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Quantification of mtDNA in Aged Skeletal Material

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QUANTIFICATION OF MTDNA IN AGED SKELETAL MATERIAL

By

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ABSTRACT

QUANTIFICATION OF MTDNA IN AGED SKELETAL MATERIAL

By

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There are times in the course of death investigations when a forensic scientist may need to analyze DNA extracted from skeletal remains. The study presented here was designed to see if the type of bone, or the level of degradation of a skeleton or bone, is related to the quantity of DNA that can be obtained from it. Knowing the best bone to use for DNA analysis, or having the ability to visually examine a bone and reliably predict if it contains enough DNA for successful analysis, would be beneficial to the forensic scientist. The skeletal material analyzed in this study came from a Pittsburgh cemetery that fell into disuse in the mid-1800s. Real time PCR was utilized to assess the mitochondrial DNA quantity contained in the skeletal material. Through this work it was determined that neither bone type nor the amount of visual degradation were significantly related to the quantity of mtDNA contained within bone.

A second goal of this study was to examine the effect PCR inhibition had on real time PCR-based estimates of DNA quantities in the bone samples. The findings indicate that for samples known to have some level of PCR inhibition, the percentage of inhibition remains relatively constant, independent of the concentration of DNA in a sample. The addition of bovine serum albumin did alleviate some inhibition, and was more effective at higher DNA concentrations. The results point to at least two types of inhibition existing, and mean that more than one strategy may be required for overcoming PCR inhibition.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

Goals of this study

It is not uncommon that bones are the sole remains recovered from a scene, in death investigations. The skeletal material may be at various stages of the degradative process, or differing types of bones may be found. The forensic scientist must decide from which bones to attempt DNA extractions, not knowing how the type of bone or its level of degradation, may affect results. The ability to predict the success of forensic DNA analysis given a bone type and its weathering condition would be of obvious use to crime laboratories.

Aged bone samples tend to harbor DNA that is at least partially degraded (Haglund, 1997), and DNA is lost over time. Environmental factors such as soil pH, moisture levels, attack of microorganisms, as well as the time the corpse had been in/on the ground may or may not have any direct effect on the DNA in the bone. Additionally, the amount of DNA that can be extracted from aged bones may or may not be related to how degraded they appear. It seems intuitive that if a bone is in poor condition visually, typable DNA is less likely to be recovered. However, no conclusive research has been done to investigate if the level of bone weathering bears a relationship to the quantity of DNA that can be extracted from it. Additionally, the structure and/or surface area of the bone itself may have an impact on the DNA quantity that can be obtained.

The amount of DNA contained within bones may also be related to the age of the individual at the time of their death. Bone changes over the course of a person's lifetime; when very young, bones are mostly cartilage, which could potentially impact the amount of DNA that can be extracted. With advancing age, bones themselves have already

started to degrade, a process which may have an effect on bone DNA (Micozzi, 1991). Additionally, skeletal density and other characteristics differ between the sexes; these factors too may be important.

The study described here had two main goals: to determine whether aged skeletal material from one or more bone types retains a greater quantity of DNA than others, and to determine whether there is a relationship between how degraded a bone appears and the amount of DNA that can be extracted from it. Two additional factors were also examined, the potential influences of the age of a person at death, as well as the sex of the individual. A tertiary goal was to examine the effect that inhibition of Polymerase Chain Reaction (PCR) has on the ability to obtain correct results when analyzing bone of forensic interest (described in more detail below).

Bone samples and skeletal degradation

Before DNA quantity can be compared to a bone's degradative state, it is necessary to classify bones in a consistent way. The bone weathering stage used in this study was a modification of the stages created by Behrensmeyer (1978). This scale has six stages, from zero to five, with zero being the least weathered and five being the most weathered. With this approach, the skeleton as a whole is placed into one of the six stages.

The skeletal material used in this study was retrieved from graves discovered in 1987 by construction workers who were in the process of building a highway in Pittsburgh, Pennsylvania. Subsequent investigation of the site determined that it had previously been the location of the cemetery of Voegtly Evangelical Lutheran Church.

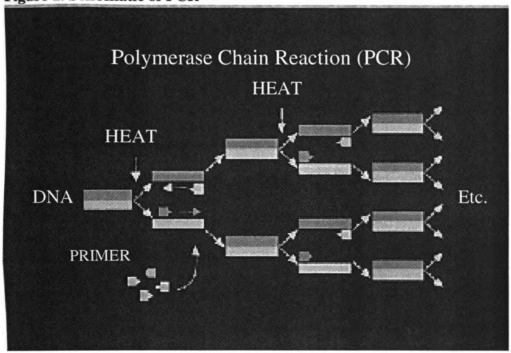
The church had buried its members in that site from 1833 to 1861, when a new cemetery was built. Only the deceased founding members of the church were moved to the new location, and the original cemetery fell into disuse. Investigation revealed 724 individuals buried at the cemetery. These samples were ideal in that the remains were all subjected to the same environment for approximately the same amount of time. These features eliminated the confounding factors described above. The history relating to the origin of the skeletal samples comes from *Human remains from Voegtly Cemetery, Pittsburgh, Pennsylvania* (Ubelaker *et al.*, 2003). The skeletal material, being unidentifiable, was donated by the Pennsylvania Department of Transportation to the Smithsonian Institution. Dr. Doug Ubelaker and co-workers from the Smithsonian classified all skeletons into one of the six weathering stages, referred to forthwith as "skeletal weathering stages." When the condition of the skeletal material permitted, they made estimations of sex and age. Smaller segments of bone from these specimens were collected for genetic study by members of the forensic biology section of the Forensic Science Program at Michigan State University. Sections of rib, femur, and pelvis were generally sampled.

While working with the skeletal material, a graduate student at Michigan State University noticed that not every type of bone sampled from an individual was degraded to the same extent. Therefore, she formulated her own weathering stages (from one to four) and categorized each individual bone into one of the four stages (Misner, 2004). These stages will forthwith be referred to as "bone weathering stages."

PCR basics

The quantity of DNA that remains in aged skeletal material is miniscule, and it would be impossible to analyze if it could not be duplicated (amplified). The standard method of DNA amplification is PCR, which was first described in 1985 (Saiki *et al.*), in which the DNA sample goes through multiple replication cycles (Figure 1). In every cycle, each segment of double stranded DNA is heated to denature it, and then cooled to a temperature where the primers (small segments of DNA that are complimentary to the boundary of the region to be amplified) can bind. After the primers anneal, two new strands of DNA are created by a DNA polymerase (an enzyme that replicates DNA). After every cycle the amount of target DNA is doubled, allowing for a great yield of DNA from a minute starting amount. The resultant DNA produced is commonly referred to as the PCR product, or an amplicon. It should be noted here that due to limiting reagents in the reaction, the quantity of DNA produced in PCR will eventually plateau. Therefore, PCR itself is not quantitative, and the amplicon quantity produced through PCR cannot be used as an indicator of the starting amount of DNA in a sample.

