Ocala, FL) in a 15 µL volume. Concentrations of ingredients were based on Lindquist et al. (2011), with slight modifications. qPCR reactions consisted of: 7.5 µL of iQ™ Supermix (Bio-Rad Laboratories), 600 nM *ATPase 8* or *MC1R* forward primer, 600 nM *ATPase 8* or *MC1R* reverse primer, 250 nM *ATPase 8* or *MC1R* probe, 1 µM IPC forward primer, 1 µM IPC reverse primer, 250 nM IPC probe, 1 µL of working concentration of IPC template DNA (1:1 billion dilution of 100 µM stock), 0.625 units of Taq DNA Polymerase (5 U/µL; Syzygy, Grand Rapids, MI), 1.325 µL of deionized water, and 1.2 µL of DNA extract or bovine DNA

standard. Bovine DNA standards were run in duplicate for each reaction. Cycling parameters were: 10 min at 95˚C, followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C.

A standard curve was created by iQ™5 Optical System Software and was used to calculate DNA concentration of the samples. In order to compare the quantity of DNA recovered by each of the extraction methods, picograms (pg) of DNA recovered per mg of bone powder was calculated. qPCR concentration (pg/µL) was multiplied by extract volume (µL) and divided by mass of bone powder (mg) to yield a value in pg of DNA/mg of bone powder.

Efficiency of Removing PCR Inhibitors

PCR Inhibitor Preparation

Calcium chloride dihydrate (J.T. Baker, Avantor Performance Materials, Center Valley, PA) was prepared at the following concentrations by dissolving in deionized water: 50 mM, 100 mM, 200 mM, 0.5 M, 1 M, and 2 M. Humic acid (Alfa Aesar, Ward Hill, MA) was dissolved in deionized water, with final concentrations of 10 ng/µL, 100 ng/µL, 1 µg/µL, and 10 µg/µL. Collagen Type I (Sigma-Aldrich) was dissolved with constant agitation in 10 mM $NaPO_4$ (pH 3.0), neutralized with 5 M NaOH until pH 7.0 was reached, and additional 10 mM $NaPO_4$ was added to yield a final concentration of 10 µg/µL.

Determination of Inhibitory Concentrations of PCR Inhibitors

PCR inhibition was assessed by spiking reactions with bovine DNA and amplifying a 126 bp region of the bovine mitochondrial *ATPase 8* gene, using primers designed by Kusama et al.

(2004). However, upon comparison to *Bos taurus* mtDNA sequence (accession NC_006853, Table 5), the reverse primer sequence was modified as shown in Table 6.

Table 5. Kusama et al. (2004) Bovine *ATPase 8* Primer Sequences
Underlined nucleotides indicate locations of forward and reverse primers. Note the discrepancy in the bolded nucleotides of the reverse primer compared to the mtDNA sequence.

Bos taurus ATPase 8 sequence (accession NC_006853)	8101 ttgagagccatatactctccttggtgacatgccgcaactagacacgtcaacatgactgac 8161 aatgatcttatcaatattcttgacccttttatcatctttcaactaaaagtttcaaaaca 8221 caacttttatcacaatccagaactgacaccaacaaaaatattaaaacaaaacacccccttg 8281 a**gaa**acaaaatgaacgaaaatttatttacctcttttattacccctgtaattttaggtctc
Forward primer	5’-ACA ATG ATC TTA TCA ATA TTC TTG-3’
Reverse primer	5’-**CCT** TCA AGG GGT GTT TTG TTT TAA-3’

Table 6. Primer Sequences for Spiked PCR Reactions

Primer Name	Sequence	Amplicon Length
F *ATPase 8*	5’-ACA ATG ATC TTA TCA ATA TTC TTG-3’	126 bp
R *ATPase 8*	5’-TTC TCA AGG GGT GTT TTG TTT TAA-3’	
F 256	5’-CAC AGC CAC TTT CCA CAC AG-3’	229 bp
R 484	5’-TGA GAT TAG TAG TAT GGG AG-3’	

Ten microliter PCR reactions with calcium chloride or humic acid included: 1 µL of GeneAMP 10x PCR Buffer II (Applied Biosystems, Carlsbad, CA), 1 µL of 25 mM $MgCl_2$ (Applied Biosystems), 0.2 µL of AmpliTaq Gold® DNA Polymerase (5 U/µL, Applied Biosystems), 2 µM F *ATPase 8* primer, 2 µM R *ATPase 8* primer, 0.2 mM deoxynucleotide 5’-triphosphates (dNTPs), 1 µL of a 1:100 dilution of bovine DNA from cow muscle, 1 µL of the

inhibitor solution, and 3 µL of deionized water. Spiked PCR reactions with collagen used primers F 256 and R 484, targeting HV II of human mtDNA (Table 6). The PCR reactions consisted of: 1 µL of GeneAMP 10x PCR Buffer II, 1 µL of 25 mM $MgCl_2$, 0.2 µL of AmpliTaq Gold® DNA Polymerase, 2 µM F 256, 2 µM R 484, 0.2 mM dNTPs, 1 µL of a 1:20 dilution of human DNA extracted from a buccal swab, and various volumes of the 10 µg/µL collagen solution and deionized water to bring the final collagen concentration to 1, 2, or 5 µg/µL. Positive controls included extra deionized water in place of inhibitor solutions. PCR cycling conditions consisted of: 94˚C for 10 min, followed by 38 cycles of 94˚C for 30 sec, 55˚C for 1 min, and 72˚C for 30 sec, with a final extension at 72˚C for 5 min. Inhibition was assessed via gel electrophoresis. Five microliters of PCR products were run on a 4% agarose gel and stained with ethidium bromide (Sigma-Aldrich). Gels were photographed with an Olympus C-4000 Zoom digital camera (Olympus, Center Valley, PA).

Addition of PCR Inhibitors to Extractions

The volumes of calcium chloride and humic acid added to each of the four extraction methods resulted in final extracts with the following concentrations, assuming that none of the inhibitor was removed: 0.5, 1, and 2 M (calcium chloride); 10, 100, and 1,000 ng/µL (humic acid). For collagen, the volume of the 10 µg/µL stock added resulted in final extracts at the following concentrations: 2.5, 5, and 12.5 µg/µL. The inhibitor solutions were added to tubes at the beginning of each extraction, using the extraction protocols described above. DNA extracts were stored at -20˚C.

PCR reactions assessing inhibition were as detailed above for calcium chloride and humic acid, using 1 µL of each extract. Collagen PCR reactions included 1.5 µL of GeneAMP 10x PCR Buffer II, 1.5 µL of 25 mM $MgCl_2$, 0.2 µL of AmpliTaq Gold® DNA Polymerase, 2 µM F 256 primer, 2 µM R 484 primer, 0.2 mM dNTPs, 1.5 µL of a 1:20 dilution of human DNA extracted from a buccal swab, and 6 µL of the collagen extract. Positive controls included 6 µL of deionized water in place of inhibitor extract. Final concentrations in PCR for the inhibitors were: 50, 100, and 200 mM (calcium chloride); 1, 10, and 100 ng/µL (humic acid); 1, 2, and 5 µg/µL (collagen). PCR cycling and gel electrophoresis were conducted as detailed above.

Alleviating Collagen Inhibition

Numerous modifications were made to the soil DNA isolation kit protocols in order to examine their effect on removing collagen. The PowerSoil® digestion was performed for 1 hr as above, 1 hr with the addition of 5 µL of proteinase K, and overnight with the addition of 5 µL of proteinase K. The SoilMaster™ incubation was performed for 10 min as above, 1 hr, and overnight. Two sets of PCR reactions were set up to assess inhibition. The first was a 15 µL reaction as detailed above for collagen. The second included the same reagent concentrations, but also included 100 ng/µL of bovine serum albumin (BSA; Thermo Fisher Scientific Inc.), and DNA used for spiking was 0.75 µL of a 1:10 dilution of human DNA extracted from a buccal swab. PCR cycling conditions were as detailed above.

Human Skeletal Remains

Optimization of SoilMaster™ Protocol

Bone powder from bovine bone 1M-3 was homogenized and subjected to different incubation and precipitation procedures for the SoilMaster™ extraction. Fifty milligrams of homogenized powder was incubated at 65˚C for 10 min, while another 50 mg was incubated overnight. The flow-through from the resin-filled column was split evenly to undergo either the kit's precipitation protocol or a sodium acetate/ethanol precipitation. One-tenth volume of 3M sodium acetate, 1/20 volume of glycogen (20 µg/µL), and two volumes of 95% ethanol were added for the modified extraction. Tubes were vortexed and centrifuged at maximum speed for 10 min. The supernatant was removed using a pipette, and pellets were washed with 180 µL of 70% ethanol. Tubes were centrifuged at maximum speed for 4 min, and supernatants were removed. The 70% ethanol wash was repeated. Pellets were vacuum-dried with a Maxima® C Plus Vacuum Pump for 15 min. Pellets were resuspended in 25 µL of low TE. DNA was quantified using the bovine mtDNA qPCR assay as detailed above.

Human Skeletal Samples

Table 7 summarizes the human skeletal remains analyzed. Right and left femora of an unidentified decedent, thought to belong to an individual last seen in August 2011, and a femur discovered on Beaver Island in Lake Michigan, potentially of Native American origin, were provided by the MSU Forensic Anthropology Laboratory. Ancient human skeletal remains were supplied by Dr. Gabriel Wrobel of the MSU Department of Anthropology. These included numerous femora and tibiae recovered from the Je'reftheel (JRH) and Actun Kabul (AKB) caves

in Belize, dating from 700 to 900 AD, which are believed to be of Maya origin. Anthropological analysis determined that several bones may originate from the same individual; these relationships are noted in Table 7.

Table 7. Summary of Human Skeletal Remains
Human skeletal remains analyzed included numerous bones recovered from the Je'reftheel (JRH) and Actun Kabul (AKB) caves in Belize, presumably of Maya origin, in addition to modern remains from an unidentified decedent (bones 14 and 15). Bones presumably originating from the same individual are indicated in the last column.

Number	Description	Type of Bone	Associated Bones
1	JRH Feature 5, Lot 19, Bone 19	Femur	-
2	JRH Feature 5, Lot 108	Femur	-
3	AKB 11-1-10	Tibia	4
4	AKB 11-1-10	Tibia	3
5	JRH 9-5-10 Feature 11	Tibia shaft	6, 12
6	JRH 9-5-10 Feature 11	Tibia shaft	5, 12
7	AKB 11-2-5	Tibia segment	8
8	AKB 11-2-5	Femur	7
9	AKB 11-0-49	Femur	-
10	JRH 07-7-10 Feature 7	Femur shaft	-
11	JRH 05-1-4 Feature 1	Tibia shaft	-
12	JRH 05-11-1 Feature 11	Femur shaft	5, 6
13	Beaver Island	Femur	-
14	Unidentified decedent	Femur	15
15	Unidentified decedent	Femur	14

Drilling Bones and DNA Extraction

Human skeletal remains were superficially cleaned by brushing with a 1% Liqui-Nox® solution (Alconox, Inc., White Plains, NY) and rinsing with water. Bones were dried, and then sanded and drilled as detailed above; however, 200 mg of bone powder was obtained and homogenized. Extractions were performed on approximately 50 mg of bone powder using the same protocols as for bovine bones, with the following modifications. Amicon® columns were

pre-treated with 10 µg of total RNA from Baker's yeast (Alfa Aesar) and 499 µL of TE. The SoilMaster™ incubation was performed at 65˚C, and the kit's DNA precipitation step was replaced with the sodium acetate/ethanol precipitation detailed above.

Quantitative PCR

Nuclear DNA was quantified from skeletal DNA extracts of the unidentified decedent (bones 14 and 15) using a Quantifiler® Human DNA Quantification Kit (Life Technologies Corporation, Carlsbad, CA). qPCR reactions followed the Quantifiler® Kits User's Manual (2012); however, the volume was scaled down from 25 µL to 15 µL. Amplification was performed on an iCycler thermal cycler, and fluorescence was detected with an iQ5 multi-color real-time PCR detection system. PCR cycling parameters were as detailed in the Quantifiler® Kits User's Manual.

DNA was quantified from extracts of ancient bones 3 – 6 using an assay targeting Alu short interspersed elements. DNA standards of known concentration were made by a serial ten-fold dilution of SRM 2372 Human DNA Quantification Standard (NIST, Gaithersburg, MD) using low TE with 20 µg/mL glycogen, resulting in final concentrations of 2 ng/µL – 0.2 fg/µL. Alu primer and probe sequences were obtained from Nicklas and Buel (2005). qPCR reactions consisted of: 7.5 µL of iQ™ Supermix, optimal Alu primer and probe concentrations determined by Jackson (2006), 2 µL of deionized water, and 1 µL of DNA extract, standard DNA, or deionized water (negative control). PCR cycling parameters were 3 min at 95˚C, and 50 cycles of 15 sec at 95˚C and 1 min at 60˚C.

Haplogroup SNP and HV I Amplification

DNAs extracted from select ancient bones and the Beaver Island femur (bone 13) were amplified with primers targeting mtDNA SNPs characteristic of haplogroups A, B, C, D, and X. Haplogroup primers were designed using Primer3 software, except for primers L 5129, H 5190, L 14440, and H 14591, whose sequences were obtained from Smith et al. (2000). Sequences of haplogroup primers are listed in Table 8. Haplogroup amplifications consisted of: 1 µL of GeneAMP 10x PCR Buffer II, 1 µL of 25 mM $MgCl_2$, 0.2 µL of AmpliTaq Gold® DNA Polymerase, 2 µM forward primer, 2 µM reverse primer, 0.2 mM dNTPs, and DNA and deionized water to a final volume of 10 µL. PCR cycling conditions were as detailed above for inhibitor experiments. If amplification was positive (assessed via gel electrophoresis), PCR products were re-amplified in a 30 µL reaction, consisting of: 3 µL of GeneAMP 10x PCR Buffer II, 3 µL of 25 mM $MgCl_2$, 0.6 µL of AmpliTaq Gold® DNA Polymerase, 2 µM forward primer, 2 µM reverse primer, 0.2 mM dNTPs, 14 µL of deionized water, and 1 µL of PCR product. PCR cycling conditions were: 94˚C for 10 min, 10 – 20 cycles of 94˚C for 30 sec, 55˚C for 5 sec, and 72˚C for 30 sec, and a final extension at 72˚C for 5 min.

Table 8. Haplogroup Primer Sequences used with Human Skeletal Remains

Primer Name	Haplogroup	Sequence	Amplicon Length
F 569	A	5'-CCC CCA CAG TTT ATG TAG CTT-3'	185 bp
R 753		5'-TGT TCC TTT TGA TCG TGG TG-3'	
F 8175	B	5'-CTG AAA TCT GTG GAG CAA ACC-3'	199 bp undeleted
R 8373		5'-TGG GGC ATT TCA CTG TAA AGA-3'	
F 13170	C	5'-ATG CTT AGG CGC TAT CAC CA-3'	201 bp
R 13370		5'-GAC CCG GAG CAC ATA AAT AG-3'	
F 5007	D	5'-GCA TAC TCC TCA ATT ACC CAC A-3'	319 bp
R 5325		5'-TGA TGG TGG CTA TGA TGG TG-3'	
L 5129	D	5'-CTA CTA CCG CAT TCC TAC TAC TCA AC-3'	108 bp
H 5190		5'-GGG TGG ATG GAA TTA AGG GTG T-3'	
L 14440	X	5'-CTG ACC CCC ATG CCT CAG GA-3'	192 bp
H 14591		5'-CTA AGC CTT CTC CTA TTT ATG G-3'	
F 6229	X	5'-TCC TAC TCC TGC TCG CAT CT-3'	227 bp
R 6455		5'-GAA GAG GGG CGT TTG GTA TT-3'	

HV I was amplified from skeletal extracts using primers F 16057 and R 16322 (Table 9). Ten microliter PCR reactions consisted of the same reagent concentrations as detailed above for haplogroup SNP amplification; however, 100 ng/µl of BSA was included. PCR cycling conditions were as detailed above for 10 µL reactions. If amplification was positive, 20 µL of deionized water was added to the PCR products, and unincorporated primers and dNTPs were removed using Diffinity RapidTips® (Diffinity Genomics, Inc., West Henrietta, NY). PCR products were re-amplified in a 30 µL reaction with the same reagent concentrations as described above for haplogroup SNP amplification, again using 100 ng/µl of BSA, and 1 – 4 µL of purified PCR product. PCR cycling conditions were as detailed above for 30 µL reactions. Extracts that failed to amplify with HV I primers after repeated attempts, including dilution of the extracts,

were tested for PCR inhibition by setting up PCR using undiluted extract and spiking with 1 µL of a 1:20 dilution of DNA extracted from a buccal swab.

Table 9. Primer Sequences for HV I Amplification from Human Skeletal Remains
Primer F 16057 begins at position 16056; however, the original nomenclature is used here.

Primer Name	Sequence	Amplicon Length
F 16057	5'-AAG TAT TGA CTC ACC CAT CA-3'	265 bp
R 16322	5'-TGG CTT TAT GTA CTA TGT AC-3'	

DNA Sequencing

Thirty microliter PCR reactions were purified with Diffinity RapidTips®. Sequencing reactions were based on either BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) or BDX64 (MCLAB, South San Francisco, CA) protocols. BigDye® reactions consisted of 2.7 µL of BigDye® Terminator Ready Reaction Mix (Applied Biosystems), 1.3 µL of BigDye® Sequencing Buffer (Applied Biosystems), 2 µM primer, and 5 µL of PCR product and deionized water. BDX64 reactions included 0.125 µL of BigDye® Terminator Ready Reaction Mix, 0.875 µL of BDX64 enhancing buffer (MCLAB), 1.5 µL of BigDye® Sequencing Buffer, 2 µM primer, and 6.5 µL of PCR product and deionized water. PCR cycling conditions for BigDye® reactions were 96˚C for 1 min, and 25 cycles of 96˚C for 10 sec, 50˚C for 5 sec, and 60˚C for 4 min, while those for BDX64 reactions were 96˚C for 3 min, followed by 30 cycles of 96˚C for 10 sec, 50˚C for 5 sec, and 60˚C for 2 min.

DNAs were precipitated by adding 1/10 volume of 3M sodium acetate, 1/10 volume of 100 mM EDTA, 1/20 volume of glycogen (20 µg/µL), and 35 µL of 95% ethanol to the 10 µL sequencing reaction products. Tubes were vortexed and centrifuged at maximum speed for 10 min. Supernatants were removed using a pipette and discarded, and pellets were washed with 180 µL of 70% ethanol. Tubes were centrifuged at maximum speed for 4 min. Supernatants were removed with a pipette, and the wash was repeated. Pellets were vacuum-dried using a Maxima® C Plus Vacuum Pump for 15 min. Pellets were resuspended in 10 µL of Hi-Di™ Formamide (Applied Biosystems) and electrophoresed on a 3500 Genetic Analyzer (Applied Biosystems). Electrophoresis parameters are listed in Table 10. Sequences were analyzed using Sequencing Analysis v5.4 (Applied Biosystems) and aligned to the rCRS using BioEdit Sequence Alignment Editor v7.0.9.0 (Hall 1999).

Table 10. Capillary Electrophoresis Parameters for Sequencing Products
Two electrophoresis protocols were used, based on the length of the DNA fragment being sequenced. Fragments longer than 300 bp included an extended run time (1400 sec) and pre-run time (90 sec).

Fragment Length	Oven Temperature (˚C)	Run Time (sec)	Run Voltage (kV)	Pre-run Time (sec)	Pre-run Voltage (kV)	Injection Time (sec)	Injection Voltage (kV)
Less than 300 bp	60	1020	19.5	60	18	8	1.6
Greater than 300 bp	60	1400	19.5	90	18	8	1.6

Statistical Tests

Statistics were conducted using R version 2.15.1. A natural logarithmic transformation was performed on both the nuclear and mtDNA quantification data from bovine bones. A linear mixed model, depicted in Figure 10, was fit to the transformed data. The models included extraction method and length of burial as fixed factors, interaction between extraction method and length of burial as a fixed factor, and bone identifier as a random factor. Assumptions for ANOVA (normal distribution of residuals with equal variance) were checked using a Shapiro-Wilk test and Levene's test. The nuclear DNA quantification model included a term to specify unequal variances of residuals grouped by length of burial (Figure 10), and nuclear DNA yields from 8M bones were removed for statistical comparisons since all extracts, except for one, failed to amplify. An ANOVA was performed on each model to determine which fixed factors had a statistically significant effect on DNA recovery. Pairwise comparisons between mean mtDNA yields for extraction methods and lengths of burial were determined using the Tukey method. Pairwise comparisons between mean nuclear DNA yields for extraction methods within each length of burial, and between lengths of burial within each extraction method were made using the 'multcomp' package in R. Statistical significance was determined for all tests at $p < 0.05$.

a) mtDNA linear mixed model

$y = \mu + \text{Method} + \text{Time} + \text{Method*Time} + \text{Bone ID} + \varepsilon$

b) Nuclear linear mixed model

$y = \mu + \text{Method} + \text{Time} + \text{Method*Time} + \text{Bone ID} + \text{Unequal Time Variance} + \varepsilon$

Figure 10. Linear Mixed Models Fit to mtDNA and Nuclear DNA Yields of Bovine Bones
Both models explain the DNA quantification (y) by a grand mean (μ), the individual bone segment as a random factor (Bone ID), residual error (ε), and the following fixed factors: extraction method (Method), length of burial (Time), and interaction between extraction method and length of burial (Method*Time). The nuclear model also specifies unequal variance of residuals grouped by length of burial (Unequal Time Variance).

RESULTS

mtDNA Quantification of Buried Cow Femora Extracts

The mtDNA yields from PowerSoil®, SoilMaster™, organic, and Qiagen extracts of buried bovine femora are listed individually in Appendix Tables A1 – A4, respectively. Yields normalized by bone mass (pg/mg) are listed in Table 11. Several trends are apparent from the mtDNA quantification results. First, as the length of burial increased, the DNA yield tended to decrease. An exception was the organic extraction of bones buried for 4 months, with a mean of 39.94 pg/mg, which was an increase from the mean for bones buried for 2 months (9.39 pg/mg); however, this can be explained by bone 4M-2 that had an unusually high yield of 151.44 pg/mg. Other bones buried for 4 months had lower yields than the 9.39 pg/mg average from bones buried 2 months: 4.85, 1.17, and 2.28 pg/mg. Note that bone 4M-2 generated the highest yield of bones buried 4 months for each extraction method, indicating that the high DNA quantity from organic extraction (151.44 pg/mg) was reliable. Second, organic extraction had the highest DNA recovery for the majority of buried bones, including those buried for 1 week (205.04 pg/mg), 1 month (20.84 pg/mg), and 4 months (39.94 pg/mg), whereas the SoilMaster™ extraction had the highest mean yield for those buried 2 months (14.39 pg/mg) and 8 months (1.81 pg/mg). qPCR IPC curves did not indicate PCR inhibition (Appendix Figures A1 and A2).

Table 11. mtDNA Yields from Buried Cow Femora
Bone identifiers are listed in the first column. "1W, 1M, 2M, 4M, and 8M" indicate length of burial in weeks or months, and "1 – 4" denote four replicate femoral segments. DNA quantities are reported as pg of DNA per mg of bone powder. Mean DNA quantities (n = 4) for each method and length of burial are bolded.

Bone Identifier	PowerSoil®	SoilMaster™	Organic	Qiagen
1W-1	57.26	251.42	286.43	89.87
1W-2	31.79	429.07	350.96	78.32
1W-3	8.98	33.67	37.85	2.73
1W-4	3.31	102.48	144.91	14.34
Mean	**25.33**	**204.16**	**205.04**	**46.31**
1M-1	0.79	4.89	5.97	0.00
1M-2	17.13	5.21	32.19	0.67
1M-3	25.37	27.53	30.16	2.60
1M-4	3.89	44.97	15.05	12.68
Mean	**11.79**	**20.65**	**20.84**	**3.99**
2M-1	1.28	2.33	3.36	0.00
2M-2	0.77	0.45	0.52	0.41
2M-3	1.30	0.21	2.75	3.57
2M-4	5.92	54.56	30.91	19.48
Mean	**2.32**	**14.39**	**9.39**	**5.86**
4M-1	3.00	3.56	4.85	2.42
4M-2	12.69	16.32	151.44	9.22
4M-3	0.80	0.77	1.17	0.82
4M-4	1.96	0.80	2.28	2.15
Mean	**4.61**	**5.36**	**39.94**	**3.65**
8M-1	2.84	2.56	1.51	1.05
8M-2	0.91	4.23	2.22	1.39
8M-3	0.73	0.11	0.69	0.56
8M-4	0.00	0.34	0.89	0.00
Mean	**1.12**	**1.81**	**1.33**	**0.75**

ANOVA of the linear mixed model fit to the natural log-transformed mtDNA yields indicated that they differed significantly across length of burial ($p = 0.0045$) and across extraction method ($p < 0.001$); however, they did not differ significantly based on the extraction method/length of burial interaction ($p = 0.1260$). Means of the raw mtDNA yields for each extraction method are depicted in Figure 11. Pairwise comparisons of the transformed mtDNA yields between extraction methods are shown in Figure 12. Organic extraction recovered significantly more mtDNA than PowerSoil® and Qiagen ($p = 0.00197$, $p < 0.001$), but not than SoilMaster™ ($p = 0.78067$). SoilMaster™ recovered significantly more mtDNA than Qiagen ($p = 0.00509$), but not PowerSoil® ($p = 0.19647$). PowerSoil® and Qiagen means were not significantly different ($p = 0.90516$).

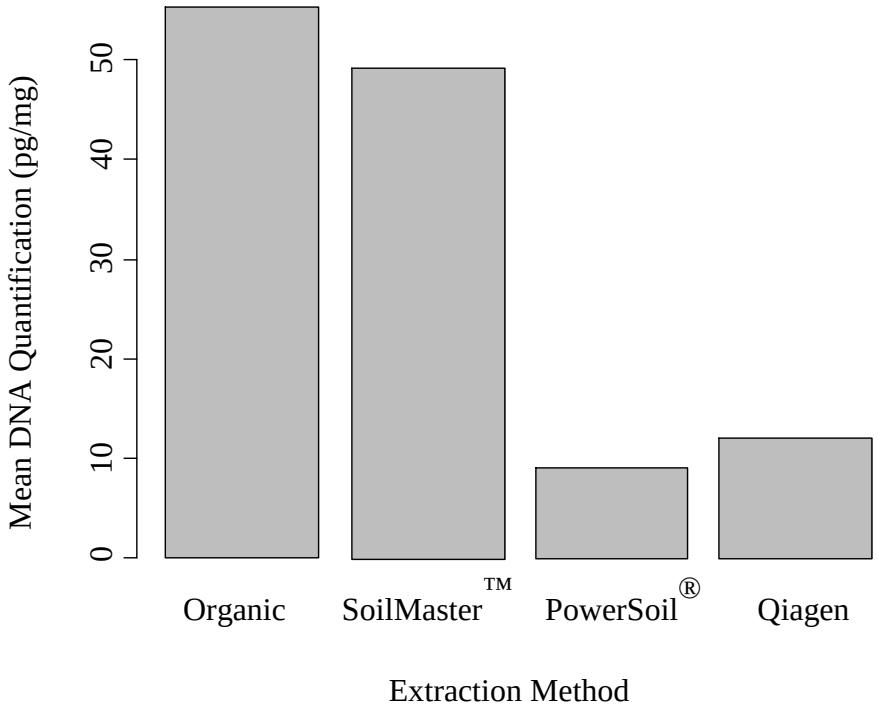


Figure 11. Mean mtDNA Yields of Extraction Methods
The x-axis lists extraction method, while the y-axis is the mean mtDNA yield from bovine bones in pg of DNA/mg of bone powder. Organic and SoilMaster™ mean yields were roughly equal and were substantially higher than those of the PowerSoil® and Qiagen kits.

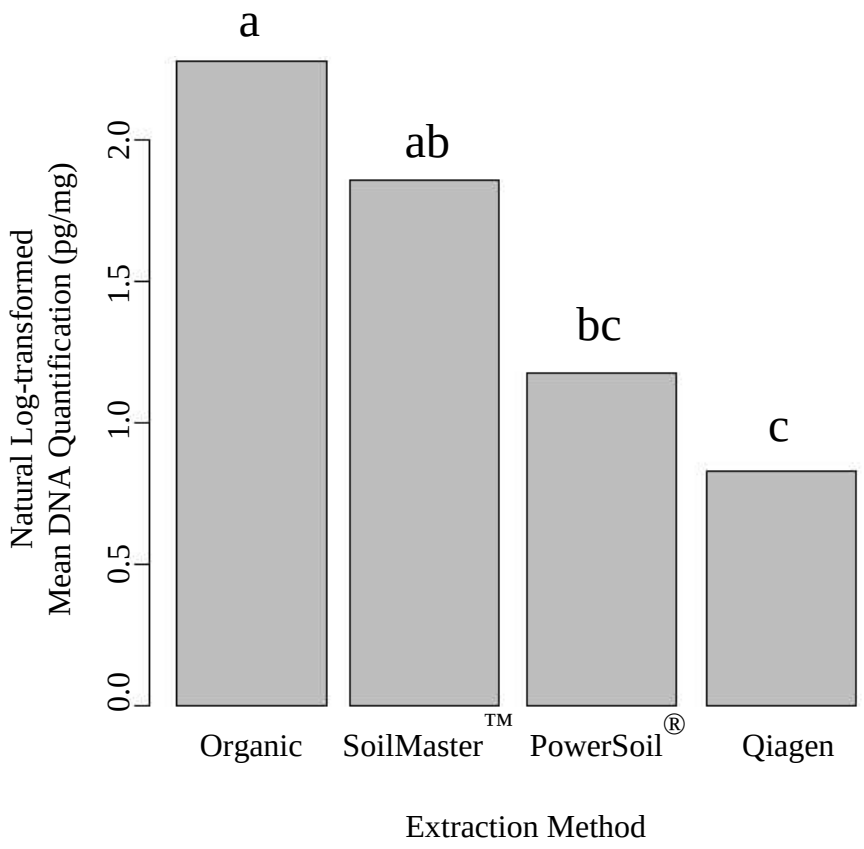


Figure 12. Pairwise Comparisons of mtDNA Yields between Extraction Methods
The x-axis lists extraction method, while the y-axis is the mean transformed mtDNA yield from bovine bones in pg of DNA/mg of bone powder. Methods that have the same letter did not have a significant difference in mtDNA yields, while methods with different letters were statistically different. Organic extraction recovered significantly more mtDNA than PowerSoil® and Qiagen. SoilMaster™ had a significantly higher mean than Qiagen. Note that transformation of the raw data altered the relationship of the PowerSoil® and Qiagen means, wherein the transformed PowerSoil® mean became higher than the Qiagen mean.

Means of the raw mtDNA yields across burial length are depicted in Figure 13. Pairwise comparisons of transformed mtDNA yields between lengths of burial are shown in Figure 14. The mean yield of bones buried for 1 week was not significantly greater than those buried 1 month ($p = 0.22154$). However, bones buried 1 week had a higher mean compared to all other lengths of burial: 2 months ($p = 0.00507$), 4 months ($p = 0.03279$), and 8 months ($p < 0.001$). All other pairwise comparisons between lengths of burial were not significantly different.

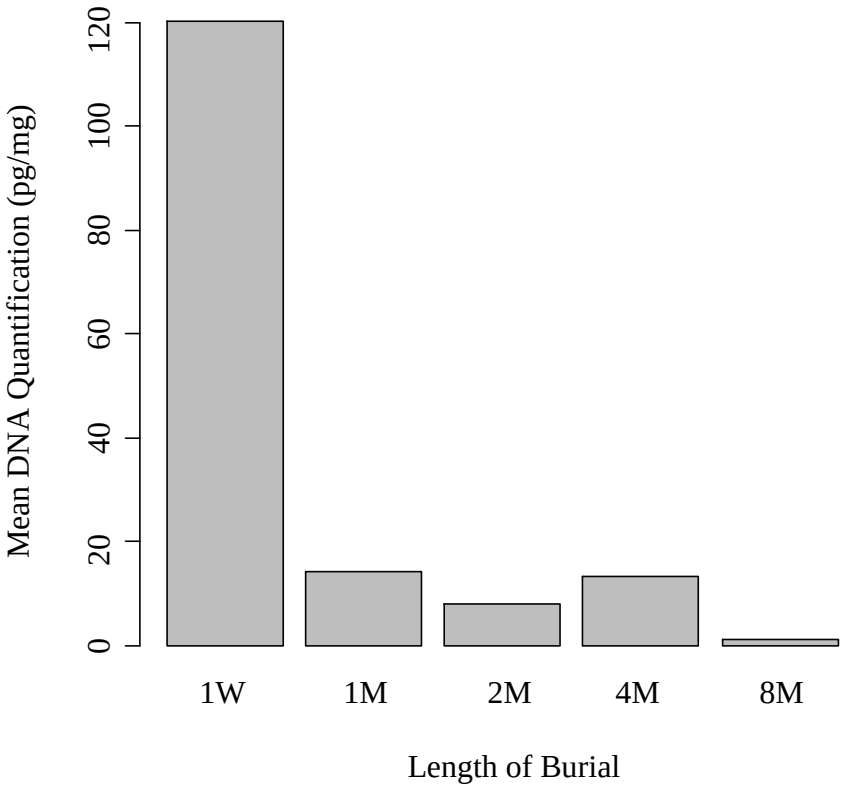


Figure 13. Mean mtDNA Yields of Bones Buried for Increasing Periods of Time
The x-axis lists length of burial in weeks or months, while the y-axis is the mean mtDNA yield from bovine bones in pg of DNA/mg of bone powder. There was a considerable decrease in mtDNA yield between 1 week and 1 month, while yields from bones buried 1 month – 8 months were roughly equal.

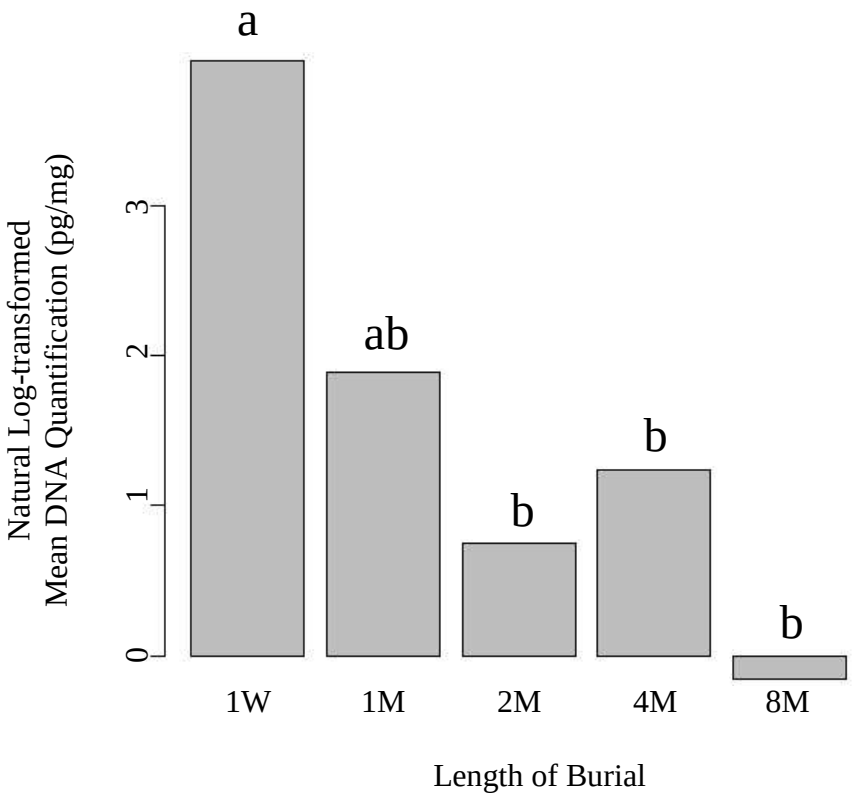


Figure 14. Pairwise Comparisons of mtDNA Yields between Lengths of Burial
The x-axis is number of weeks or months bovine bones were buried, while the y-axis is the mean transformed mtDNA quantification in pg of DNA/mg of bone powder. Burial lengths that have the same letter did not have a significant difference in mtDNA yields, while those with different letters were statistically different. Bones buried for 1 week had a higher mean than those buried 2, 4, or 8 months. Note that an artifact of the transformation resulted in the mean of bones buried 8 months to become negative.

Nuclear DNA Quantification of Buried Cow Femora Extracts

The nuclear DNA quantification results for PowerSoil®, SoilMaster™, organic, and Qiagen extractions are listed individually in Appendix Tables A5 – A8, respectively, and a summary of nuclear DNA yields is shown in Table 12. As length of burial increased, the DNA quantity generally decreased. An exception was the mean yield of organic extracts of bones buried 4 months (84.02 pg/mg), which was higher than that of those buried for 2 months (68.08

pg/mg). This can be explained by the higher DNA yield from bone 4M-2 (333.23 pg/mg) compared to the others buried 4 months (1.13, 1.07, and 0.66 pg/mg), which artificially increased the mean. It should be noted that, like the mtDNA yields, bone 4M-2 produced the highest nuclear yield of bones buried 4 months across every extraction method. Another exception to the decreased yield over time was the mean of the Qiagen extraction of bones buried for 1 month (16.37 pg/mg) compared to means from those buried 2 months and 4 months (35.27 and 18.84 pg/mg). This is likely due to bone 1M-3, which quantified as 0 pg/mg from two separate qPCR runs, while there were no bones that had a 0 pg/mg yield in the 2 or 4 month burial groups. Removing the 0 pg/mg data point resulted in a mean of 21.83 pg/mg, which better fit the trend of decreased DNA yield over time.