Figure 1. Schematic of PCR



The steps required for PCR amplification. First, heat causes the strand of DNA to denature. In the second step, heat is reduced and the primers anneal. Then the polymerase creates the new strands. (Figure taken from Powledge, 2004)

PCR Inhibition

A potential problem in analyzing forensic samples is the presence of PCR inhibitors that affect the DNA replication process (Butler, 2001) and can result in a lowered yield of DNA, or potentially no DNA production. The type of inhibition that affects the sample generally stems from the sample itself, and the environment to which it was subjected. Wilson (1997) proposed three major ways in which inhibitors work: disruption in the process of breaking open the cell and isolating the DNA, hindering the activity of the DNA polymerase used in PCR, or interference by degrading nucleic acids. Common inhibitors include components of body fluids (e.g., hemoglobin, urea, heparin), food constituents (e.g., glycogen, fats, calcium), environmental compounds (e.g., phenolic compounds, humic acids, and heavy metals), bacteria, and non-target DNA

(Wilson, 1997). The inhibition may be alleviated depending on the type of inhibitor present, but often the type is not known. If that is the case, common approaches are to dilute the sample (thus diluting the inhibitor), purify the DNA extensively to remove inhibitors, or adding a compound with a higher affinity for the inhibitor than the component it inhibits (Wilson, 1997). Bovine serum albumin (BSA) is one such compound.

Mitochondrial DNA background

Mitochondria are cytoplasmic organelles that provide energy for the cell. They contain their own circular DNA (mtDNA) that is about 16,569 base pairs in length. Often, with degraded samples, insufficient nuclear DNA remains for analysis. MtDNA seems to survive the degradation process better than nuclear DNA, potentially due to superior strength of the mitochondrial membrane (as compared to the nuclear membrane). Therefore, mtDNA is often used in the analysis of ancient bone samples (Gilbert *et al.*, 2003). Additionally, there can be thousands of mitochondria in each cell, so each cell can have thousands of mtDNA copies. Due to their role in energy production, properly functioning mitochondria are essential to life function, and thus their proteins are highly conserved, and most of the mtDNA sequence does not differ among individuals. The emphasis of human genetic analysis of ancient samples resides in a non-coding control region (Gilbert *et al.*, 2003). This section of DNA contains two regions with high variability among individuals—hypervariable region I and II (HVI, and HVII).

Methods of DNA quantification

Forensic laboratories employ multiple methods of DNA quantification, including optical density measurements, use of a slot blot, or real time PCR. DNA quantity can be estimated spectrophotometrically by measuring the ultraviolet light absorbance of the DNA at a wavelength of 260 nanometers. There are two problems with this approach however: forensic samples often do not contain enough DNA to obtain an accurate measurement, and the amount of contaminating DNA (e.g. bacterial) is included in the measurement as it cannot be differentiated from the DNA of interest.

The QuantiBlot® Human DNA Quantification Kit (Perkin Elmer, 2004) is a common slot blot method used in forensic science. The sample DNA is captured on a nylon membrane, and then a 40 base pair (bp) primate specific DNA probe is added. When the probe finds its complimentary sequence on the sample DNA, a chemiluminescent or colorimetric signal is produced. The signal intensity emitted by the sample is compared to the signal intensity produced by serial dilutions of known concentrations of DNA to give an estimate of quantity (Butler, 2001). Slot blots are widely used in forensic science, but have three main disadvantages: they are labor intensive, time consuming, and subjective (Richard *et al.*, 2003). Because quantification is determined by visually comparing band intensities with standards, it allows for different interpretations among researchers. In addition, quantification is performed before an amplification step, so no signal may be derived, or a substantial portion of the sample may be used in trying to generate one. This leaves the possibility that after quantification there will not be enough DNA remaining for analysis. Another potential

drawback in using QuantiBlot® is that it only quantifies nuclear DNA. Therefore it is not the best option for estimating the amount of mitochondrial DNA in a sample.

Real time PCR (also called quantitative PCR or QPCR) is a specific type of PCR that allows for detection of amplicons as they are produced. What is actually detected is not the DNA strand, but a fluorescent dye that is associated with the DNA. The main advantage of real-time PCR is that minute quantities of DNA may be analyzed. Additionally, multiple samples can be analyzed simultaneously (see below for details on real time PCR).

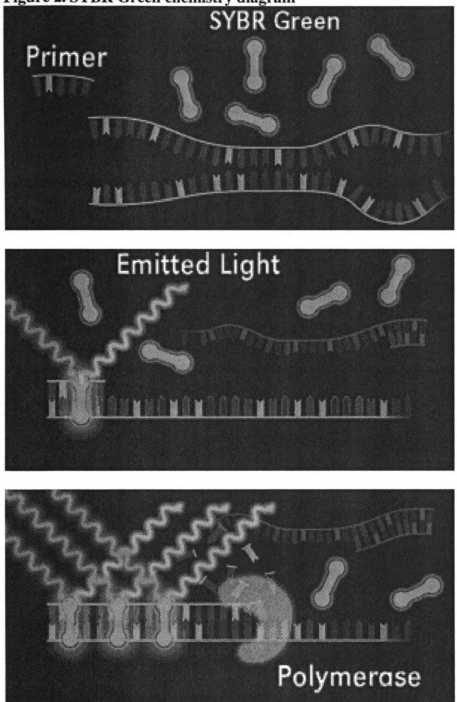
Accurate PCR quantification is complicated by the presence of inhibition. This is because the control DNA that is used for standards is free of inhibiting contaminants, while the sample DNA is not. Therefore, quantification of the sample using real time PCR may give an underestimated value. It is of scientific interest to know how much of an underestimate is occurring.

Real time PCR detection methods

In real time PCR, the detection device measures the fluorescent emission produced by the association of DNA with a dye. The more DNA produced, the greater the signal intensity emitted. The quantity of DNA in the sample can then be correlated with the amount of fluorescence. There are two main tools for detecting the PCR amplification product: DNA binding agents and fluorescent probes. DNA binding agents are fluorescent dyes that intercalate into double-stranded DNA, and are used for non-specific amplification detection. The most commonly used dye is SYBR Green which exhibits little fluorescence when free in solution, but produces a strong fluorescent signal

when it is bound to double-stranded DNA (Dorak, 2004; see Figure 2). The advantage of using a dye is that it is much cheaper than using a probe. It can be used to quantify all double stranded DNA without knowledge of sequence. The main disadvantage is it quantifies non-specific amplicons in addition to the target sequence (Dorak, 2004).