Organic extraction had the highest nuclear DNA recovery for the majority of lengths of burial: 1 week (9,520.45 pg/mg), 1 month (80.71 pg/mg), 2 months (68.08 pg/mg), and 4 months (84.02 pg/mg). SoilMaster™ extractions resulted in the second highest yield for bones buried for 1 week (6,445.37 pg/mg), 1 month (66.73 pg/mg), 2 months (52.04 pg/mg), and 4 months (23.74 pg/mg), and the highest yield for bones buried 8 months (2.26 pg/mg). qPCR IPC curves did not indicate PCR inhibition (Appendix Figures A3 and A4).

Table 12. Nuclear DNA Yields from Buried Cow Femora

Bone identifiers are listed in the first column. "1W, 1M, 2M, 4M, and 8M" indicate length of burial in weeks or months, and "1 – 4" denote four replicate femoral segments. DNA quantities are pg of DNA per mg of bone powder. Mean DNA quantities (n = 4) for each method and length of burial are bolded.

Bone Identifier	PowerSoil®	SoilMaster™	Organic	Qiagen
1W-1	440.53	3,867.11	10,216.50	636.92
1W-2	405.52	19,698.14	22,757.65	576.83
1W-3	26.19	463.17	1,636.06	43.02
1W-4	24.71	1,753.07	3,471.60	150.90
Mean	**224.24**	**6,445.37**	**9,520.45**	**351.92**
1M-1	10.53	17.19	8.29	21.74
1M-2	38.97	17.63	79.06	39.38
1M-3	8.58	17.50	11.01	0.00
1M-4	0.45	214.60	224.47	4.36
Mean	**14.63**	**66.73**	**80.71**	**16.37**
2M-1	2.59	17.53	3.59	6.27
2M-2	7.51	6.65	1.69	42.17
2M-3	16.49	17.45	1.94	6.80
2M-4	16.03	166.51	265.08	85.84
Mean	**10.66**	**52.04**	**68.08**	**35.27**
4M-1	16.98	34.36	1.13	12.34
4M-2	17.09	41.66	333.23	57.18
4M-3	6.43	6.02	1.07	4.37
4M-4	0.78	12.92	0.66	1.45
Mean	**10.32**	**23.74**	**84.02**	**18.84**
8M-1	0.00	0.00	0.00	0.00
8M-2	0.00	9.03	0.00	0.00
8M-3	0.00	0.00	0.00	0.00
8M-4	0.00	0.00	0.00	0.00
Mean	**0.00**	**2.26**	**0.00**	**0.00**

Means of the nuclear DNA yields for each combination of extraction method and length of burial are shown in Figure 15. ANOVA of the linear mixed model fit to the natural log-transformed nuclear DNA yields indicated that they differed significantly by length of burial ($p = 0.0008$) and by extraction method ($p < 0.0001$); however, there was also a significant effect of the interaction between extraction method and length of burial ($p < 0.001$). Pairwise comparisons between extraction methods within a single length of burial, and comparisons between lengths of burial within each extraction method are shown in Figures 16 and 17, respectively. Organic extraction recovered significantly more nuclear DNA from bones buried 1 week than did Qiagen and PowerSoil® ($p < 0.001$, $p < 0.001$), but the organic mean was not significantly higher than SoilMaster™ ($p = 0.403$). Similarly, the SoilMaster™ kit recovered more nuclear DNA from bones buried 1 week than the Qiagen and PowerSoil® extractions ($p < 0.001$, $p < 0.001$). PowerSoil® and Qiagen nuclear DNA yields did not differ from bones buried 1 week ($p = 0.424$). All pairwise comparisons between extraction methods within the other time points—1, 2, and 4 months—did not differ significantly.

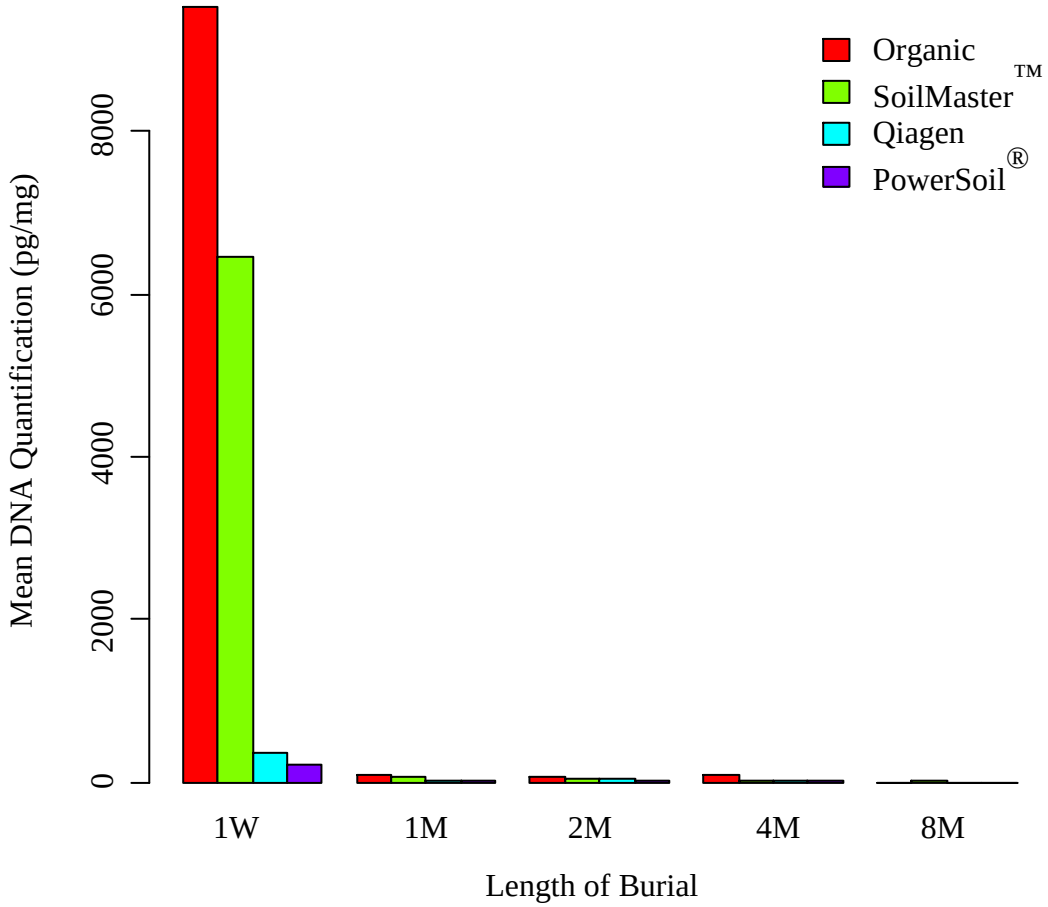


Figure 15. Mean Nuclear DNA Yields of Extraction Methods across Length of Burial
The x-axis is length of burial in weeks or months, while the y-axis is mean nuclear DNA yield from bovine bones in pg of DNA/mg of bone powder. Note the sharp decline in nuclear DNA yields from 1 week to 1 month.

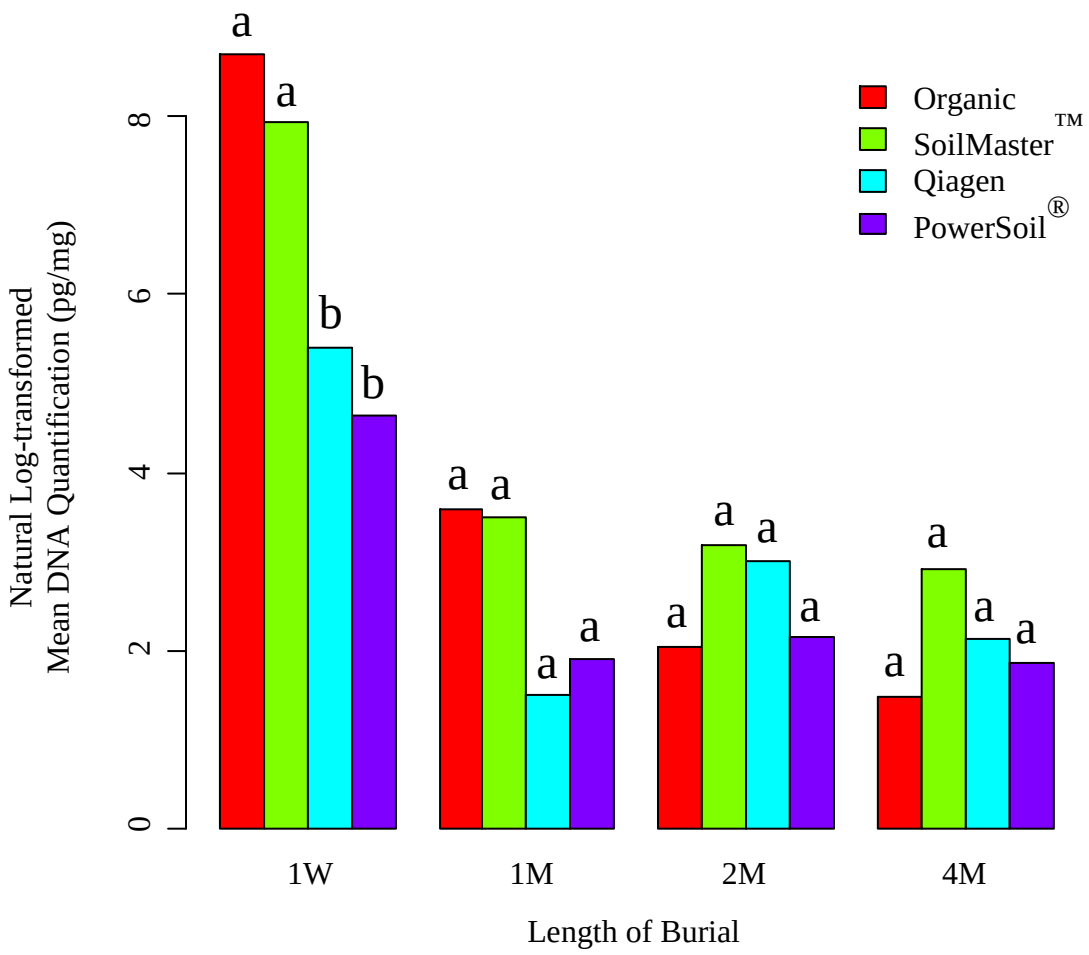


Figure 16. Pairwise Comparisons of Nuclear DNA Yields between Extraction Methods The x-axis is length of burial in weeks or months, while the y-axis is mean transformed nuclear DNA yield from bovine bones in pg of DNA/mg of bone powder. Significant differences between extraction methods within each length of burial are noted by different letters. Bones buried for 8 months were not included in statistical comparisons because the majority of data points were 0 pg/mg, which did not allow for meaningful comparisons between methods. Organic extraction had a higher nuclear DNA yield from bones buried 1 week than Qiagen and PowerSoil® extractions. The SoilMaster™ mean was also higher than Qiagen and PowerSoil® for bones buried 1 week.

Bones buried for 1 week had a significantly higher mean nuclear DNA yield than those buried 1 month from organic, SoilMaster™, and Qiagen extractions ($p < 0.01$, $p < 0.01$, and $p = 0.0371$, respectively), but not from PowerSoil® extraction ($p = 0.3585$). Mean yields from bones buried 1 week were significantly higher than those buried 2 months for organic and SoilMaster™ extractions ($p < 0.01$, $p < 0.01$), but not for PowerSoil® and Qiagen ($p = 0.2215$, $p = 0.2648$). Bones buried for 1 week had a higher mean than those buried 4 months from organic, SoilMaster™, and Qiagen extractions ($p < 0.01$, $p < 0.01$, and $p = 0.0427$, respectively), but not from PowerSoil® extraction ($p = 0.1478$). There were no significant pairwise differences in nuclear DNA yields from bones buried for 1, 2, or 4 months within any extraction method.

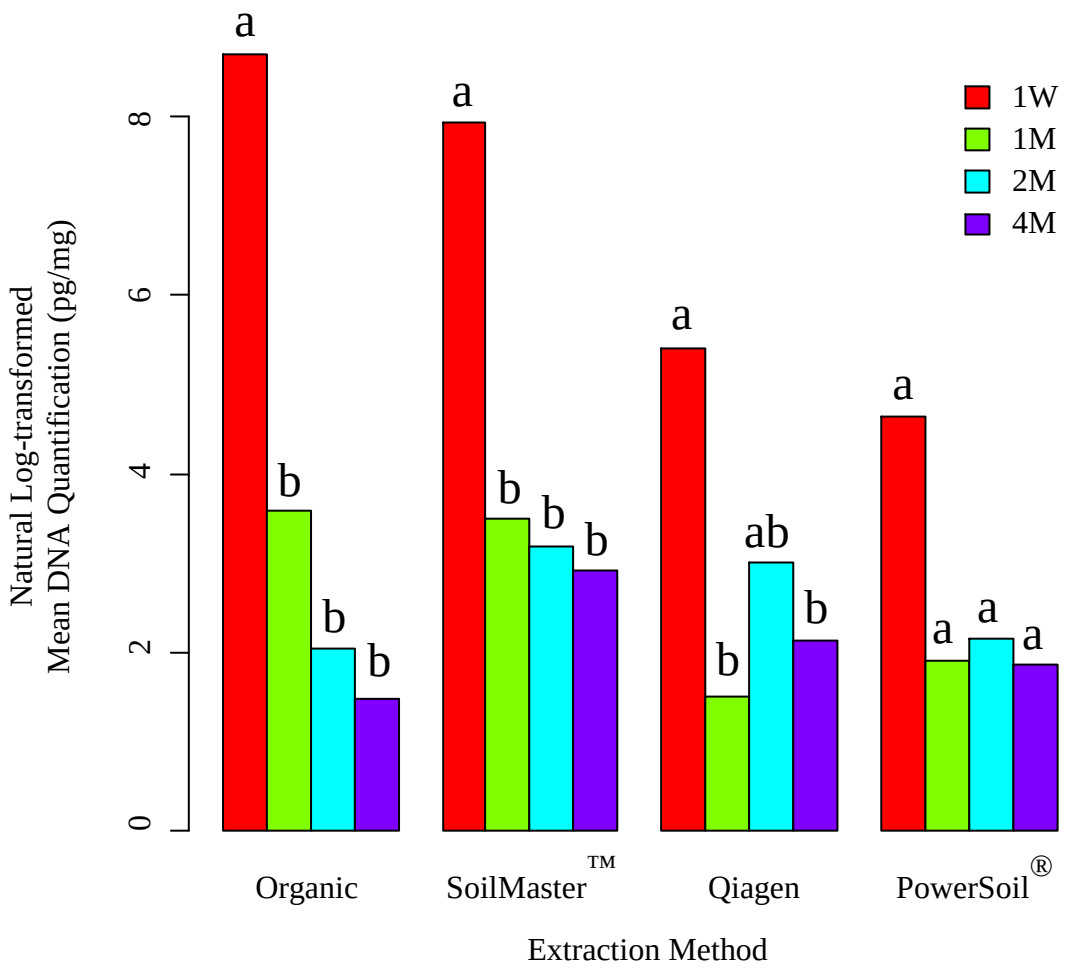


Figure 17. Pairwise Comparisons of Nuclear DNA Yields between Lengths of Burial
The x-axis is extraction method, while the y-axis is mean transformed nuclear DNA yield from bovine bones in pg of DNA/mg of bone powder. Significant differences between lengths of burial within each extraction method are noted by different letters. Bones buried for 8 months were not included in statistical comparisons because nearly all values were 0 pg/mg. Bones buried for 1 week had a higher yield than those buried 1 month for organic, SoilMaster™, and Qiagen extractions. The mean yield from bones buried 1 week was also higher than those buried 2 months for organic and SoilMaster™ extractions, and it was higher than the mean of 4 months for organic, SoilMaster™, and Qiagen extractions.

Efficiency of Removing PCR Inhibitors

Table 13 depicts concentrations of each PCR inhibitor that resulted in inhibition. Calcium chloride was inhibitory at the higher concentrations tested (50, 100, and 200 mM), and partial inhibition was apparent at lower concentrations (5, 10, and 20 mM). Humic acid and collagen were inhibitory at all concentrations tested (1, 10, 100, and 1,000 ng/µL; 1, 2, and 5 µg/µL). Three inhibitory concentrations were selected for each PCR inhibitor and used in subsequent experiments: calcium chloride (50, 100, and 200 mM), humic acid (1, 10, and 100 ng/µL), and collagen (1, 2, and 5 µg/µL).

Table 13. Evaluation of PCR Inhibition at Various Inhibitor Concentrations
Several concentrations of PCR inhibitors were tested to determine if the concentration was inhibitory to PCR. Values listed were the final concentration in the PCR reaction. "-" indicates PCR was inhibited (no amplification). "+/-" indicates that partial inhibition occurred, wherein the DNA band was fainter than that of the positive control.

Calcium chloride	**5 mM**	**10 mM**	**20 mM**	**50 mM**	**100 mM**	**200 mM**
	+/-	+/-	+/-	-	-	-
Humic acid	**1 ng/µL**	**10 ng/µL**	**100 ng/µL**	**1,000 ng/µL**		
	-	-	-	-		
Collagen	**1 µg/µL**	**2 µg/µL**	**5 µg/µL**			
	-	-	-			

PCR inhibition results when calcium chloride, humic acid, or collagen were added to extractions are shown in Figures 18, 19, and 20, respectively. Amplification was successful at every concentration of calcium chloride for each extraction method (Figure 18). At the lowest concentrations of humic acid (1 and 10 ng/µL), all extraction methods resulted in amplification.

However, at the highest concentration (100 ng/µL), the organic and Qiagen PCR reactions were inhibited, while the soil DNA isolation kits resulted in amplification (Figure 19). PCR reactions of organic and Qiagen extractions were not inhibited at any concentration of collagen, whereas SoilMaster™ PCR reactions were inhibited at all concentrations. At 1 µg/µL of collagen, PowerSoil® PCR reactions had amplification, but inhibition occurred at 2 and 5 µg/µL (Figure 20). Further experiments with the soil DNA isolation kits indicated that lengthening the proteinase K incubation to 1 hr or overnight in the SoilMaster™ protocol did not alleviate collagen PCR inhibition. Similarly, when proteinase K was added and/or the digestion was lengthened in the PowerSoil® protocol, all PCR reactions were still inhibited. However, when PCR was repeated in the presence of 100 ng/µL of BSA, amplification was successful from all PowerSoil® extracts, although all SoilMaster™ extracts were still inhibited (Figure 21).

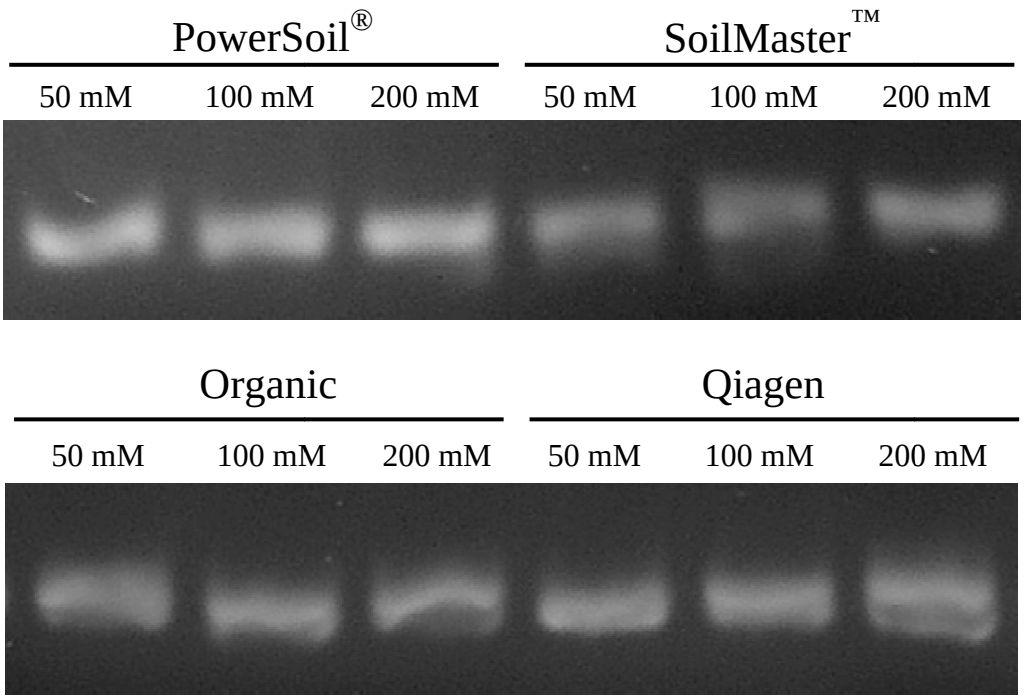


Figure 18. Ability of Extraction Methods to Overcome Calcium PCR Inhibition
Extraction method and concentration of calcium chloride are shown above each lane. Each extraction method was capable of removing calcium chloride at every concentration, as indicated by successful amplification of the bovine DNA used for spiking.

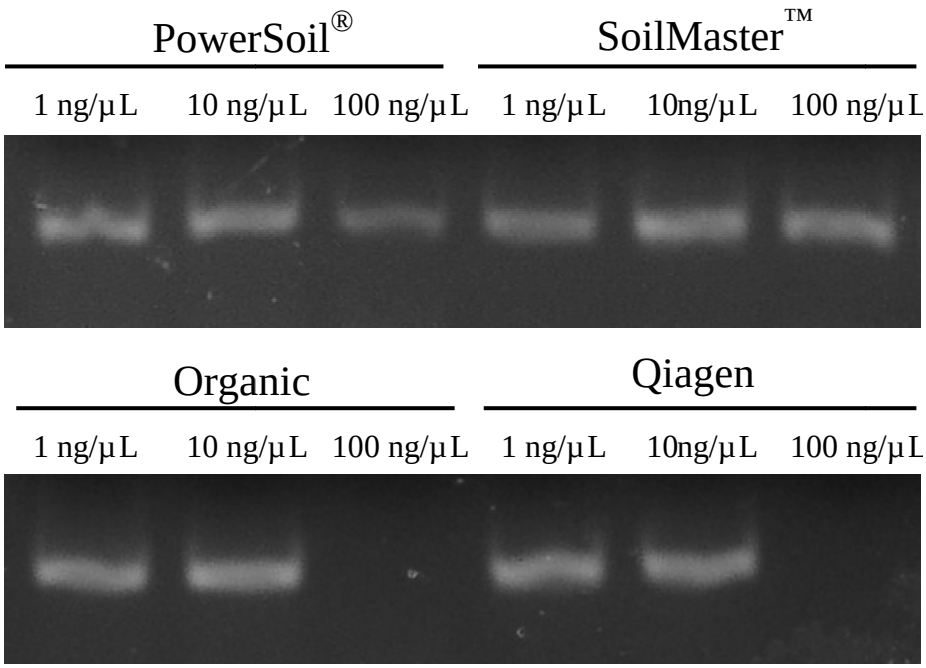


Figure 19. Ability of Extraction Methods to Overcome Humic Acid PCR Inhibition
Extraction method and concentration of humic acid are shown above each lane. The soil DNA isolation kits were capable of removing humic acid at every concentration, as indicated by successful amplification of the spiked bovine DNA. Organic and Qiagen extractions resulted in successful amplification at the lower concentrations of humic acid (1 and 10 ng/µL), but PCR was inhibited at 100 ng/µL of humic acid.

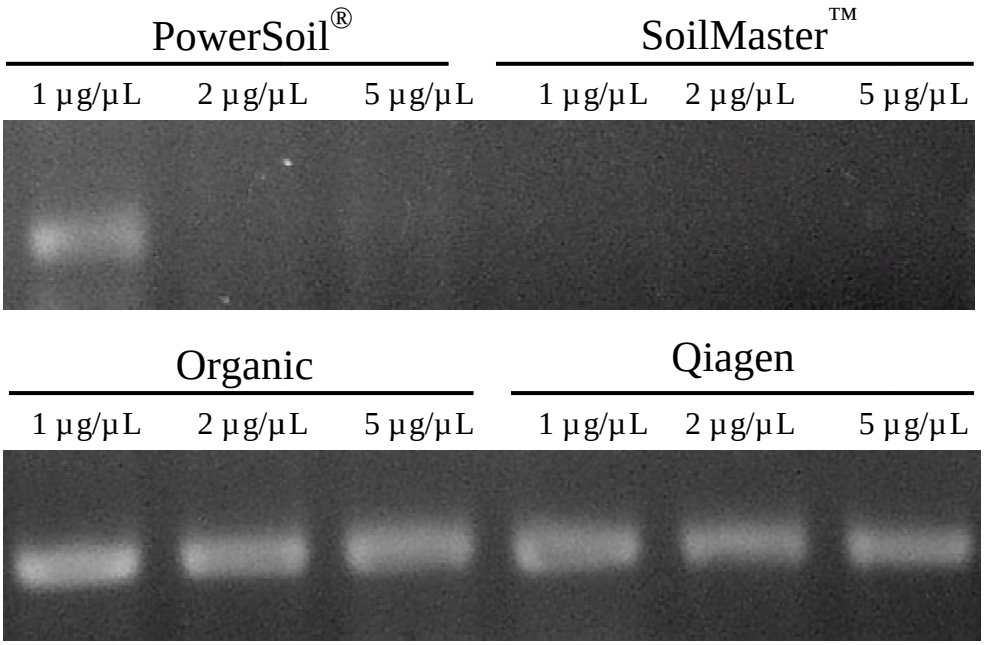
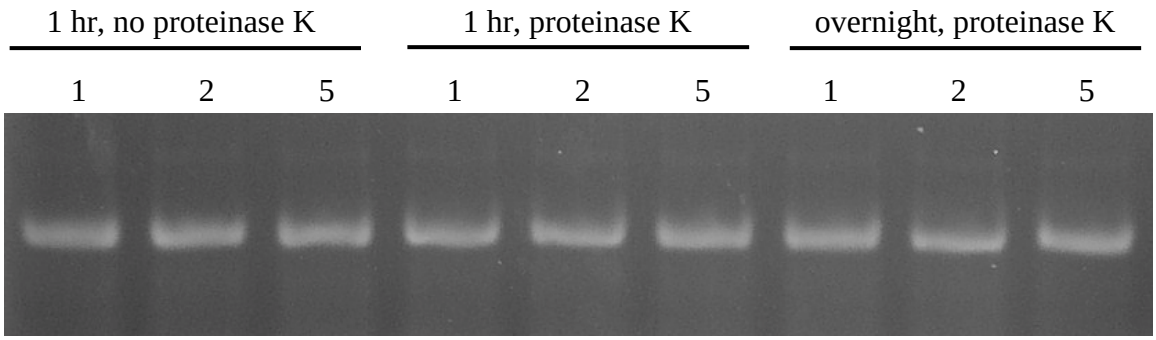


Figure 20. Ability of Extraction Methods to Overcome Collagen PCR Inhibition

Extraction method and concentration of collagen are indicated above each lane. The PowerSoil® kit removed collagen at the lowest concentration, as indicated by amplification of the spiked human DNA. Higher concentrations resulted in PCR inhibition using the PowerSoil® kit. The SoilMaster™ kit was incapable of removing collagen, while organic and Qiagen extractions removed collagen at every concentration.

a) PowerSoil®



b) SoilMaster™

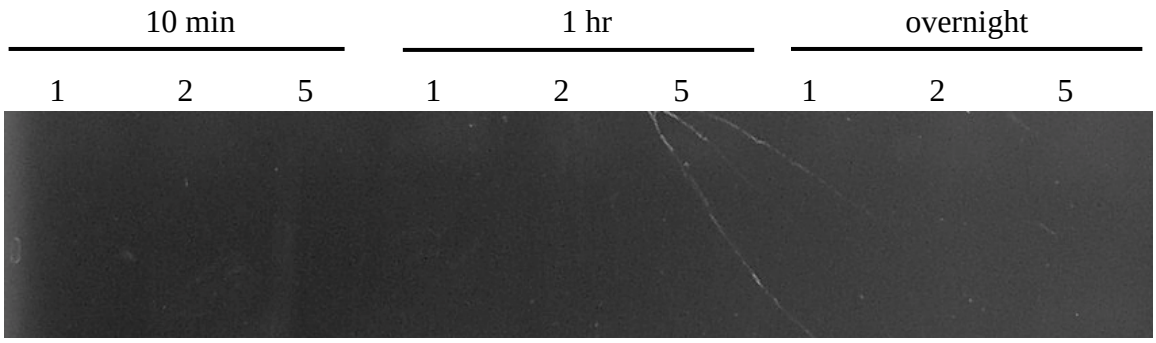


Figure 21. Effect of Bovine Serum Albumin on Collagen PCR Inhibition using Soil DNA Isolation Kits

(a) Spiked PCR reactions when collagen was added to PowerSoil® extractions. (b) Spiked PCR reactions when collagen was added to SoilMaster™ extractions. The modified extraction protocol (length of digestion and use of proteinase K) is indicated above each lane, as are the concentrations of collagen in µg/µL. BSA alleviated collagen PCR inhibition from all PowerSoil® extracts, whereas all SoilMaster™ extracts were still inhibited.

Human Skeletal Remains

SoilMaster™ Kit Optimization

mtDNA yields from a bovine bone using variations of the SoilMaster™ extraction are listed in Table 14. The 10 min incubation resulted in higher DNA yields than overnight incubation, and the modified sodium acetate/ethanol precipitation increased DNA recovery compared to the SoilMaster™ kit precipitation. The highest yield was produced from a combination of a 10 min incubation and sodium acetate/ethanol precipitation (27.70 pg/mg), and this protocol was used for subsequent SoilMaster™ extractions from human skeletal remains.

Table 14. mtDNA Yields from SoilMaster™ Protocol Optimization
DNA yields are in pg of DNA per mg of bone powder from bone 1M-3. The combination of incubation time and precipitation that generated the highest yield was 10 min and sodium acetate/ethanol precipitation.

Incubation	Precipitation	mtDNA quantification (pg/mg)
10 min	kit	8.23
10 min	sodium acetate/ethanol	27.70
overnight	kit	0.62
overnight	sodium acetate/ethanol	2.84

Quantitative PCR

Nuclear DNA yields from extracts of modern skeletal remains (bones 14 and 15) are listed in Table 15. None of the extraction methods recovered enough nuclear DNA to meet the recommended 1 ng input in the AmpFlSTR® Identifiler® PCR Amplification Kit User Guide

(2012) for successful STR analysis. The Qiagen kit recovered 0 pg/mg from both bones. PowerSoil® and SoilMaster™ extractions yielded 9.78 and 2.25 pg/mg from bone 14, while they did not recover nuclear DNA from bone 15. Organic extraction was the only method that recovered nuclear DNA from both bones: 6.65 and 2.26 pg/mg (bones 14 and 15, respectively).

DNA quantification results for the Alu qPCR assay of organic and SoilMaster™ extracts of Maya bones 3 – 6 are shown in Table 16. Extracts of bones 3 and 4 contained 0 – 9.21 fg/µL of DNA; however, the negative control also quantified in the same range (1.57 fg/µL). Organic and SoilMaster™ extracts of bones 5 and 6 contained 0 fg/µL of DNA.

Table 15. Nuclear DNA Yields from Modern Skeletal Remains
DNA quantities are in pg of DNA per mg of bone powder. Amounts of nuclear DNA recovered from bones 14 and 15 were insufficient for STR analysis. Organic extraction recovered DNA from both femora, whereas the other methods recovered DNA from only bone 14, or in the case of the Qiagen kit, no nuclear DNA was recovered from either bone.

Bone Number	PowerSoil®	SoilMaster™	Organic	Qiagen
14	9.78	2.25	6.65	0
15	0	0	2.26	0

Table 16. Alu qPCR Results for Ancient Skeletal Remains

"O" and "S" represent organic and SoilMaster™ extracts. DNA quantities were determined based on comparison to DNA standards ranging from 2 ng/µL to 0.2 fg/µL. Note that the skeletal samples contained 0 – 9.21 fg/µL of DNA, which is not much higher than the DNA quantity detected in the negative control of deionized water (1.57 fg/µL).

Bone Extract	DNA Quantification (fg/µL)
3 O	9.21
3 S	8.53
4 O	1.59
4 S	0
5 O	0
5 S	0
6 O	0
6 S	0
Negative control	1.57

Haplogroup Analysis

Haplogroup results are summarized in Table 17. Bones 1 and 2 did not have SNPs indicative of haplogroups A, B, C, or D. Bone 3 was excluded as haplogroup A from the SoilMaster™ extract, while bone 4 failed to amplify with haplogroup A primers. Bone 5 was negative for haplogroup A from the SoilMaster™ extract, although the organic extract had an ambiguous result, wherein sequence indicated that both nucleotides A and G were present at site 663. Bone 6 was excluded as haplogroup A from an organic extract, and the SoilMaster™ extract failed to amplify. Sequence from a SoilMaster™ extract of bone 9 excluded it as haplogroup A. Bone 12 was negative for haplogroup A from organic and PowerSoil® extracts.

Bone 13 was excluded as haplogroups A, B, C, D, or X from an organic extract. A Qiagen extract of bone 13 also produced a negative haplogroup A result.

Table 17. Haplogroup Analysis of Ancient Skeletal Remains

"O", "S", "P", and "Q" indicate organic, SoilMaster™, PowerSoil®, and Qiagen extracts. Shaded cells represent haplogroups that were not tested. "n/a" signifies that no amplification occurred or DNA sequence was indecipherable due to poor resolution. "negative" indicates that the haplogroup-specific SNP was not present in the DNA sequence. The presence of both the rCRS nucleotide and the haplogroup SNP is designated as "ambiguous."

Bone Extract	Haplogroup A	Haplogroup B	Haplogroup C	Haplogroup D	Haplogroup X
1 O	negative	negative	negative	negative	
2 O	negative	negative	negative	negative	
3 O	n/a				
3 S	negative				
4 O	n/a				
4 S	n/a				
5 O	ambiguous				
5 S	negative				
6 O	negative				
6 S	n/a				
7 S	n/a				
9 S	negative				
12 O	negative				
12 S	n/a				
12 P	negative				
12 Q	n/a				
13 O	negative	negative	negative	negative	negative
13 S	n/a				
13 P	n/a				
13 Q	negative				

HV I Haplotypes

HV I polymorphisms from DNA extracts of bones 1 – 15 are listed in Appendix Table A9, while a summary of concordance among sequences obtained from each bone is shown in Table 18. Haplotypes from bones 1 – 3 were not concordant within each bone. The SoilMaster™ extract of bone 4 generated a sequence with no differences from the rCRS; however, no amplification occurred with the other extracts. PowerSoil® and Qiagen extracts of bone 5 generated a concordant haplotype, but the SoilMaster™ and organic extracts had two different haplotypes. Concordant sequences for bone 6 were obtained from PowerSoil® and Qiagen extracts, while the SoilMaster™ extract produced a different sequence. No sequences from bone 7 were concordant with each other. Repeated attempts to amplify DNA from bone 8 failed. Sequence was obtained from the SoilMaster™ extract of bone 9, but it could not be confirmed from other extracts. PowerSoil® and organic extracts of bone 10 had a concordant haplotype. Sequences obtained from bone 11 were not concordant. The haplotype obtained from a Qiagen extract of bone 12 could not be confirmed by other extracts. PowerSoil® and organic extracts of bone 13 were concordant, while SoilMaster™ and Qiagen extracts generated two different haplotypes. Sequences obtained from all extracts of bones 14 and 15 were concordant within each bone and between them. Figure 22 illustrates the DNA sequences obtained from bones 14 and 15, each containing the polymorphisms 16134 T and 16234 T.

Table 18. Concordance of HV I Haplotypes of Human Skeletal Remains
"n/a" indicates no amplification or sequence that was not interpretable. "C" designates extracts that generated concordant haplotypes from the same bone. "NC" indicates that the sequence obtained was not concordant to other sequences obtained from the bone. If sequence was generated from only one extraction method, it is designated as "uninformative," since polymorphisms could not be confirmed from other extracts.