Figure 2. SYBR Green chemistry diagram

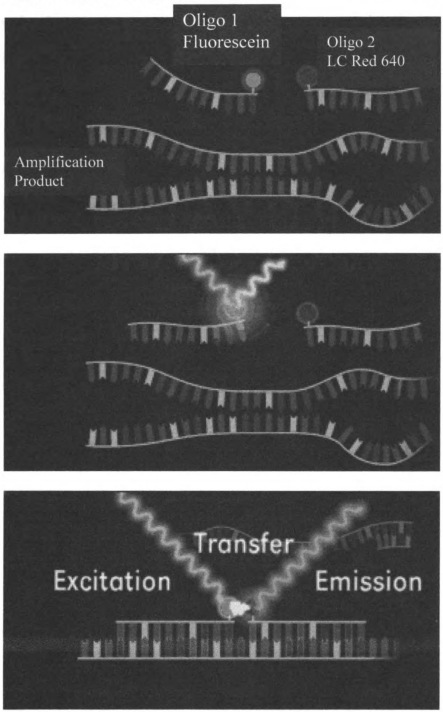


A representation of the action of SYBR Green when in contact with double-stranded DNA. The DNA is separating and the SYBR Green is free in solution. Next, the primer binds to the DNA, and SYBR Green intercalates where the DNA is double stranded. Then, SYBR green intercalates after the polymerase extends the DNA strand. (Figure taken from LightCycler Principles, 2004)

The second method for quantification is the use of a fluorescent probe. Scorpion probes, Molecular Beacons, Roche Hybridization probes, and TaqMan® probes are commonly used (Dyes and Fluorescence Detection Chemistry in qPCR, 2004). Probes are single stranded stretches of DNA that anneal to the target DNA between the primers sites. These probes all are sequence specific and rely on fluorescence resonance energy transfer (FRET). FRET works on the principle that when a donor dye molecule (also referred to as the anchor, or fluorophore) is excited by a laser and it is in close proximity to an acceptor dye molecule (also referred to as the reporter or quencher), the donor will transfer energy to the acceptor (Figure 3). There are two ways that FRET can be utilized for use in real time PCR. The amount of fluorescent light emitted by the acceptor when it is in close contact with the donor can be measured. This is the approach taken by Roche Hybridization probes. Another option is to measure the amount of fluorescence emitted by the donor when it is not in close proximity to the acceptor, and thus not quenched by it. This is the method used by Scorpion probes, Molecular Beacons, and TaqMan® probes. The real time instrument (described in more detail below) is set to measure the light emitted at a particular wavelength. Therefore, the dyes chosen as the donor and acceptor must emit light at different wavelengths.

Use of Roche Hybridization probes requires two probes that bind next to each other at the target site on the amplicon. One probe is the anchor and is labeled with a dye on its 3′ end (in Figure 3 this is depicted as the right end); the other probe is the reporter and is labeled on its 5′ end (the left end). When the two dyes are bound, FRET occurs and the fluorescent light emitted by the acceptor can be measured. Figure 3 is a representation of the described mode of action of Roche Hybridization probes.

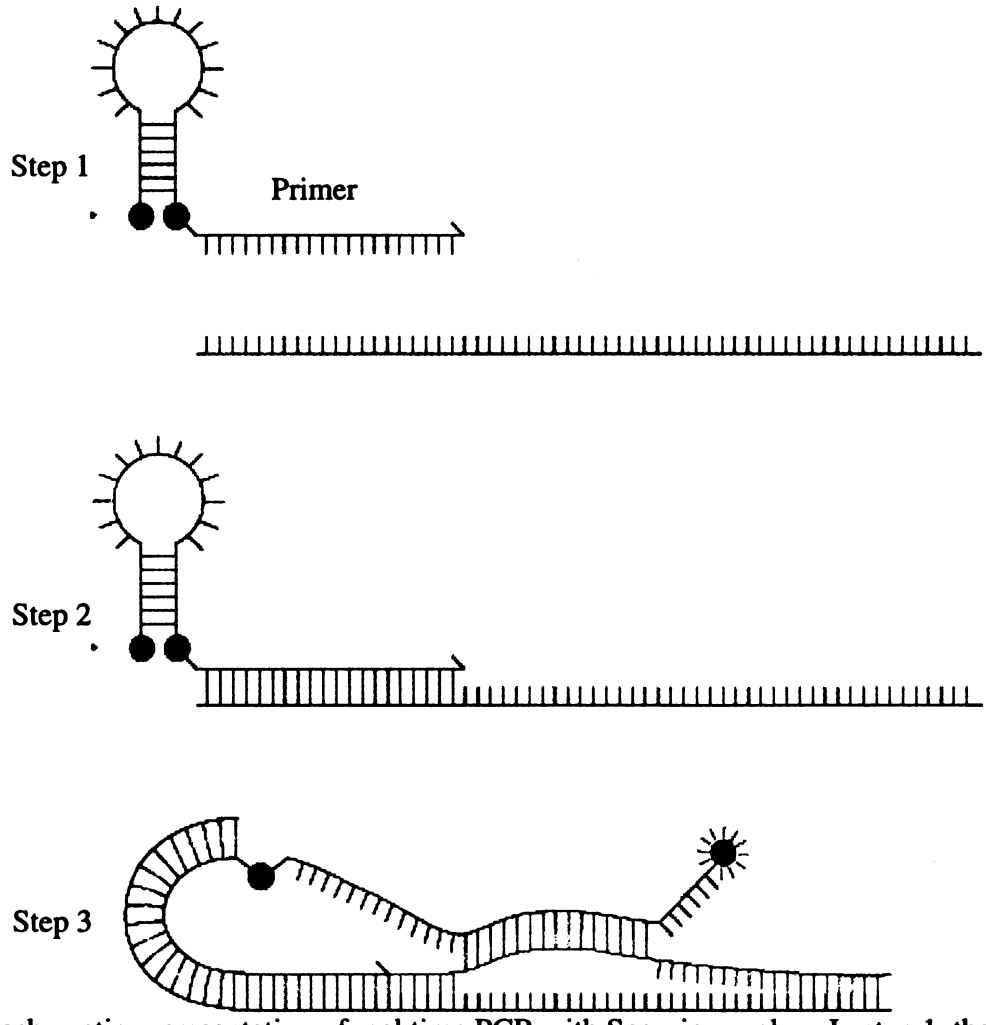
Figure 3. Hybridization Probe diagram



A diagram of the action of Hybridization probes during PCR. In the figure, Fluorescein is the anchor, and LC Red 640 is the reporter. When the two are in close contact, the Fluorescein transfers energy to the LC Red 640. The emission of LC Red 640 is detected. (Figure taken from LightCycler Principles, 2004)

Scorpion probes form a hairpin-loop structure and have a fluorophore on the 5′ end and a quencher on the 3′ end. The 5′ end is connected to the PCR primer. The 3′ end is complimentary to a section of the DNA target sequence. Once the polymerase extends the DNA, the probe hybridizes to the sequence and the hairpin opens allowing measurement of the light emitted by the fluorophore (Dorak 2004). Figure 4 is a drawing that shows the mechanism of action of Scorpion Probes.