Bone Number	PowerSoil®	SoilMaster™	Organic	Qiagen
1	NC	n/a	NC	NC
2	NC	n/a	NC	NC
3	n/a	NC	NC	NC
4	n/a	uninformative	n/a	n/a
5	C	NC	NC	C
6	C	NC	n/a	C
7	NC	NC	NC	n/a
8	n/a	n/a	n/a	n/a
9	n/a	uninformative	n/a	n/a
10	C	NC	C	n/a
11	NC	NC	n/a	NC
12	n/a	n/a	n/a	uninformative
13	C	NC	C	NC
14	C	C	C	C
15	C	C	C	C

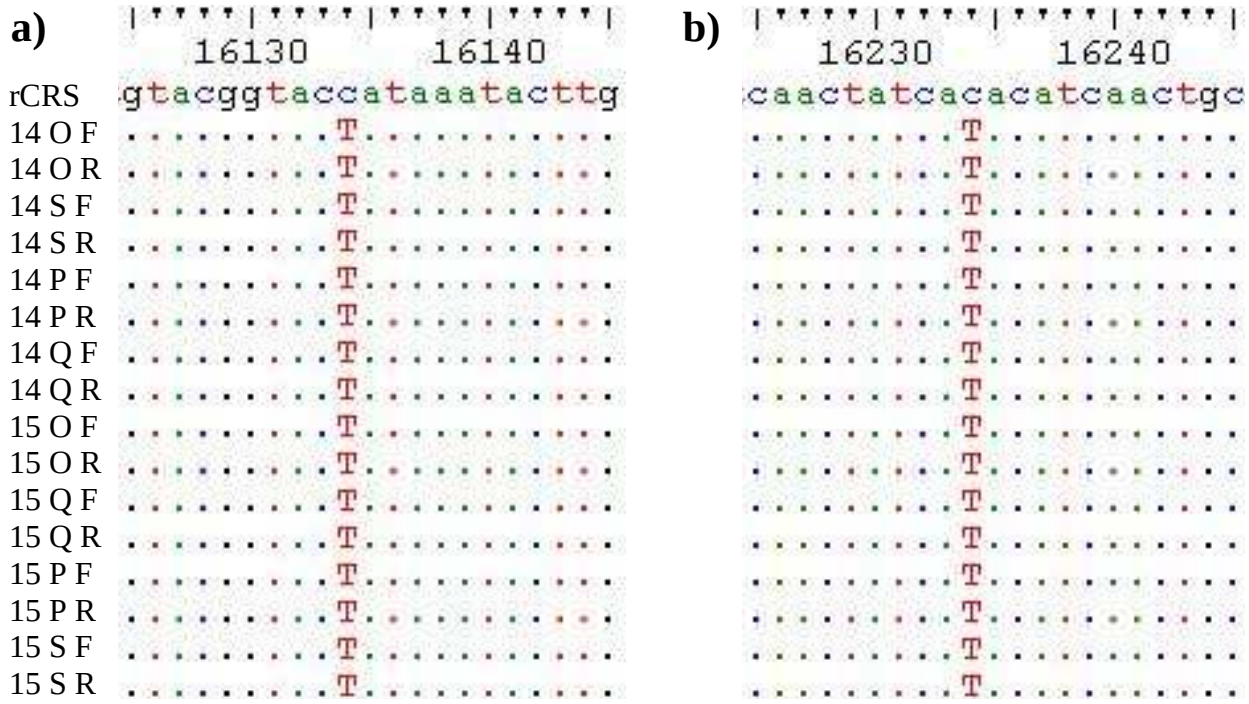


Figure 22. HV I Sequences from Modern Skeletal Remains (Bones 14 and 15)
(a) A portion (16125 – 16145 bp) of HV I from bones 14 and 15, femora of an unidentified decedent. (b) A portion (16225 – 16245 bp) of HV I from bones 14 and 15. "O", "S", "P", and "Q" represent organic, SoilMaster™, PowerSoil®, and Qiagen extractions of each bone, while "F" and "R" indicate sequences obtained from primers F 16057 and R 16322. Complete concordance was found among sequences obtained from soil DNA isolation kit extractions and standard DNA extraction methods for modern skeletal remains. Complete concordance was also found between both femora. The HV I haplotype of this individual was 16134 T, 16234 T.

PCR Inhibition

A roughly equal percentage of PowerSoil®, SoilMaster™, organic, and Qiagen extracts failed to amplify with HV I primers after repeated attempts (samples indicated by "n/a" in Table 18). Spiking PCR of undiluted extracts indicated that the SoilMaster™ extract of bone 1 was the only sample that was inhibited, while all others generated amplification of the DNA used for spiking.

DISCUSSION

DNA identification of decedents is essential in instances when remains are highly decomposed or skeletonized and other identification methods are not possible, or when results are indeterminate. DNA analysis of skeletal remains can be compromised due to the presence of PCR inhibitors in both bone and the material that the remains come into contact with (e.g., soil and plant matter). An optimal DNA extraction technique for skeletal remains would simultaneously remove PCR inhibitors and maximize DNA recovery. A wide variety of DNA extraction protocols are used with skeletal remains; however, PCR inhibition has not been completely eliminated. Therefore, there is a need to further investigate best techniques for DNA isolation from bone.

Numerous commercial DNA extraction kits have been designed to obtain DNA from various substrates, such as buccal swabs, blood, hair, bone, feces, and soil. Soil DNA isolation kits, typically used in microbial DNA analyses, have the ability to remove PCR-inhibitory humic substances. The aims of this study were to evaluate the utility of soil DNA isolation kits to extract DNA from bone and remove PCR inhibitors, and to subsequently compare the performance of these kits to standard skeletal DNA extraction procedures. The findings of this research indicate that soil DNA isolation kits can be used to successfully extract amplifiable DNA from skeletal material and that their performance is equivalent to currently used techniques.

Preliminary work, in which bone powder for each extraction was obtained from different holes drilled on the same bovine femoral segment, revealed that areas within a segment were not always similar in DNA quantity. DNA yields from a given bone showed inconsistencies across

extraction methods. For instance, it might be expected that the bone segment that was the richest source of DNA using a particular DNA extraction method would generate the highest yields among all extraction methods; however, this was not the case (data not shown). Instead, several femur sections had the highest mtDNA yields using one (or more) extraction methods, but generated the lowest yield using a different extraction method, which is counterintuitive. For example, bone 1M-2 had the highest yields using organic, PowerSoil®, and SoilMaster™ extractions, but generated the lowest yield among one month bones from Qiagen extractions. This indicates that DNA quantities in bone powder obtained from separate drillings was not equal, hindering valid comparison of extraction techniques. Other skeletal DNA researchers created bone powder through homogenization using a blender, grinder, or other technique prior to comparison of extraction procedures (Fisher et al. 1993, Davoren et al. 2007, Loreille et al. 2007, Lee et al. 2010, Amory et al. 2012), which would help alleviate the problem of bones not generating consistent DNA quantities among extractions. Because entire bones or bone fragments were not homogenized in this research, it was determined that a bone needed to be drilled and the resultant powder homogenized before distributing it for each extraction method to ensure equivalent quantity and quality of DNA input.

It was also noted that some extracts produced DNA yields that appeared atypical. Homogenized powder from bones 1W-1 and 1W-2 generated unusually high nuclear DNA yields from all extractions, which may have resulted from the presence of soft tissue in bone powder obtained during drilling. The majority of soft tissue was removed prior to burial of bones, although some did remain, which may not have degraded before the bones were unearthed. The cleanest area of the bone without soft tissue was sanded and drilled, but occasionally an area of soft tissue needed to be sanded away, as was true for bones buried for one week. Further

examination of bone 1W-3, which had the lowest nuclear DNA yield of those buried for one week, revealed that less soft tissue remained compared to others, suggesting that yields from one week bones may be artificially high due to the presence of soft tissue. Alternatively, bones buried for one week were fresher than the other bones tested, so they may simply be a richer source of DNA. This could be investigated by completely defleshing the bones buried one week (currently stored at -20˚C), re-drilling them, and extracting and quantifying DNA.

Bone 4M-2 also had an unusually high DNA yield compared to other bones buried for four months, most notably from organic extraction, but this was likely not a result of soft tissue since most of it had decomposed by that time. Instead, the high yield could result from irregularity in the DNA content of bone. Others have reported variable DNA profiling success rates based on the type of bone analyzed, and also unequal DNA quantities in different regions of bone. Edson et al. (2004) noted that, of the long bones, weight-bearing bones such as femora and tibiae were most successful for mtDNA sequencing, while metatarsals and ribs also generated sequence in 80% of skeletal remains. DNA analysis from World Trade Center victims was more successful with lower limbs, especially patellae, and less so with upper limbs, bones from the central axis of the skeleton, and the head (Mundorff et al. 2009). In general, denser bone tends to correlate with a higher DNA typing success rate (Miloš et al. 2007, Misner et al. 2009), and since different areas of the same bone vary in density (Atkinson et al. 1962), each cow femoral segment was likely unequal in DNA quantity. Segments 2M-2, 2M-4, 4M-2, and 8M-3 spanned both the diaphysis and metaphysis of the femur, indicated by a widening at one end of the segment, while other segments originated from the diaphysis. Interestingly, the high relative DNA yields of 2M-4 and 4M-2, and low yields of 2M-2 and 8M-3 may correlate with what region of the segment was drilled; 2M-4 and 4M-2 were drilled near the metaphyseal end,

while the diaphyseal region of 2M-2 and 8M-3 was drilled. Longitudinal bone growth occurs at the ends of long bones by deposition of new bone between the epiphyseal cartilage and end of the diaphysis, as well as between the epiphysis and articular cartilage (Bisgard and Bisgard 1935). Therefore, bone located closer to epiphyseal plates would be relatively newer than diaphyseal bone and may be a richer source of DNA. Yamaguchi and Yamaguchi (1986) measured DNA content in weanling rat femoral diaphysis and epiphysis and found a higher DNA quantity in the epiphysis (avg. 40 – 45 mg/g) than diaphysis (avg. 15 – 20 mg/g). Yamaguchi et al. (2003) also reported higher DNA quantities from metaphyseal (avg. 2.5 – 3 mg/g) than diaphyseal (avg. 1 – 1.5 mg/g) regions of rat femora. These authors extracted and quantified DNA using methods atypical of forensic casework (i.e., sodium hydroxide extraction and spectrophotometry of the color reaction between DNA and indole) and used fresh bone, making it difficult to extrapolate these findings to instances of skeletal remains buried for long periods of time. However, it does necessitate further research that evaluates the difference in DNA content within a bone (diaphysis, epiphysis, and metaphysis). If DNA quantities also vary in skeletal remains with long postmortem intervals depending on what region is sampled, then this is problematic when comparative studies are performed. The region of bone that powder originates from—whether obtained by drilling, removing a fragment and grinding/blending it, or other methods—would be a confounding factor that affects other variables examined in the study. Therefore, comparisons made between bone types, extraction methods, or other variables should be performed on the same region of bone.

Overall, nuclear and mtDNA recoveries from buried cow femora were not significantly different between organic and SoilMaster™ extractions or between Qiagen and PowerSoil® kits, which suggests that soil DNA isolation kits recover equivalent amounts of DNA from bone as

standard DNA extraction methods. Interestingly, the silica-based kits (Qiagen and PowerSoil®) recovered significantly less DNA from buried bone than did the other methods, with one exception. The PowerSoil® mtDNA yield was not significantly lower than the SoilMaster™ yield. Rucinski et al. (2012) reported lower nuclear DNA yields from exhumed skeletal remains buried an average of 37 months using a modified protocol of the silica-based Qiagen Blood Maxi Kit than organic extraction (avg. 7.57 and 44.3 ng/g, respectively). However, Davoren et al. (2007), who published the modified silica protocol Rucinski et al. (2012) used, found higher nuclear DNA yields using the silica kit from skeletal remains of individuals killed during armed conflicts between 1992 and 1995 (avg. 1.94 ng/g) than organic extraction (avg. 0.68 ng/g). This discrepancy could be explained by differences in the protocols for centrifugal filter concentration of organic extracts or by different DNA quantification kits used. However, the major difference was that Rucinski et al. (2012) demineralized bone powder, whereas Davoren et al. (2007) did not include demineralization (discussed below). In contrast, Rohland and Hofreiter (2007) reported no statistical difference in DNA recovery from bone using numerous silica-based kits and an organic extraction; however, the yields varied widely both within and among extraction methods. The authors examined remains over 20,000 years old and quantified mtDNA, which is not an ideal comparison to skeletal DNA extraction methods for forensic purposes. The results from Rucinski et al. (2012) and Davoren et al. (2007) using recently deceased individuals were more applicable to forensic casework and to the DNA yields from bovine bones presented in this research. Based on these studies, silica extractions recovered less DNA than demineralization coupled with organic extraction. When demineralization was not performed, silica kits recovered more DNA than organic extraction, a finding that was opposite of the current study.

Low DNA recovery using silica-based kits in the current research may have resulted from various factors, such as insufficient cell lysis, DNA not binding to the silica membranes, or DNA failing to elute from the membrane. In order for DNA to bind to silica, sufficient concentration of a chaotropic salt must be present, in addition to a buffer for maintaining the pH (Melzak et al. 1996). Rohland and Hofreiter (2007) reported that mtDNA yields from a silica-based extraction of ancient bone were dependent on the pH of the binding solution, wherein pH 4 resulted in the highest recovery, and lower yields were obtained when the pH was 6 or 8. Similarly, Melzak et al. (1996) demonstrated that DNA bound silica most efficiently at pH 3 – 5, and binding was less efficient at pH 6 – 8. The pH of binding solution Buffer AL used in the Rucinski et al. (2012) and Davoren et al. (2007) studies, as well as in Qiagen extractions in the current study, is not disclosed by the manufacturer. However, Buffer AL was determined to be at pH 7 using pH paper. Similarly, the pH of binding solution C4 from the PowerSoil® kit is not disclosed but was determined to be 5 – 6. Bone powder may have contained organic acids from exposure to soil; therefore, pH was tested after addition of soil from a buried bone to determine if it decreased the pH. No change was observed in pH, so these may be less than optimal binding conditions based on the previous findings (Melzak et al. 1996, Rohland and Hofreiter 2007). On the other hand, quality control of silica-based kits likely includes optimization of all solutions, including their pH. For instance, the QIAquick® Spin Handbook (2012) states that the optimal pH for binding DNA to QIAquick silica membranes is less than or equal to 7.5, and a pH indicator is included in the binding buffer that changes color when the pH exceeds 7.5. Although this was not the Qiagen kit used in this research or the previously mentioned studies comparing silica and organic extractions, it suggests that manufacturers do determine the optimal pH for

their binding solutions. The optimal pH for Qiagen binding buffer may be higher than those determined by Melzak et al. (1996) and Rohland and Hofreiter (2007) since those studies incorporated silica particles, not a column with a silica membrane, and it is unclear what other proprietary additives are in the binding solutions of kits that may alter optimal pH. Therefore, optimizing the pH of binding solutions from commercial silica-based kits is not necessary, although when DNA quantity is limited, it is worth considering.

After DNA binds to silica, it must be eluted effectively, or DNA recovery will be reduced. Hebda et al. (in press) recovered DNA from each of four 20 µL elutions of Qiagen DNA Investigator silica columns, while the QIAamp DNA Investigator Handbook (2010) recommends only performing one elution. In the current study, three 20 µL elutions were performed using the Qiagen kit instead of the single elution. Similarly, the PowerSoil® DNA Isolation Kit Instruction Manual (2013) recommends a single 100 µL elution. The PowerSoil® elution used here was based on work done in a microbiology laboratory at MSU, where researchers found that two elutions using 75 µL of TE warmed to 55˚C increased DNA recovery (Dr. Tom Schmidt, personal communications). Despite these optimized elutions, the PowerSoil® and Qiagen kits still generated significantly lower DNA yields from buried bovine bones.

Further comparison of silica-based kit DNA yields to those from other methods revealed that the silica kits' performance was more in line with the other methods as bones were buried for longer periods of time. Organic extraction, which generated the highest nuclear DNA yields, recovered 42.5, 5.5, 6.4, and 8.1-fold more nuclear DNA than the PowerSoil® kit from bones

buried one week, one month, two months, and four months, respectively, and 27.1, 4.9, 1.9, and 4.5-fold more than the Qiagen kit from the same bones. The apparent increase in the silica kits' efficiency with longer burial, or decrease in the other methods' efficiencies, may be due to the limited binding capacity of the silica membranes. Unlike organic or SoilMaster™ extractions, which are not constrained by the amount of DNA that is isolated, the PowerSoil® silica column binds up to 20 µg of DNA (MO BIO Laboratories, Inc. 2010), and the Qiagen kit yields up to 3 µg of eluted DNA (QIAGEN 2013). The highest total yield of nuclear DNA—1.93 µg (Appendix Table A7)—came from an organic extract of bone 1W-2. This value is below the binding capacities of the silica columns; therefore, it seems that either the actual binding capacity is lower than reported, resulting in DNA being lost in the flow-through, or a large amount of DNA bound to the silica was not eluted. These could be tested by binding a known amount of DNA to silica columns, retaining and concentrating the flow-through, performing several elutions that are collected separately, and quantifying DNA from the flow-through and eluates. If DNA is detected in the flow-through, it supports the limited binding capacity hypothesis. If DNA is present in eluates beyond the recommended number of elutions, then it seems that DNA loss occurs from inefficient elution. It is also possible that both scenarios occur, as they are not mutually exclusive.

Alternatively, the organic and SoilMaster™ extractions could be decreasing in their efficiency of recovering DNA from bones buried for longer lengths of time. For instance, it has been shown that when back extraction is performed during organic extraction, wherein the phenol phase is combined with aqueous solution and phases are separated a second time, additional DNA is recovered that was residing in the phenol layer (Webb and Knapp 1990).

Therefore, it is plausible that lower molecular weight DNA from bones buried longer entered the organic phase and resulted in decreased recovery, or simply lower quantities of DNA present in those bones resulted in a greater percentage of DNA loss in the organic phase.

Another observation from the DNA quantification results was the rapid degradation of DNA between one week and one month of burial. After death, endogenous enzymes and microorganisms decompose tissues (Vass 2001), resulting in DNA degradation in tissues within hours or days postmortem (Bär et al. 1988). Exogenous bacteria in soil are also capable of degrading DNA (Antheunisse 1972). Johnson and Ferris (2002) reported that nuclear DNA from porcine liver and kidney was thoroughly fragmented without identifiable nuclei via single-cell gel electrophoresis by three hours postmortem. However, the rate of nuclear DNA degradation was organ-dependent, as nuclei were discernible in skeletal muscle from 3 to 56 hours postmortem, and DNA fragmentation increased gradually during that time. DNA in bone also degrades in a time-dependent manner. Hochmeister et al. (1991) documented DNA degradation in femora of decedents (12 hours postmortem) subjected to various environmental conditions for three months, including outdoors in the summer (avg. temperature 25˚C), wrapped in plastic outdoors, submerged in a river and maintained at 25˚C, and buried in soil. The control group of femora was kept at -70˚C. Average DNA recovery of bones buried in soil relative to the control group was the lowest at 0.4% (50 ng/g), while bones placed outdoors resulted in an average DNA recovery of 7.1% (900 ng/g). Kaiser et al. (2008) reported an increase in DNA fragmentation from human bones buried 1 to 34 years in the same environmental conditions (a cemetery in Munich), which was assessed by amplification of various sized PCR products. Amplification of a 763 bp region of nuclear DNA was successful from remains up to 8 years postmortem, while a 150 bp fragment was amplified from all bones except two: 30 and 200+

years postmortem. These results differ from those found in this study, where nuclear DNA was not detected in bone buried for 8 months based on a qPCR assay; however, the nuclear target was a single copy gene, whereas Kaiser et al. (2008) amplified a multicopy nuclear gene (β-actin). The burial environment could also explain this difference. Misner et al. (2009) found a wide range of skeletal weathering and DNA degradation of remains that were recovered from the Voegtly Cemetery in northern Pittsburgh, Pennsylvania. Because these remains experienced the same temperatures, soil type, and precipitation levels, Misner et al. (2009) proposed that the DNA differences were heavily impacted by environmental micro-habitats. Given that environmental conditions and microbial communities can fluctuate within the same geographic area, it is very likely that they differed substantially between the Kaiser et al. (2008) study and this research, wherein bovine bones were buried in direct contact with fertile top soil, which may have accelerated nuclear DNA degradation.

It is interesting that degradation between one week and one month was more pronounced in nuclear DNA than mtDNA. This may have resulted from cellular location, which has a large influence on DNA degradation. Foran (2006) showed that nuclear DNA degraded faster than mtDNA in whole tissues, but when tissues were homogenized, mtDNA degraded as fast or faster than nuclear DNA. The mitochondrion itself may protect DNA from degradation, while the nuclear membrane breaks down and exposes nuclear DNA to nucleases in the cell and environmental factors that accelerate degradation. Steadman et al. (2006) found that nuclear DNA was more susceptible to degradation from maceration than mtDNA. Fresh porcine ribs were treated using various maceration techniques and nuclear and mtDNAs were amplified. A set of ribs was manually defleshed using a scalpel as a control, which did not impede nuclear or mtDNA amplification. Conversely, no nuclear DNA was recovered from bones processed with

10% bleach, EDTA/papain solution, or detergent/sodium carbonate solution followed by a degreasing solution. mtDNA amplification was successful when any of the maceration techniques were employed, indicating that nuclear DNA was more susceptible to degradation from the treatments than mtDNA. Rennick et al. (2005) reported similar results, wherein nuclear DNA amplification from skeletal remains (several weeks postmortem) was unsuccessful when three maceration techniques were used: boiling for 4 hours in either water, a 3% bleach solution, or detergent/carbonate solution. However, mtDNA was amplified in all cases, and detergent/carbonate solution was least detrimental to DNA. These results mirror what was found in this study with buried bones, wherein nuclear DNA levels decreased significantly between one week and one month of burial, with little to no nuclear DNA detected after eight months. In contrast, mtDNA was present in bones after eight months of burial. A decrease in mtDNA levels between one week and one month of burial was also apparent, although the difference was not statistically significant. Since mtDNA is protected by the mitochondrial membrane, it was likely better shielded from environmental factors than nuclear DNA, potentially explaining the sharper decrease in nuclear DNA between one week and one month. Further research investigating nuclear and mtDNA degradation in bone between the day of burial and one month by including more sampling points would be worthwhile to better grasp the rate of DNA degradation. It would also be interesting to examine degradation among bones buried in various soil types to study the effects of texture (clay, silt, sand), microbial communities, pH, organic content, and mineral content on the rate of degradation.

An aspect of skeletal DNA extraction that was not examined in the current study is demineralizing the bone powder. This is usually achieved by incubating it in a high concentration EDTA solution, which chelates divalent cations such as Ca^{2+} and partially or

completely dissolves the powder. Salamon et al. (2005) reported that DNA is present in crystal aggregates in bone; therefore, as demineralization breaks down the mineral matrix it presumably releases any DNA residing there. This hypothesis is supported by several studies that demonstrated increased DNA yields when demineralization was used. Loreille et al. (2007) of the Armed Forces DNA Identification Laboratory (AFDIL) found that dissolving bone powder significantly increased mtDNA yields and the number of STR alleles generated from degraded skeletal remains, based on an organic extraction. Specifically, the total demineralization protocol increased DNA recovery approximately 5-fold compared to the protocol without demineralization using 0.6 – 1.21 g of bone powder input. Similarly, Amory et al. (2012) obtained equivalent or greater nuclear DNA yields in 90% of bones tested using silica-based kits when 0.5 g of bone powder was completely demineralized than when 2 g of the same homogenized bone powder was partially demineralized.

Conversely, Fisher et al. (1993), also of AFDIL, found that demineralization resulted in lower DNA yields compared to non-demineralized bone (avg. 20 µg/g and 40 µg/g, respectively). However, their demineralization included several EDTA washes, wherein the supernatant was tested for the presence of Ca^{2+} and discarded. These washes could have resulted in DNA loss, and significant quantities of DNA have indeed been detected in EDTA wash solution (Loreille et al. 2007). The studies that determined demineralization increased DNA yield did not use washes; rather, a high concentration of EDTA was a component of the lysis buffer, presumably eliminating DNA loss during the demineralization procedure. The organic extraction used in the current study, which outperformed the other techniques in recovering DNA, included 50 mM EDTA in the digestion buffer, which was the same EDTA concentration of digestion buffers in the protocols not including demineralization implemented

by Loreille et al. (2007) and Amory et al. (2012). It is unknown whether the extraction kits used in the current study contain EDTA in the digestion solutions, but even if EDTA was present, the short incubation times (10 minutes and 1 hour) used with the soil DNA isolation kits were too brief for substantial demineralization to occur.

Evaluation of the PCR inhibitor removal ability of the four DNA extraction techniques revealed that each removed PCR-inhibitory concentrations of calcium chloride efficiently. This makes sense as the charge and size of Ca^{2+} allow it to be easily removed during extraction. First, EDTA in the digestion buffer used during organic extraction chelates Ca^{2+} (as mentioned above, it is unclear whether EDTA exists in digestion solutions used in the kits, as manufacturers only denote the presence of organic and inorganic salts). Second, Ca^{2+} is much smaller than collagen or humic acid molecules. Washes of the silica and Amicon® columns are effective at desalting solutions (PowerSoil® DNA Isolation Kit Instruction Manual 2010, Amicon Ultra-0.5 Centrifugal Filter Devices User Guide 2011); hence, any Ca^{2+} initially contained in the extractions passed through the columns. Calcium ions are also small enough to be held up in the pores of Sephadex G-100 beads in the SoilMaster™ kit columns, which have a molecular weight limit of 100 kDa (GE Healthcare Life Sciences 2012). Thus, no extraction method was disadvantageous for overcoming calcium PCR inhibition.

On the other hand, the four extraction methods were not equally efficient at overcoming collagen inhibition. Soil DNA isolation kits were less effective than a Qiagen kit, which is surprising considering that the PowerSoil® kit includes silica spin filters like the Qiagen kit, and

the SoilMaster™ protocol involves a protein precipitation step. In inhibitor experiments, the amount of collagen added to extractions resulted in 1, 2, and 5 µg/µL of collagen in PCR, assuming that none was removed during extraction. The SoilMaster™ kit did not effectively remove collagen and resulted in PCR inhibition at all concentrations. Eilert and Foran (2009) found collagen to be inhibitory in PCR at 1 µg/µL or greater, but it was not inhibitory at 0.5 µg/µL; therefore, the SoilMaster™ kit likely removed less than half, if any, of the collagen present in extractions. The protein precipitation step should theoretically remove proteins from solution. Published protocols for isolating type I collagen consist of salting-out precipitations like that employed in the SoilMaster™ protocol; however, the SoilMaster™ kit uses ammonium acetate, whereas others used sodium chloride (Stark et al. 1972, Nagai and Suzuki 2000, Kittiphattanabawon et al. 2005), ammonium sulfate (Messent et al. 1998), or a combination of both (Nalinanon et al. 2011). Collagen that did not precipitate during salting-out was unlikely to be removed using the Sephadex G-100-filled column due to its size. The collagen tripeptide is approximately 300 kDa (Zhang et al. 2006), while the Sephadex pore size prevents molecules greater than 100 kDa from entering the beads. Organic extraction removes proteins and lipids using organic solvents, which is likely why collagen was removed effectively. The silica-based extractions also removed collagen efficiently through washes of the DNA bound to the column; however, the PowerSoil® kit was less efficient than the Qiagen kit. This may be due to the single wash of the column in the PowerSoil® protocol compared to three washes in the Qiagen kit protocol.

The fact that lengthening the digestion step for SoilMaster™ and PowerSoil® extractions did not improve collagen removal suggests that the shorter incubation relative to the standard methods does not explain the soil kits' inferior performances. Addition of proteinase K to the PowerSoil® digestion also did not improve results; however, collagen is fairly resistant to proteolysis due to extensive cross-linking (Hollander 2001), which may indicate that proteinase K is not an effective protease for digesting collagen. Li (2009) compared a proteinase K digestion protocol with a protocol incorporating both collagenase and proteinase K digestion. The author found that collagenase treatment was not detrimental to STR analysis, and it increased DNA yield from homogenized bone powder nearly two-fold compared to treatment with proteinase K alone (avg. 35 µg/g vs. 20 µg/g). The increase in DNA yield from collagenase treatment is hypothesized to stem from the release of additional DNA embedded in the protein matrix of bone after cleavage of the collagen tripeptide (Li 2009). Given this, collagenase digestion may be useful in DNA extraction from skeletal remains to increase DNA yields and minimize PCR inhibition by cleavage of collagen.

The extraction methods also differed in their effectiveness in removing humic acid. It is not surprising that PCR inhibition did not occur when humic acid was added to soil DNA isolation kits because they are specifically designed to remove humic substances. The PowerSoil® kit contains two reagents that precipitate humic substances, while the SoilMaster™ kit's Sephadex column removes them. In contrast, PCR of organic and Qiagen extracts was inhibited at 100 ng/µL of humic acid, indicating that these standard extraction methods are incapable of removing high concentrations of humic acid. Because humic substances have a similar molecular weight and charge to DNA (Holben 1994), copurification of humics with DNA

often occurs. Humic acid was not removed during organic extraction since it is soluble in the aqueous phase. Purification using 30 kDa Amicon® filters may have removed humic acid molecules less than 30 kDa in size, although humic acids larger than 30 kDa have been documented (Lobartini et al. 1997, Harry et al. 1999), and these larger molecules were likely retained during filtration. Organic extracts resulting from addition of low concentrations of humic acid were not discolored; however, slight discoloration was present at the highest concentration. Harry et al. (1999) found that humic acids remained in the eluate after DNA purification using a silica membrane, which they proposed was due to humic acid directly binding to the silica or forming a complex with DNA, which then bound to the silica. At the lower concentrations of humic acid, the Qiagen extraction was effective at removing humic acid, resulting in extracts that were not discolored. At the highest concentration however, some humic acid may have adhered to the silica column and eluted with DNA, as these extracts were also discolored.

Although experiments adding known inhibitors to extractions resulted in PCR inhibition, DNA analysis of human skeletal remains revealed that PCR inhibition was infrequent. All extraction methods removed any PCR inhibitors present in the femora of the recently deceased individual effectively since DNA amplified successfully. On the other hand, some SoilMaster™ extracts from ancient remains required dilutions for amplification and appeared slightly discolored, suggesting the presence of PCR inhibitors. Dilutions were not necessary when the other extraction techniques were used, and those extracts were not discolored. When ancient DNA did not amplify despite repeated dilutions, reactions with the undiluted extracts were spiked with high quality DNA to assess PCR inhibition. This revealed that one SoilMaster™

extract was inhibited, whereas extracts using the other methods were not. Given these results, PowerSoil®, Qiagen, and organic extractions were superior at removing PCR inhibitors present in ancient skeletal material than a SoilMaster™ kit. Dineen et al. (2010) also encountered PCR inhibition when using a SoilMaster™ kit. Extracts of loam were diluted ten-fold to overcome PCR inhibition indicated by the qPCR IPC. The authors proposed that the inhibition was due to humic substances because loam had the highest organic content (8.5%) of all soils tested. However, in the research presented here, the SoilMaster™ kit was found to be efficient at removing humic acid, and the Maya remains appeared to be quite clean, therefore it is unclear what PCR inhibitor(s) were present in the SoilMaster™ extracts. Despite PCR inhibition from SoilMaster™ extracts of ancient bone, the lack of inhibition from PowerSoil® extracts of ancient and modern bone accentuates the utility of soil DNA isolation kits to handle PCR inhibitors associated with skeletal remains. It would be interesting to examine the frequency of PCR inhibition when using each extraction method on human skeletal remains that are in less than pristine condition and have previously demonstrated inhibition.

DNA extracts of modern human skeletal remains using both the standard methods and soil DNA isolation kits produced HV I sequences that were used to positively identify a decedent (approximately two years postmortem). Given that all techniques generated concordant HV I sequences of the decedent, there does not appear to be a clear advantage of one technique over another when skeletal remains are relatively fresh. However, the silica-based kits (i.e. PowerSoil® and Qiagen) are not ideal for maximizing DNA recovery, owing to the low DNA

recovery from relatively fresh bone detailed above. An advantage to the soil DNA isolation kits was the quicker extraction time (approximately 1 to 2 hours), while the standard methods included an overnight incubation in addition to 1 to 2 hours needed for extraction. Therefore, given the above considerations, the SoilMaster™ kit seems to be most useful for forensic identification because it provided accurate results while maximizing DNA recovery and reducing turnaround time.

Conversely, extracts of ancient skeletal remains had few instances of HV I concordance, making it difficult to compare the various DNA extraction methods. Ideally, DNA analysis of ancient skeletal remains is performed on multiple bones known to originate from the same skeleton to ensure concordance. Rennick (2005) obtained sets of three separate bones derived from the same individuals buried in the Kamenica tumulus in Albania ($7^{th} – 6^{th}$ century BC), which assisted with confirmation that the mtDNA haplotypes obtained actually originated from skeletal DNA. Unfortunately, the ancient skeletal remains analyzed in this research originated from caves in which they were commingled, making it difficult to confidently obtain multiple bones from the same individual. However, certain bones analyzed may have originated from the same person based on anthropological analysis. Comparison of HV I haplotypes indicated that bones 3 and 4—right and left tibiae that were of similar length—were not from the same individual. Bones 5 and 6, right and left tibiae that were similar in length, were potentially from the same person as extracts of both contained the haplotype 16126 C, while bone 12, a femur possibly associated with the tibiae, did not appear to originate from the same individual. A relationship between bones 7 and 8, a tibia and femur discovered next to each other, could not be established due to failed DNA amplification from bone 8.

The ancient DNA haplotypes obtained in this study were not generally concordant among different extractions of the same bone, highlighting that replication of results is essential in ancient DNA analysis in order to ensure that DNA sequences originated from the bone itself. As such, guidelines have been developed among ancient DNA researchers to ensure authenticity of results, including the use of negative controls, reproducible results from multiple extractions and/or PCR, and isolation of work areas (Cooper and Poinar 2000). Even when researchers follow recommendations, Pääbo et al. (2004) advised that this does not guarantee ancient DNA results are accurate. For instance, a 30,000-year-old tooth originating from a bear in China reproducibly generated human DNA sequences when primers targeting the mtDNA control region were used, and cloning of the PCR products revealed the presence of 20 different human sequences (Hofreiter et al. 2001). Clearly, ancient DNA analysis requires methodical laboratory procedures to avoid modern DNA contamination as well as replication to enhance the authenticity of results.

Genetic haplogroup determination can be used to strengthen the notion that DNA sequences obtained are from ancient bone, especially in cases when the haplogroups of analysts are different than that of the bone. Various control region polymorphisms that tend to correlate with haplogroups can be examined. Torroni et al. (1993) found that certain HV I polymorphisms were consistent with specific Native American/Asian haplogroups of modern subjects. In their study, all haplogroup A individuals had polymorphisms 16290 T and 16319 A, which were not present in haplogroup B, C, or D individuals. HV I polymorphisms among Native Americans that were indicative of haplogroup B included 16189 C and 16217 C, but 16189 C was seen in one haplogroup A individual and 16217 C was not present in all haplogroup B individuals. 16298 C and 16327 T were specific to haplogroup C, although 16298 C was not present in all

haplogroup C individuals. The authors did not find HV I polymorphisms specific to haplogroup D.

Due to primer positioning, the 16319 A polymorphism could not be determined from the ancient skeletal remains in this study; however, 16290 T was present in extracts from bones 3, 5, 6, 7, 9, and 13, meaning these remains are potentially haplogroup A. Bones 1, 3, 5, 6, 7, 9, 11, 12, and 13 could also belong to haplogroup B due to the presence of 16189 C. No HV I polymorphisms pointed to haplogroup C. Before using these HV I polymorphisms to assign haplogroups, it is important to note that some of them have been documented in non-Native American/Asian populations. 16189 C and 16298 C were present in British Caucasians (Piercy et al. 1993) and other Europeans (Torroni et al. 1998), and 16189 C has also been found in African Americans (Budowle et al. 1999). Therefore, these polymorphisms are not absolutely diagnostic for haplogroups, but since certain ones are present in all or most haplogroup A, B, or C individuals, their presence provides some support for what haplogroups remains most likely belong to.

Haplogroups A, B, C, and D have previously been documented in ancient and contemporary Maya populations (Torroni et al. 1992, Merriwether et al. 1997, González-Oliver et al. 2001). Haplogroups A and B were most prevalent in contemporary and ancient Maya from the Yucatán Peninsula of Mexico. Haplogroups A and B composed 84% and 4% of ancient Maya analyzed by González-Oliver et al. (2001) and 51.9% and 22.2% of contemporary Maya sampled by Torroni et al. (1992). Haplogroups A and B were absent in ancient Maya from Copán, Honduras; rather, haplogroups C and D were most prevalent: 89% and 11%, respectively (Merriwether et al. 1997). The sample size of Merriwether et al. (1997) was small (n = 9), so the high prevalence of haplogroup C is less reliable than the percentage of haplogroup A individuals

from González-Oliver et al. (2001) and Torroni et al. (1992), which had sample sizes of 25 and 27, respectively. The contrast in haplogroup frequencies in ancient Maya could also be due to the different geographical populations sampled (Mexico vs. Honduras). The higher percentage of haplogroup B in modern Maya sampled by Torroni et al. (1992) compared to ancient Maya of roughly the same geographic region could be explained by lack of random sampling. The authors noted that their sample was limited due to the remoteness of the village and relatedness of the inhabitants; most individuals in the village were related at least at the second cousin level. This is problematic because all maternal relatives share the same mtDNA and hence the same mtDNA haplogroup, resulting in haplogroup frequencies that may deviate from those documented in ancient Maya.

Despite this, haplogroup A is predominant among North and Central American Amerinds (Torroni et al. 1993); therefore, haplogroup analysis of the ancient Maya remains in this study focused mainly on haplogroup A. No positive assignments to haplogroups A, B, C, D, or X were made from the ancient remains, although bone 5 is potentially haplogroup A, as nucleotides consistent with both the rCRS and the haplogroup SNP were present at position 663. Note that numerous bones were only tested for the haplogroup A SNP, leaving the possibility that they are haplogroup B, C, D, or X. Another individual in the MSU Forensic Biology Laboratory is currently continuing the haplogroup analysis of these skeletal remains, which should help to resolve whether the mtDNA haplotypes produced originated from the skeletal material.