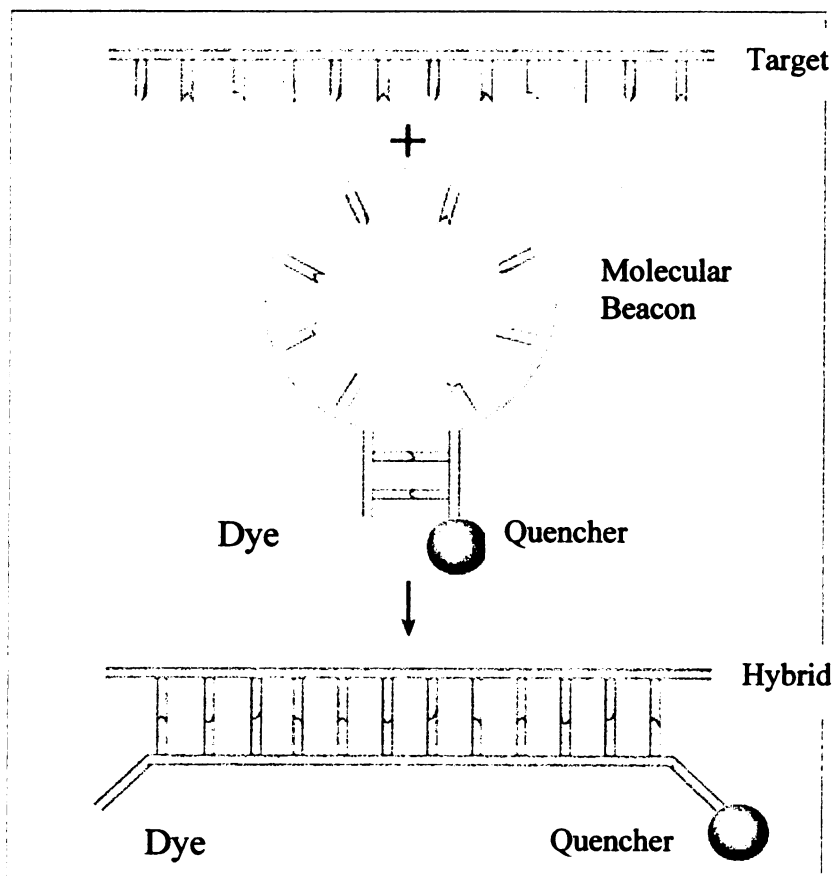
Figure 4. Scorpion probe diagram



A schematic representation of real time PCR with Scorpion probes. In step 1, the DNA is denatured. In step 2, the primer anneals to the single stranded DNA. Step 3 shows as the DNA is extended the probe can hybridize to its complementary sequence on the newly formed strand. This separates the fluorophore and the quencher. (Figure taken from Molecular Probes, 2002)

Molecular Beacons contain a donor dye on one end, a quencher on the other end, and the DNA complimentary to the target sequence in the stem (Public Health Research Institute, 2004). The probe forms a stem-loop structure when free in solution, bringing the donor dye and the quencher in close proximity (Figure 5). During the annealing phase of PCR, the beacon encounters its DNA target sequence and binds to it. When the probe binds, FRET no longer occurs and the donor dye fluoresces. Figure 5 is a drawing of the action of a Molecular Beacon when the target sequence is encountered.

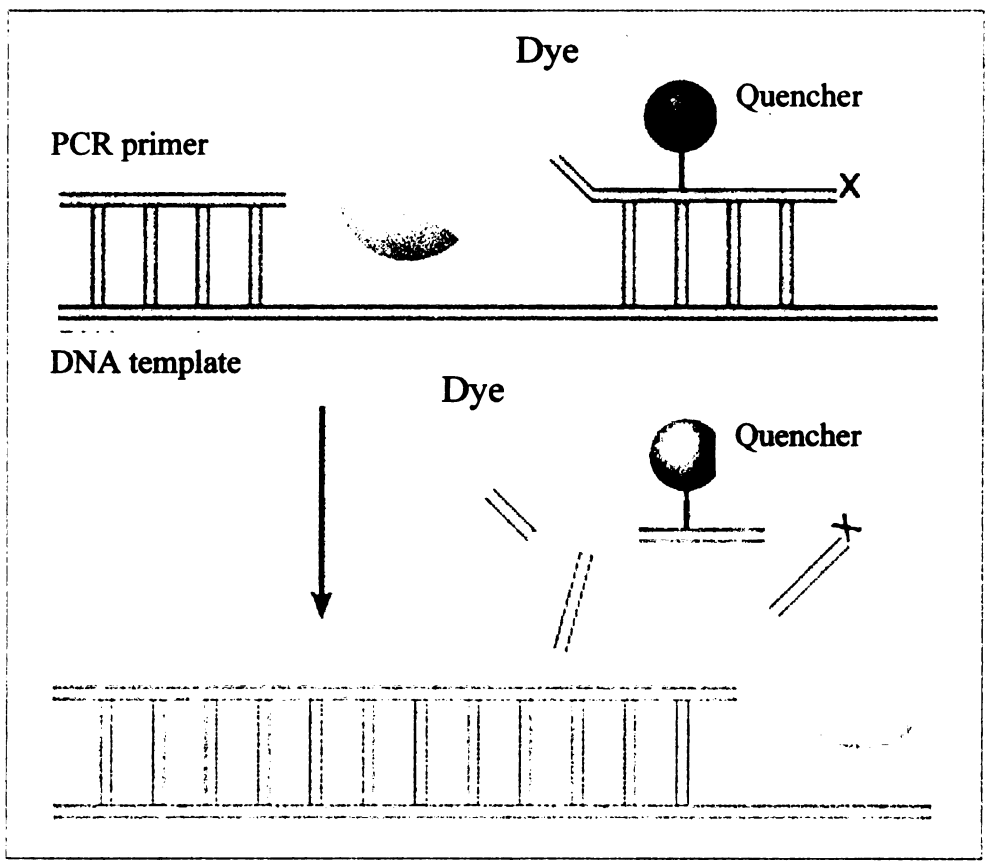
Figure 5. Molecular Beacons diagram



An artist's rendering of a Molecular Beacon. (Figure taken from Dyes and Fluorescence Detection Chemistry in qPCR, 2004) The beacon binds to its complementary DNA sequence, and the donor dye (no longer near the quencher) fluoresces.