Another factor that hindered ancient DNA analysis was that reference DNA samples from all individuals who handled the remains were unavailable. Thus, contamination could not be ruled out as the reason that Native American/Asian haplogroups could not be confirmed and that little concordance existed among HV I haplotypes. However, if contamination occurred due to

handling bones or from laboratory personnel, then the same haplotypes would be expected in multiple extracts of a bone, which was not apparent with any of the ancient material. Interestingly, the SoilMaster™ kit generated sequences that were not concordant with other extraction methods, which potentially means that the kit was contaminated. Further, the same control region polymorphisms were present in SoilMaster™ extracts from bones 3, 5, 6, 7, and 9 (Appendix Table A9), a trend that was not apparent with other extraction methods. If the kit was contaminated, the SoilMaster™ reagent blank would have DNA amplification, which did not occur in the current study. Despite this, contamination is still a concern since the soil DNA isolation kits are not designed for human DNA purposes, and the production quality control procedures are likely different that those of kits designed for forensic use. Solutions used in the soil DNA isolation kits were not UV irradiated as it was not known whether this treatment would negatively affect the proprietary ingredients in them, meaning that if DNA did contaminate the solutions, it was not destroyed prior to extraction. To eradicate this problem, any negative effect of UV irradiation on each solution could be tested individually, and pending the results, solutions that are not negatively affected should be UV irradiated before extraction.

Factors besides the ability to extract amplifiable DNA that may influence the choice of extraction method are cost, ease of use, and time. Each SoilMaster™ extraction costs approximately $4, while a Qiagen DNA Investigator extraction is slightly more expensive. A PowerSoil® extraction costs around $5, but with the additional Amicon® column concentration step used in this study, it is closer to $8. Another drawback of the PowerSoil® kit is that the

protocol is not user-friendly and contamination is a concern. At one point, the 2 mL tube is filled almost completely with solution, so if this kit is to be implemented for forensic purposes, tubes that accommodate a larger volume should be used. Furthermore, the silica columns must be manually removed from tubes numerous times, which is not ideal when extracting DNA from forensic samples, especially if contamination-sensitive mtDNA analysis will be conducted. On the other hand, the SoilMaster™ extraction is easy to execute, although there are numerous long incubations and/or centrifugations that create down-time for the laboratory analyst. Despite these drawbacks, samples can be digested and extracted the same day when soil DNA isolation kits are used, which is not true of the standard methods. The Qiagen kit protocol, which is also user-friendly, suggests an overnight incubation for DNA extraction from bone, and an overnight digestion was also used for organic extraction. However, the effect of shorter digestion times on DNA recovery was not tested for either organic or Qiagen extractions, so it is possible that those extractions could be reduced to a single day procedure without adversely affecting DNA yields. Based solely on cost, ease of use, time required for the protocols employed in this research, and DNA yields, the SoilMaster™ kit is the most cost-effective and efficient extraction method among those examined in this study.

CONCLUSION

Based on the research presented here, soil DNA isolation kits can act as effective tools for purifying DNA from modern skeletal remains. These kits produced DNA yields from buried skeletal remains equivalent to standard skeletal DNA extraction techniques without using an overnight incubation step, thus speeding up the process of DNA extraction. However, DNA recovery was significantly lower when silica-based kits were used. Low DNA recovery is a concern that impacts choice of extraction method when minimal amounts of DNA are likely present in evidentiary items, including degraded skeletal remains. Extraction methods also varied in their ability to overcome PCR inhibition from inhibitors associated with skeletal remains. Despite these differences, the extraction methods tested were equally effective when they were implemented to identify a modern decedent through mtDNA analysis. Comparison of the techniques for use in ancient DNA extraction could not be made due to inconsistencies in DNA haplotypes, which were not confirmed to originate from skeletal DNA by haplogroup analysis. However, PowerSoil®, Qiagen, and organic extractions removed PCR inhibitors in ancient bone more effectively than a SoilMaster™ kit. DNA contamination of the soil DNA isolation kits during manufacturing is a concern since they are not intended for human DNA applications, and possible contamination was detected in the SoilMaster™ extracts of ancient skeletal remains. Overall, the techniques in this study that resulted in maximal DNA recovery, minimal PCR inhibition, and successful sequencing of mtDNA from modern skeletal remains were organic and SoilMaster™ extractions.

APPENDIX

Table A1. mtDNA Quantification of PowerSoil® Extracts of Buried Bovine Bones
Bone identifiers are listed in the first column. "1W, 1M, 2M, 4M, and 8M" indicate length of burial in weeks or months, and "1 – 4" denote four replicate femoral segments. Volume of PowerSoil® extract, as well as mg of bone powder, was considered in order to normalize DNA yields. Normalized values are reported as pg of DNA per mg of bone powder.

Bone Identifier	PowerSoil® Quantification (pg/µL)	Extract Volume (µL)	Total DNA Recovered (pg)	Bone Powder Input (mg)	Total DNA Normalized (pg/mg)
1W-1	160.00	34.0	5440.00	95	57.26
1W-2	89.80	30.8	2765.84	87	31.79
1W-3	25.50	33.8	861.90	96	8.98
1W-4	11.20	30.4	340.48	103	3.31
1M-1	2.52	32.6	82.15	104	0.79
1M-2	45.60	33.8	1,541.28	90	17.13
1M-3	65.80	37.4	2,460.92	97	25.37
1M-4	10.60	33.4	354.04	91	3.89
2M-1	3.56	33.0	117.48	92	1.28
2M-2	1.96	37.2	72.91	95	0.77
2M-3	3.02	38.0	114.76	88	1.30
2M-4	17.10	34.6	591.66	100	5.92
4M-1	12.00	29.0	348.00	116	3.00
4M-2	39.60	33.0	1,306.80	103	12.69
4M-3	2.68	31.4	84.15	105	0.80
4M-4	6.60	31.4	207.24	106	1.96
8M-1	10.10	27.6	278.76	98	2.84
8M-2	2.85	30.4	86.64	95	0.91
8M-3	1.86	33.6	62.50	86	0.73
8M-4	0.00	30.2	0.00	84	0.00

Table A2. mtDNA Quantification of SoilMaster™ Extracts of Buried Bovine Bones

Bone identifiers are listed in the first column. "1W, 1M, 2M, 4M, and 8M" indicate length of burial in weeks or months, and "1 – 4" denote four replicate femoral segments. Volume of SoilMaster™ extract, as well as mg of bone powder, was considered in order to normalize DNA yields. Normalized values are reported as pg of DNA per mg of bone powder.

Bone Identifier	SoilMaster™ Quantification (pg/µL)	Extract Volume (µL)	Total DNA Recovered (pg)	Bone Powder Input (mg)	Total DNA Normalized (pg/mg)
1W-1	967.00	23.4	22,627.80	90	251.42
1W-2	1,500.00	24.6	36,900.00	86	429.07
1W-3	119.00	23.2	2,760.80	82	33.67
1W-4	282.00	29.8	8,403.60	82	102.48
1M-1	19.60	25.2	493.92	101	4.89
1M-2	20.40	25.8	526.32	101	5.21
1M-3	108.00	26.0	2,808.00	102	27.53
1M-4	142.00	26.6	3,777.20	84	44.97
2M-1	8.22	25.8	212.08	91	2.33
2M-2	1.90	25.6	48.64	107	0.45
2M-3	0.88	26.2	23.00	109	0.21
2M-4	198.00	24.8	4,910.40	90	54.56
4M-1	16.60	20.8	345.28	97	3.56
4M-2	53.40	27.2	1,452.48	89	16.32
4M-3	3.47	25.0	86.75	112	0.77
4M-4	3.73	22.6	84.30	106	0.80
8M-1	11.90	22.4	266.56	104	2.56
8M-2	18.70	22.4	418.88	99	4.23
8M-3	0.50	22.6	11.21	105	0.11
8M-4	1.71	21.8	37.28	109	0.34

Table A3. mtDNA Quantification of Organic Extracts of Buried Bovine Bones
Bone identifiers are listed in the first column. "1W, 1M, 2M, 4M, and 8M" indicate length of burial in weeks or months, and "1 – 4" denote four replicate femoral segments. Volume of organic extract, as well as mg of bone powder, was considered in order to normalize DNA yields. Normalized values are reported as pg of DNA per mg of bone powder.

Bone Identifier	Organic Quantification (pg/µL)	Extract Volume (µL)	Total DNA Recovered (pg)	Bone Powder Input (mg)	Total DNA Normalized (pg/mg)
1W-1	1,490.00	19.8	29,502.00	103	286.43
1W-2	1,320.00	22.6	29,832.00	85	350.96
1W-3	160.00	22.0	3,520.00	93	37.85
1W-4	448.00	26.2	11,737.60	81	144.91
1M-1	20.30	29.4	596.82	100	5.97
1M-2	144.00	22.8	3,283.20	102	32.19
1M-3	128.00	23.8	3,046.40	101	30.16
1M-4	49.80	26.6	1,324.68	88	15.05
2M-1	16.00	20.4	326.40	97	3.36
2M-2	2.39	22.8	54.49	105	0.52
2M-3	11.90	22.4	266.56	97	2.75
2M-4	138.00	22.4	3,091.20	100	30.91
4M-1	23.20	18.6	431.52	89	4.85
4M-2	625.00	25.2	15,750.00	104	151.44
4M-3	7.37	16.8	123.82	106	1.17
4M-4	9.48	22.4	212.35	93	2.28
8M-1	7.78	20.8	161.82	107	1.51
8M-2	11.00	21.0	231.00	104	2.22
8M-3	3.48	19.4	67.51	98	0.69
8M-4	3.29	21.0	69.09	78	0.89

Table A4. mtDNA Quantification of Qiagen Extracts of Buried Bovine Bones
Bone identifiers are listed in the first column. "1W, 1M, 2M, 4M, and 8M" indicate length of burial in weeks or months, and "1 – 4" denote four replicate femoral segments. Volume of Qiagen extract, as well as mg of bone powder, was considered in order to normalize DNA yields. Normalized values are reported as pg of DNA per mg of bone powder.

Bone Identifier	Qiagen Quantification (pg/µL)	Extract Volume (µL)	Total DNA Recovered (pg)	Bone Powder Input (mg)	Total DNA Normalized (pg/mg)
1W-1	145.00	56.4	8,178.00	91	89.87
1W-2	114.00	58.4	6,657.60	85	78.32
1W-3	4.84	58.0	280.72	103	2.73
1W-4	20.20	58.2	1,175.64	82	14.34
1M-1	0.00	59.0	0.00	102	0.00
1M-2	1.17	57.8	67.63	101	0.67
1M-3	4.52	58.6	264.87	102	2.60
1M-4	19.00	57.4	1,090.60	86	12.68
2M-1	0.00	57.0	0.00	104	0.00
2M-2	0.73	56.0	40.94	100	0.41
2M-3	5.23	58.0	303.34	85	3.57
2M-4	32.90	59.2	1,947.68	100	19.48
4M-1	3.77	56.5	213.01	88	2.42
4M-2	16.80	56.0	940.80	102	9.22
4M-3	1.59	54.4	86.50	106	0.82
4M-4	3.81	53.0	201.93	94	2.15
8M-1	1.83	56.2	102.85	98	1.05
8M-2	2.60	54.6	141.96	102	1.39
8M-3	1.01	54.8	55.35	98	0.56
8M-4	0.00	56.0	0.00	81	0.00

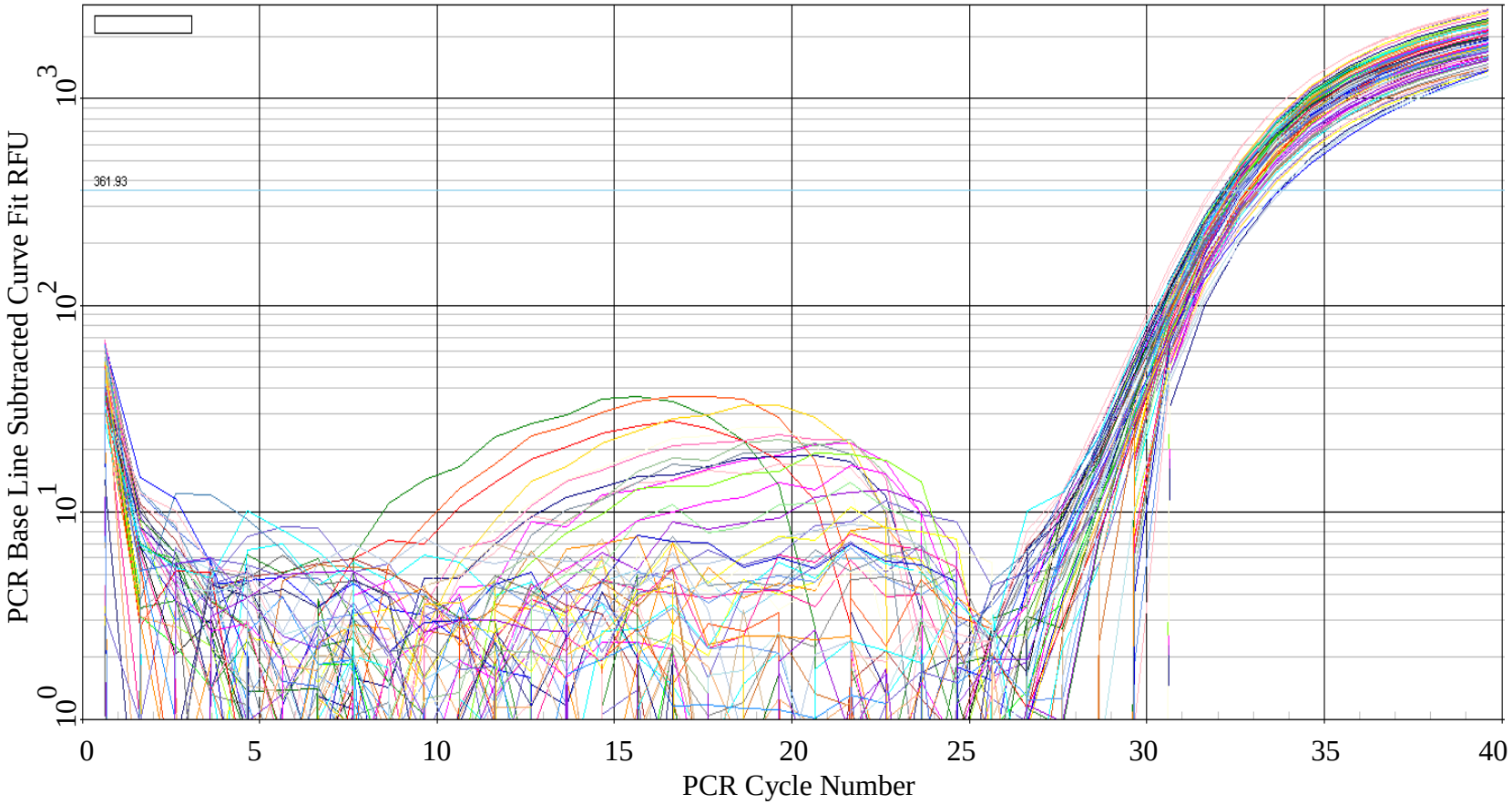


Figure A1. mtDNA qPCR IPC Curves of 1W, 1M, 2M, and 4M Bovine Bones

The x-axis is PCR cycle number, and y-axis is a logarithmic scale of RFU. The IPC amplified in all samples at a similar C_t value, indicating that no PCR inhibition occurred.

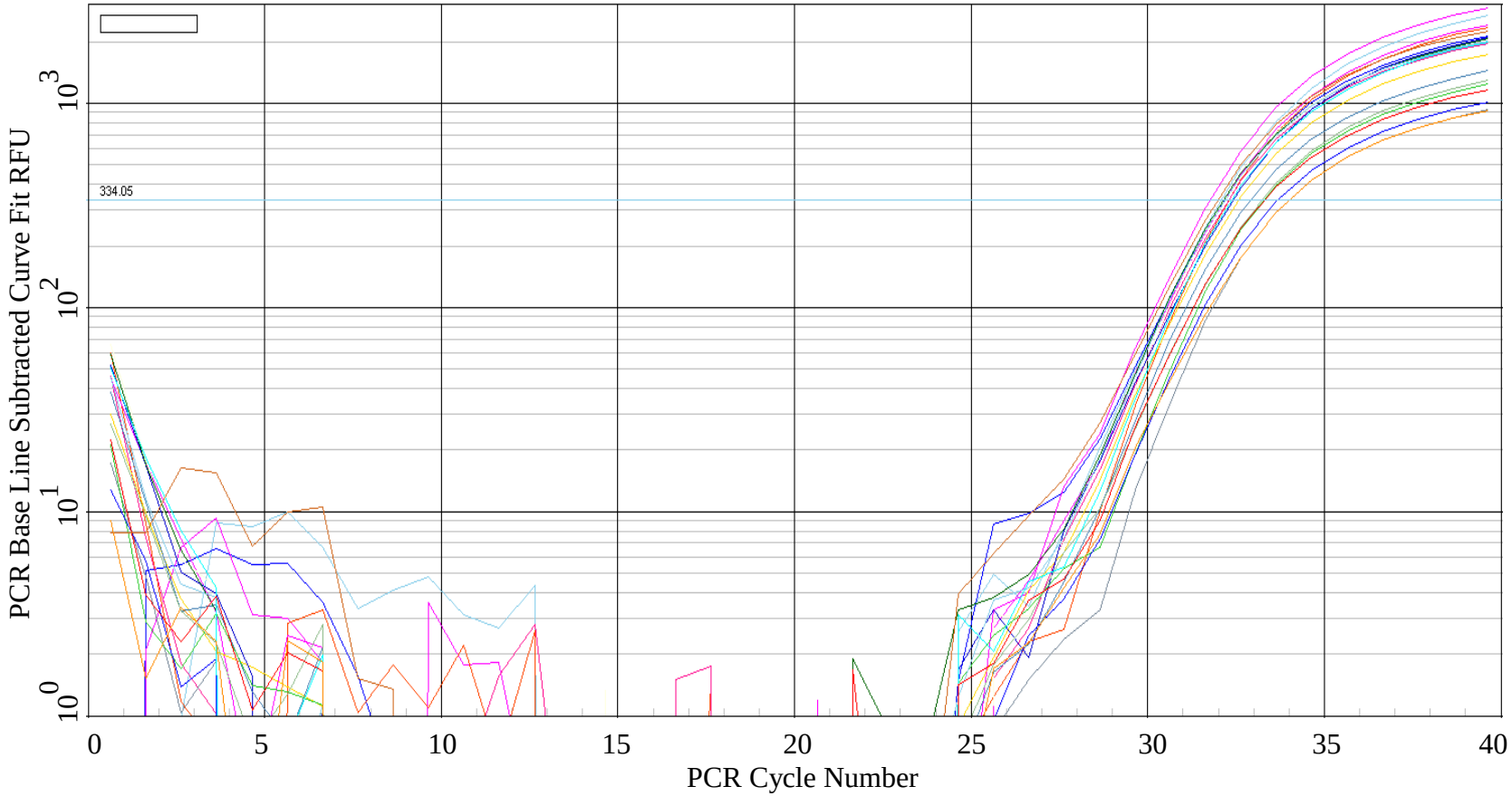


Figure A2. mtDNA qPCR IPC Curves of 8M Bovine Bones

The x-axis is PCR cycle number, and y-axis is a logarithmic scale of RFU. The IPC amplified in all samples at a similar C_t value, indicating that no PCR inhibition occurred.