TaqMan® probes also bind between the primer sites. The probe contains a fluorescent reporter dye on the 5′ end, and a quencher dye on the 3′ end. If the fluorophore is excited and is in close proximity to the quencher, the fluorescent dye will transfer energy to the quenching dye and fluorescence will not be detected. While the DNA is being replicated during PCR, the 5′ nuclease activity of DNA polymerase cleaves the probe (Figure 6). Once the two dyes are separated, the fluorescent dye emits light that is detected by the real time instrument allowing quantification of the amount of DNA. A TaqMan® probe was chosen for these experiments as they work well with ABI instruments (the instrument used in this study, due to its accessibility).

Figure 6. TaqMan probe diagram



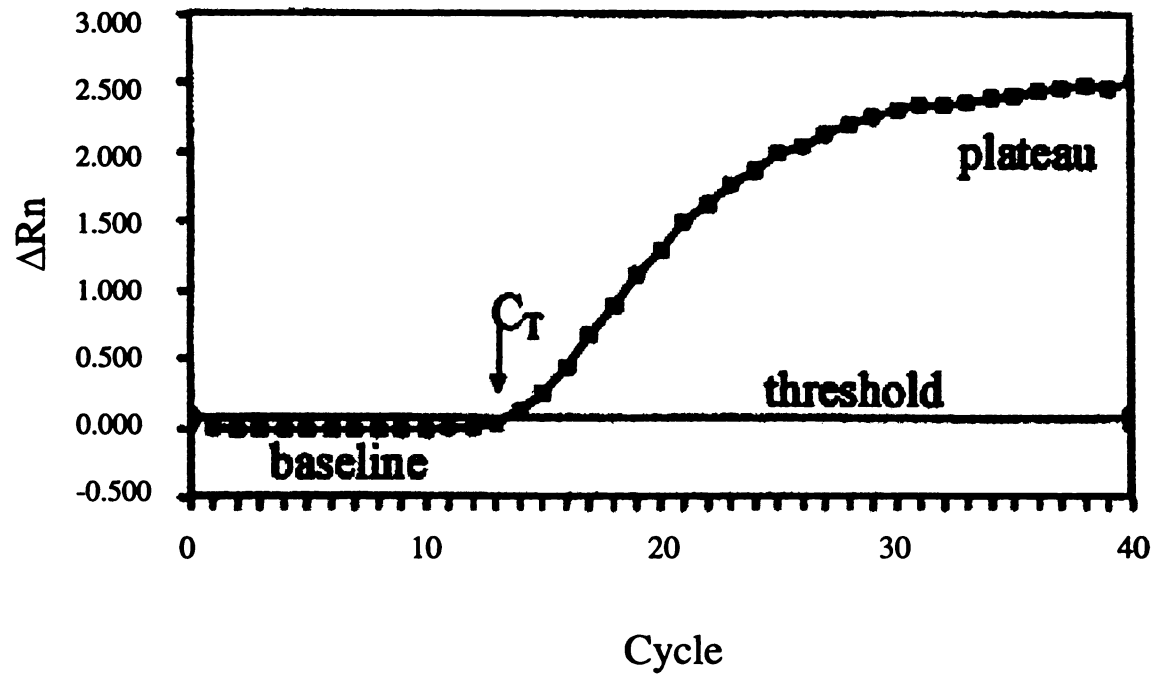
A representation of a TaqMan probe. The figure shows the nuclease activity of the polymerase as it extends the DNA strand. (Figure taken from Dyes and Fluorescence Detection Chemistry in qPCR, 2004)

Real time PCR quantification methods

There are several different kinds of real-time machines available on the market today. ABI has four (ABIPrism™ 5700, 7000, 7700, 7900), while others include the ICycler™, Engine Opticon™, Corbett Rotorgene™, LightCycler®, MX4000™, and the SmartCycler®. Each machine has a thermal cycler, computer, data acquisition and analysis software, and a laser light source (Real time PCR goes Prime Time, 2004). The laser excites the sample and a charged-coupled device (CCD) camera measures the fluorescence intensity and spectrum (ABI Sequence Detector, 2004).

During the first few cycles of PCR, insufficient product accumulates to cause a measurable increase in fluorescence. It is during these cycles that the computer software determines a baseline (or background) fluorescence level. The computer also calculates the threshold level, which is, by default, the value where the fluorescence is ten standard deviations above the mean of the baseline. One can manually adjust the baseline and the threshold if necessary. Once the baseline and the threshold are determined it is possible to find the threshold cycle (Ct), which is the number of PCR cycles at which the reaction's fluorescent emission crosses the threshold (and is therefore above the baseline). Figure 7 shows an example of an amplification plot.

Figure 7. Theoretical amplification plot

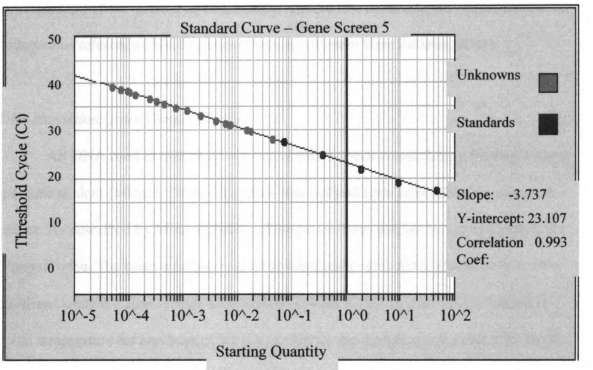


A graphical representation of the concepts of baseline, Ct, and threshold. The signal intensity is on the y-axis and the cycle number on the x-axis. ΔRn is essentially a measure of the fluorescent emission intensity of the sample after the background has been subtracted away. (Figure from Martell *et al.*, 1998)