Table A5. Nuclear Quantification of PowerSoil® Extracts of Buried Bovine Bones
Bone identifiers are listed in the first column. "1W, 1M, 2M, 4M, and 8M" indicate length of burial in weeks or months, and "1 – 4" denote four replicate femoral segments. Volume of PowerSoil® extract, as well as mg of bone powder, was considered in order to normalize DNA yields. Normalized values are reported as pg of DNA per mg of bone powder.

Bone Identifier	PowerSoil® Quantification (pg/µL)	Extract Volume (µL)	Total DNA Recovered (pg)	Bone Powder Input (mg)	Total DNA Normalized (pg/mg)
1W-1	1,350.00	31.0	41,850.00	95	440.53
1W-2	1,200.00	29.4	35,280.00	87	405.52
1W-3	82.70	30.4	2,514.08	96	26.19
1W-4	101.00	25.2	2,545.20	103	24.71
1M-1	35.10	31.2	1,095.1	104	10.53
1M-2	111.00	31.6	3,507.6	90	38.97
1M-3	23.00	36.2	832.6	97	8.58
1M-4	1.17	34.8	40.72	91	0.45
2M-1	7.50	31.8	238.50	92	2.59
2M-2	19.60	36.4	713.44	95	7.51
2M-3	39.00	37.2	1,450.80	88	16.49
2M-4	51.70	31.0	1,602.70	100	16.03
4M-1	67.90	29.0	1,969.10	116	16.98
4M-2	55.70	31.6	1,760.12	103	17.09
4M-3	22.50	30.0	675.00	105	6.43
4M-4	2.76	29.8	82.25	106	0.78
8M-1	0.00	27.6	0.00	98	0.00
8M-2	0.00	30.4	0.00	95	0.00
8M-3	0.00	33.6	0.00	86	0.00
8M-4	0.00	30.2	0.00	84	0.00

Table A6. Nuclear DNA Quantification of SoilMaster™ Extracts of Buried Bovine Bones
Bone identifiers are listed in the first column. "1W, 1M, 2M, 4M, and 8M" indicate length of burial in weeks or months, and "1 – 4" denote four replicate femoral segments. Volume of SoilMaster™ extract, as well as mg of bone powder, was considered in order to normalize DNA yields. Normalized values are reported as pg of DNA per mg of bone powder.

Bone Identifier	SoilMaster™ Quantification (pg/µL)	Extract Volume (µL)	Total DNA Recovered (pg)	Bone Powder Input (mg)	Total DNA Normalized (pg/mg)
1W-1	15,400.00	22.6	348,040.00	90	3,867.11
1W-2	74,300.00	22.8	1,694,040.00	86	19,698.14
1W-3	2,110.00	18.0	37,980.00	82	463.17
1W-4	6,040.00	23.8	143,752.00	82	1,753.07
1M-1	80.40	21.6	1,736.64	101	17.19
1M-2	74.20	24.0	1,780.80	101	17.63
1M-3	75.00	23.8	1,785.00	102	17.50
1M-4	777.00	23.2	18,026.40	84	214.60
2M-1	71.20	22.4	1,594.88	91	17.53
2M-2	31.20	22.8	711.36	107	6.65
2M-3	84.90	22.4	1,901.76	109	17.45
2M-4	892.00	16.8	14,985.60	90	166.51
4M-1	165.00	20.2	3,333.00	97	34.36
4M-2	180.00	20.6	3,708.00	89	41.66
4M-3	34.40	19.6	674.24	112	6.02
4M-4	67.80	20.2	1,369.56	106	12.92
8M-1	0.00	22.4	0.00	104	0.00
8M-2	39.90	22.4	893.76	99	9.03
8M-3	0.00	22.6	0.00	105	0.00
8M-4	0.00	21.8	0.00	109	0.00

Table A7. Nuclear DNA Quantification of Organic Extracts of Buried Bovine Bones
Bone identifiers are listed in the first column. "1W, 1M, 2M, 4M, and 8M" indicate length of burial in weeks or months, and "1 – 4" denote four replicate femoral segments. Volume of organic extract, as well as mg of bone powder, was considered in order to normalize DNA yields. Normalized values are reported as pg of DNA per mg of bone powder.

Bone Identifier	Organic Quantification (pg/µL)	Extract Volume (µL)	Total DNA Recovered (pg)	Bone Powder Input (mg)	Total DNA Normalized (pg/mg)
1W-1	61,900.00	17.0	1,052,300.00	103	10,216.50
1W-2	104,000.00	18.6	1,934,400.00	85	22,757.65
1W-3	7,110.00	21.4	152,154.00	93	1,636.06
1W-4	14,800.00	19.0	281,200.00	81	3,471.60
1M-1	48.20	17.2	829.04	100	8.29
1M-2	448.00	18.0	8,064.00	102	79.06
1M-3	54.00	20.6	1,112.40	101	11.01
1M-4	837.00	23.6	19,753.20	88	224.47
2M-1	18.90	18.4	347.76	97	3.59
2M-2	9.17	19.4	177.90	105	1.69
2M-3	8.48	22.2	188.26	97	1.94
2M-4	1,410.00	18.8	26,508.00	100	265.08
4M-1	6.07	16.6	100.76	89	1.13
4M-2	1,520.00	22.8	34,656.00	104	333.23
4M-3	6.03	18.8	113.36	106	1.07
4M-4	2.75	22.2	61.05	93	0.66
8M-1	0.00	20.8	0.00	107	0.00
8M-2	0.00	21.0	0.00	104	0.00
8M-3	0.00	19.4	0.00	98	0.00
8M-4	0.00	21.0	0.00	78	0.00

Table A8. Nuclear DNA Quantification of Qiagen Extracts of Buried Bovine Bones
Bone identifiers are listed in the first column. "1W, 1M, 2M, 4M, and 8M" indicate length of burial in weeks or months, and "1 – 4" denote four replicate femoral segments. Volume of Qiagen extract, as well as mg of bone powder, was considered in order to normalize DNA yields. Normalized values are reported as pg of DNA per mg of bone powder.

Bone Identifier	Qiagen Quantification (pg/µL)	Extract Volume (µL)	Total DNA Recovered (pg)	Bone Powder Input (mg)	Total DNA Normalized (pg/mg)
1W-1	1,150.00	50.4	57,960.00	91	636.92
1W-2	898.00	54.6	49,030.80	85	576.83
1W-3	79.70	55.6	4,431.32	103	43.02
1W-4	230.00	53.8	12,374.00	82	150.90
1M-1	39.60	56.0	2,217.60	102	21.74
1M-2	75.90	52.4	3,977.16	101	39.38
1M-3	0.00	54.2	0.00	102	0.00
1M-4	6.92	54.2	375.06	86	4.36
2M-1	12.30	53.0	651.90	104	6.27
2M-2	78.10	54.0	4,217.40	100	42.17
2M-3	10.10	57.2	577.72	85	6.80
2M-4	171.00	50.2	8,584.20	100	85.84
4M-1	20.80	52.2	1,085.76	88	12.34
4M-2	108.00	54.0	5,832.00	102	57.18
4M-3	9.20	50.4	463.68	106	4.37
4M-4	2.73	50.0	136.50	94	1.45
8M-1	0.00	56.2	0.00	98	0.00
8M-2	0.00	54.6	0.00	102	0.00
8M-3	0.00	54.8	0.00	98	0.00
8M-4	0.00	56.0	0.00	81	0.00

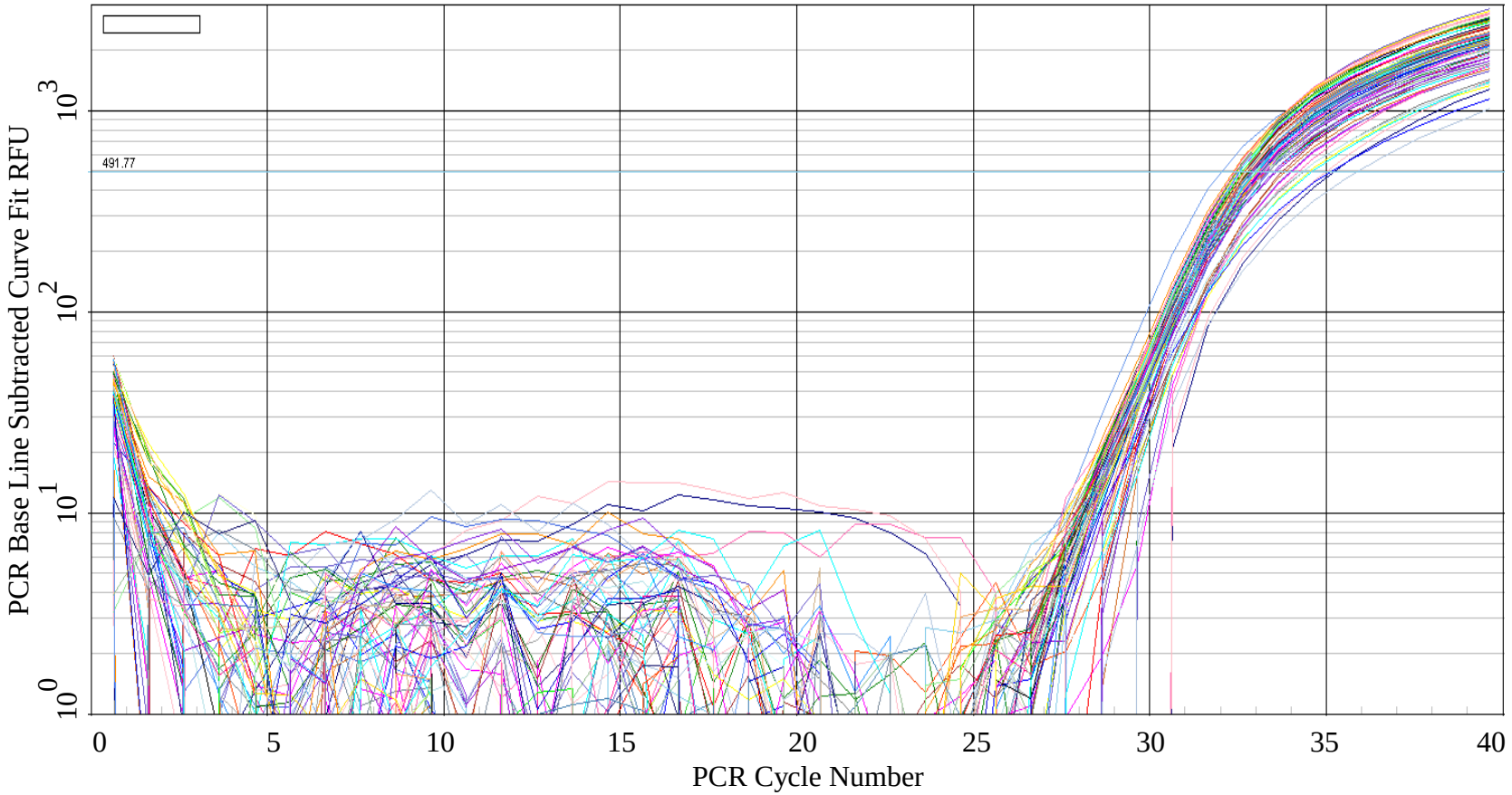


Figure A3. Nuclear qPCR IPC Curves of 1W, 1M, 2M, and 4M Bovine Bones

The x-axis is PCR cycle number, and y-axis is a logarithmic scale of RFU. The IPC amplified in all samples at a similar C_t value, indicating that no PCR inhibition occurred.

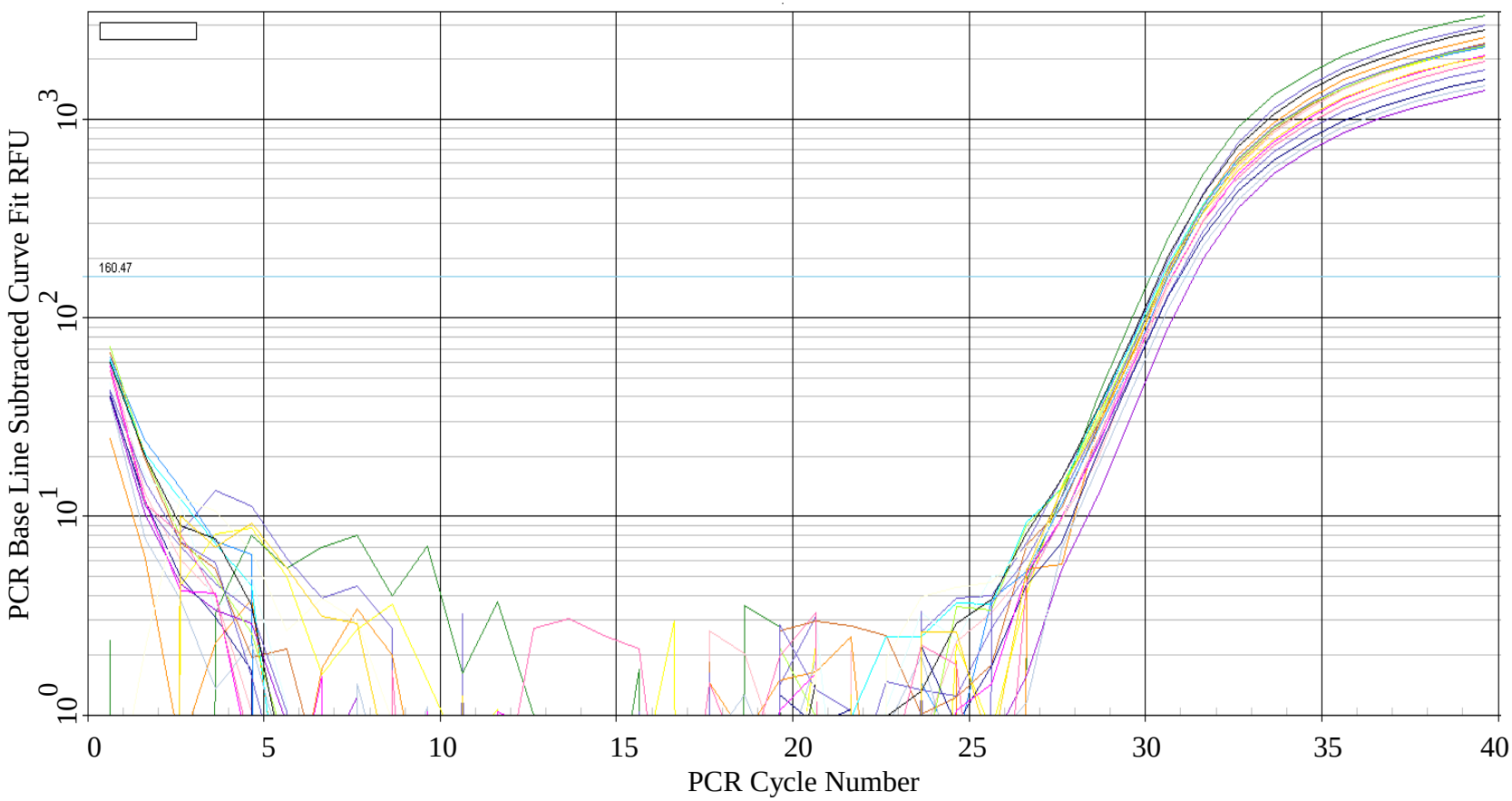


Figure A4. Nuclear qPCR IPC Curves of 8M Bovine Bones

The x-axis is PCR cycle number, and y-axis is a logarithmic scale of RFU. The IPC amplified in all samples at a similar C_t value, indicating that no PCR inhibition occurred.

Table A9. HV I Polymorphisms from Human Skeletal Remains
Shaded cells represent sequences that were concordant among extraction methods within a bone. "n/a" represents no amplification or sequence obtained that was of poor quality. ".1" and ".2" denote nucleotide insertions, while a position followed by "del" was a deletion. Sequences with no differences from the rCRS were noted by "rCRS." "*" designated a polymorphism that fell within the primer sequence.

	Bone 1	Bone 2	Bone 3	Bone 4	Bone 5	Bone 6
PowerSoil®	16154 C 16176 C/T 16183 C 16189 T/C 16193.1 C 16234 T	16145 A 16176 G 16223 T	n/a	n/a	16126 C	16126 T/C
SoilMaster™	n/a	n/a	16189 C 16290 T	rCRS	16189 C 16193 del 16290 T	16070 A/C* 16095 C/T 16149 A/C 16175 A/C 16189 C 16265 A/C 16290 T 16293 A/C
Organic	16145 A 16176 G 16223 T	16126 C	16086 C 16148 T 16223 T 16259 T 16278 T	n/a	16164 A/G 16179 C/T 16248 C/T	n/a
Qiagen	rCRS	16248 T	16189 C 16193.1 C 16193.2 C	n/a	16095 C/T 16126 C	16126 C

Table A9 (cont'd)

	Bone 7	Bone 8	Bone 9	Bone 10	Bone 11	Bone 12
PowerSoil®	rCRS	n/a	n/a	16126 C	16189 C 16193.1 C 16193.2 C	n/a
SoilMaster™	16189 C 16290 T	n/a	16095 C/T 16103 A/T 16104 C/T 16189 C 16290 T	16126 C 16132 A/G 16168 T 16193 T 16199 T/G 16278 T	16126 T/C 16256 C/T	n/a
Organic	16189 C 16193.1 C 16225 C/A 16230 A/T	n/a	n/a	16126 C	n/a	n/a
Qiagen	n/a	n/a	n/a	n/a	16063 T/G* 16078 A/G 16086 C 16098 A/G 16129 A 16187 T 16189 C 16199 T/G 16223 T 16241 A/G 16278 T 16284 G 16293 G 16294 T	16189 C 16193.1 C

Table A9 (cont'd)

	Bone 13	Bone 14	Bone 15
PowerSoil®	rCRS	16134 T 16234 T	16134 T 16234 T
SoilMaster™	16223 T 16255 G/A	16134 T 16234 T	16134 T 16234 T
Organic	rCRS	16134 T 16234 T	16134 T 16234 T
Qiagen	16189 C 16290 T	16134 T 16234 T	16134 T 16234 T

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