It is the calculated Ct value that the software uses to estimate the starting amount of DNA. There are three methods for doing this: the standard curve method, the relative standard curve method, and the comparative Ct method. In this study, the standard curve method was used to obtain the estimate of DNA quantity. With this method, known concentrations of DNA (the standards) are run on the same reaction plate as the samples of unknown concentration. During the PCR run, the computer determines the Ct of all samples. Once the run is finished, the computer creates a graph of the calculated Ct values for the standards, versus their DNA quantity (see Figure 8). The sequence detection system (SDS) software determines an equation for a standard curve of the

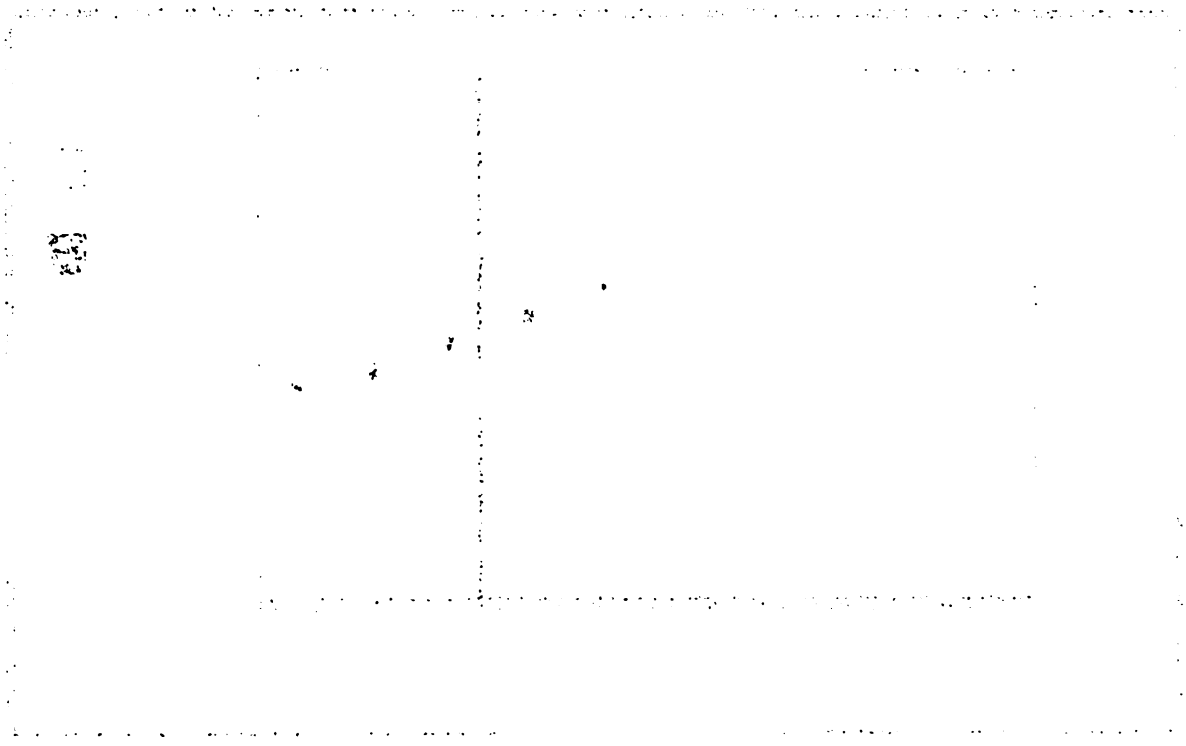
knowns, then puts the Ct value of the unknown into the equation to solve for the unknown DNA quantity.

Figure 8. Typical standard curve plot



The threshold cycle is shown on the y-axis, and the starting quantity of DNA on the x-axis. (Figure taken from Bartram *et al.*, 2003). The equation of the line best fitting the standard samples (black dots) is used to solve for the quantity of DNA in the unknown samples (red dots).

Another quantification approach is the relative standard curve method. In this case the DNA quantity is determined relative to a reference sample, such as a sample that has undergone some treatment versus an untreated sample (ABI Prism Sequence Detection System User Bulletin, 2001). For example, a researcher can compare RNA levels before and after a drug is administered to a patient. In this application the researcher would not be interested in an absolute quantity; they would merely want to know if there is an increase or decrease in gene expression as compared to the normal state of the gene.



The final approach to quantification is the comparative Ct method. It works in a similar fashion as the relative standard curve method, but instead of a standard curve a mathematical algorithm is used. It also gives relative quantification, allowing for a determination of the amount of target DNA, relative to a calibrator, and normalized to an endogenous reference (ABI Sequence Detection System User Bulletin, 2001).

DNA extractions from Voegtly samples

All DNA used in this study was extracted for thesis research by a Michigan State graduate student (Misner, 2004). The DNA was isolated from crania, femurs, ribs, and pelves. As described by Misner (2004), a Dremel tool was used to cut approximately 5–20mg of bone. The bone was then soaked in wash buffer (1% SDS, 25mM EDTA, filter sterilized using a 0.2μm syringe filter) and proteinase K (0.1 mg/ml) and incubated at room temperature for one hour. After the incubation the sample was washed with sterile dH_2O six times. The sample was dried using compressed air and ground to a powder using an IKA A11 Basic Grinder. The sample was then incubated at 56°C overnight in digestion buffer (20mM Tris, 100mM EDTA, 0.1% SDS) and proteinase K (0.4mg/ml). Following incubation, phenol was added and the sample was vortexed and centrifuged. The aqueous layer was removed, chloroform was added, and the sample was again vortexed and centrifuged. The aqueous layer was removed and the DNA precipitated using sodium acetate and EtOH then incubated for at least an hour at -20°C. The sample was centrifuged, the supernatant removed, and the pellet dried. The sample was resuspended in TE (10mM Tris, 1mM EDTA) buffer based on the starting mass of bone processed.

Materials and Methods

Designing of primers and probes

The primers used in the real time PCR reactions in this study were designed in house using Primer Express™ software to amplify a 118 base pair (bp) region in hypervariable region 1 (HV1) of human mitochondrial DNA. F16400 was chosen as the forward primer (5'-ACCATCCTCCGTGAAATCAA-3'), and D-loop was chosen as the reverse primer (5'-ACCCTGAAGTAGGAACCAGA-3'). For these experiments a TaqMan® probe (5'-CCTCGCTCCGGGCCCATAAC-3') was designed to sit between the forward and reverse primer sites. All reactions were run in a 96-well plate (MicroAmp®) with optical adhesive covers and compression pad. An ABI Prism 7700 was the real time thermal cycler used for quantification.

PCR Optimization

The primers and probes were optimized according to the TaqMan® Universal PCR Master Mix protocol (2002). Two and one-half microliters of human control DNA (20ng/uL) was used for the optimization. The forward and reverse primers were diluted so that their final concentrations in the reaction were 500nM, 200nM, or 50nM. The ratio of forward primer concentration to reverse primer concentration was tested according to the conditions set forth in Table 1. All reactions were run in triplicate. The standard protocol states the primer combination with the lowest Ct is optimal (TaqMan® Universal PCR Master Mix protocol, 2002).

Table 1. Concentrations for optimization of primers.

	50nM	200nM	500nM
50nM	50:50	200:50	500:50
200nM	50:200	200:200	500:200
500nM	50:500	200:500	500:500

The primer concentrations are stated as a ratio of forward primer to reverse primer. The primers used for optimization are given in nanomolar (nM) concentrations.

In order to optimize the probe concentration, it was diluted to 250nM, 200nM, 150nM, 100nM, and 50nM. Triplicates were run at each probe concentration using the optimal primer concentrations. Two and one-half microliters of human control DNA (20ng/uL) was used for the optimization. The optimal primer concentration was determined to be 250nM.

The universal parameters recommended for quantitative TaqMan® assays (as described in the TaqMan® Universal PCR Master Mix protocol, 2002) were utilized, except for reaction volume and cycle number. A 50μL reaction is suggested, however, 10μL and 25μL reaction volumes were tested to see if a lower volume would suffice. One microliter and 2.5μL of standard DNA (described below) from $6x10^8 - 6x10^2$ copies were run in triplicate in a 10μL reaction volume and 25μL volume (respectively) on a reaction plate. The Ct values for each DNA concentration at each reaction volume were compared. Based on these results, all subsequent reactions were run using a 10μL reaction volume.

For each 10 μL reaction volume, 1μL of bone DNA extract was used, as was 4.35μL of TaqMan® Universal PCR Master Mix (PE Biosystems) which contains: AmpliTaq Gold DNA Polymerase, dNTPs with dUTP, AmpErase Uracil-N-glycosylase ((UNG) (which is used to prevent contamination from carry-over PCR products, by

degrading dU-containing DNA), Passive Reference, and optimized buffer components (TaqMan® Universal PCR Master Mix Protocol, 2002).

The recommended thermal cycling conditions were used: a 50°C hold for 2 minutes to incubate the UNG, followed by a 95°C hold for 10 minutes to activate the AmpliTaq Gold, and inactivate the UNG. After the holds, the amplification cycles began with a 15 second denaturation step at 95°C, and a 1 minute annealing/extension step at 60°C (TaqMan® Universal PCR Master Mix Protocol, 2002). Forty cycles is the default cycle number for the ABI 7700, however, Andreasson *et al.* (2003) found success on degraded samples using 50 cycles. Therefore, in this experiment the denaturation and annealing/extension steps were repeated for 50 cycles. In addition to the standards (see below) and the unknowns, each plate contained a no-template control (NTC, consisting of all of the components necessary for real-time PCR, without the addition of DNA) which was run in triplicate to check for DNA contamination.

Generating the known DNA concentrations for use as standards

Using the standard curve method, the ABI 7700's sequence detection system (SDS) software uses the values input for DNA of known concentration in order to quantify the unknown DNA samples. A 118 bp fragment of human mtDNA was amplified from human DNA using standard PCR to create the standards. The estimation of PCR product concentration was determined spectrophotometrically based on absorbance at 260nm. The absorbance value produced at this wavelength multiplied by the volume of the sample and a conversion factor (50ng/uL for double-stranded DNA) gave the concentration of DNA. The number of each base pair (A:T, and C:G) in the 118

bp sequence was counted and the molecular weight of each base pair was added up to determine the molecular weight of the entire 118bp segment. Using Avogadro's number, the molecular weight of the DNA strand, and the concentration of the DNA estimated spectrophotometrically, an estimate of the number of DNA molecules per microliter was obtained. Once the concentration of the human control DNA was determined, the DNA was diluted to $6x10^7$copies/microliter. Ten-fold dilutions of DNA from $6x10^7$ copies/μL down to 6 copies/μL were made for use as standards. For every PCR reaction, each standard was run in triplicate. All standards were set up for a 30μL reaction volume, then 10μL was dispensed to each of three wells. After all PCR runs were completed it came to light that the concentrations of the standards had been miscalculated and were off by a factor of 60, so the standards and all measured DNA quantities were multiplied by 60 to adjust for the miscalculation.

Dilution of the Voegtly samples

Previous experiments showed that a dilution of the Voegtly cemetery samples was necessary for successful amplification (Misner, 2004). To determine the best concentration to use in a PCR reaction, eight DNA samples, the maximum number that can be tested on an amplification plate, were each diluted 1:10, 1:20, and 1:100. The DNA was then quantified using the real time PCR parameters detailed above. The calculated values were the highest for the 1:20 and 1:100 DNA dilutions. It appeared the optimal dilution fell between the two, thus a 1:50 dilution of the DNA was used for all subsequent quantification. All quantification results were multiplied by the dilution factor to estimate the starting amount of mtDNA in the bone extraction.

Voegtly samples and addition of BSA

After the results were obtained for the eight samples analyzed above, it was decided that an additive that could minimize inhibition might result in more accurate estimates of DNA concentration. A stock concentration (10ng/uL) of de-acetylated BSA (FisherBiotech) was diluted to 5ng/uL, 2ng/uL, and 1ng/uL. To determine the optimal concentration of BSA, 1μL of each concentration was added to two Voegtly samples that were then amplified in a standard PCR reaction. The real time thermal cycling parameters described above were used for this reaction. Five microliters of the PCR product was electrophoresed on a 3% agarose gel and then stained with ethidium bromide. The 2mg/mL dilution appeared to give the best results.

Quantification reactions are typically done in triplicate (Richard *et al., 2003)*, or quadruplicate (TaqMan® Universal PCR Master Mix Protocol, 2002). Preliminary quantification was done in triplicate, but owing to the variation seen in the DNA concentration results, it was decided that for these samples it would be beneficial to have a higher number of replicates. Each Voegtly sample was thus run in replicates of five. Femurs, ribs, and pelves were the bone types chosen for analysis. Crania were not analyzed due to the low sample number (2).

Effects of PCR inhibition

In this study, the effects of inhibition when estimating DNA concentration using real time PCR on aged skeletal material were examined. Three categories (a total of 23 samples) were tested: a mix of samples that had previously shown inhibition, samples that showed no indication of inhibition, and those that were possibly inhibited. These

determinations were based on results obtained by Misner (2004). Samples were considered inhibited if no amplification bands or primer dimers could be observed on an agarose gel after electrophoresis. If a sample gave no results for Misner (2004), but a quantity value was obtained, it was considered possibly inhibited.

In the first set of quantification experiments, 1μL of DNA extracted from bone was spiked with 1μL of the mtDNA standards (in ten-fold dilutions from 6,000,000 – 60 copies/μL) without the addition of BSA to address how the known quantity of DNA might change in the presence of the ancient sample and any inhibitors present.

Eleven of the 23 samples appeared inhibited (at least two-thirds of the quantity values obtained in the experiment described in the previous paragraph fell below the amount of standard added to the reaction), and were tested further. These samples were spiked with the standard mtDNA (from 6,000,000 – 60 copies/μL), and 1μL of BSA was added.

In the final series of inhibition experiments, the bone DNA was spiked with the standard DNA, (from 6,000,000 – 60 copies/μL) and an enhancer (TaqMaster® 5x PCR Enhancer (Eppendorf)) was added to test the role of a commercial and proprietary product for reducing PCR inhibition. Previous study showed that 2μL of Enhancer in a 10μL reaction was optimal (personal correspondence, Lisa Misner). In all cases, samples were run in triplicate using the PCR conditions described above.

Statistical analysis

Analysis of variance (ANOVA) was used to analyze the data generated in the Voegtly experiments, comparing skeletal weathering, individual bone weathering, bone type, age, and sex to mtDNA concentrations. In order to analyze the ages at death they

were put into six categories: infant (less than 1 year old), child (1–13 years), adolescent (14–17 years), young adult (18–24 years), adult (25–49 years), and older adult (50+ years). ANOVA compares the within-sample variation to the between-sample variation to determine if the samples in a group have a significantly different result as compared to other groups (Ott, 2001). Results were considered significant when $p<0.05$. A chi-square test was performed to determine if there was a statistical difference between the known amount of mtDNA added to a spiked sample, and the mtDNA yield calculated by the SDS software. A two-tailed paired t-test was performed to compare the means obtained for spiked samples without any anti-inhibitory additions (BSA or Enhancer), and the means for spiked samples with anti-inhibitory addition. Results were considered significant when $p<0.05$. All statistical analysis was performed using Microsoft Excel XP.

Results

Primer optimization

In order to optimize primers, differing ratios of forward to reverse primer concentrations were run in a real time PCR reaction. The optimal concentrations were found to be 200nM for the forward primer and 500nM for the reverse primer, which were consequently used for all further reactions. The results for the primer optimization are shown in Table 2.

Table 2. Primer optimization

Ratio of forward to reverse primer (nM)	Average Ct
50:50	21.0
50:200	21.2
50:500	20.1
200:50	19.4
200:200	18.6
200:500	18.3
500:50	19.4
500:200	18.6
500:500	18.6

The average Ct obtained for each primer concentration. The primers are given in nanomolar (nM) concentrations.

Probe optimization

The probe was optimized using the probe concentrations shown in Table 3. The optimal probe concentration was 250nM, and was consequently used in all reactions.

Table 3. Probe optimization

Probe concentration (nM)	Average Ct
250	20.2
200	20.3
150	20.4
100	20.5
50	21.0

The average Ct value for each probe concentration. The probe in nanomolar (nM) concentrations.

Reaction volume experiment

The validity of using a 10μL reaction volume, as opposed to a 25μL reaction volume, was tested. Standard DNA (at varying concentrations) was run in each of the two volumes. Since the percent differences were not larger than 5% (Table 4), it was decided that a 10μL reaction was valid for use in these experiments.

Table 4. Reaction volume optimization

Standard concentration (copies/μL) in the reaction	Average Ct for 25μL reaction	Average Ct for 10μL reaction	Percent difference between the reaction volumes
60,000,000 copies	15.3	15.3	0%
6,000,000 copies	15.4	15.4	0%
600,000 copies	17.0	17.0	0%
60,000 copies	20.4	21.3	4%
6,000 copies	23.7	24.0	1%
600 copies	27.2	26.9	1%

The average Ct values are given for 25μL and 10μL reaction volumes at each DNA concentration. Note the small percentage difference between the two volumes, indicating that the reaction volume does not affect amplification.

Dilution experiments

An experiment was performed to determine the DNA dilution that would maximize quantity results, such that the most replicates of a sample were successfully amplified. For samples where all replicates amplified at more than one concentration, the dilution factor that gave the highest average quantity values was chosen. Table 5 gives the results of the dilution experiment. Four samples gave the best results with a dilution of 1:100, and three samples with a dilution 1:20. It seemed that the optimal value fell somewhere between the two dilution factors, so a 1:50 dilution of DNA was chosen for subsequent reactions.

Table 5. Dilution experiment results

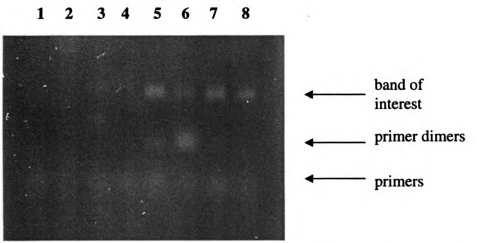
Sample	1:10	1:20	1:100
704R			√
704F			√
704P			√
124R		√	
124P	√		
124F		√	
381R			√
381F		√	

The sample name is the burial number and the bone type, R=rib, F=femur, and P=pelvis. The columns to the right of the sample column are the dilution factors of 1:10, 1:20, and 1:100. Checkmarks indicate the optimal dilution for each sample.

BSA optimization

Two samples were amplified in a PCR reaction with differing levels of BSA to determine the optimal BSA concentration (Figure 9). The brightest band appeared at 2ng/μL; therefore that concentration was used in all subsequent experiments.

Figure 9. Gel with different concentrations of BSA



Lane 1 is the negative control. Lane 2 is sample 704P with 2ng/μL of BSA. Lane 3 is sample 704P with 1ng/μL BSA. Lane 4 is 704P with a 5ng/μL BSA. Lane 5 is 381F with 2ng/μL BSA. Lane 6 is 704P with 5ng/μL BSA. Lane 7 is 704P with 1ng/μL BSA. Lane 8 is the positive control. The lack of distinct bands in lanes 1 – 4 indicate that the DNA did not amplify well.

Voegtly samples

Each mtDNA quantity obtained was multiplied by both the dilution factor and the factor necessary to correct for the miscalculation performed when preparing the standards. Six samples (rib samples 27, 409B, 448, and 704, femur samples 349 and 448) were found to give non-reproducible results and were excluded from the final analysis.

Each bone sample was analyzed individually, with two exceptions: the average of all the bone types available for each burial was used for the age and sex analysis. In this way, skeletons with several bones tested would not be over-represented.

Skeletal weathering stages

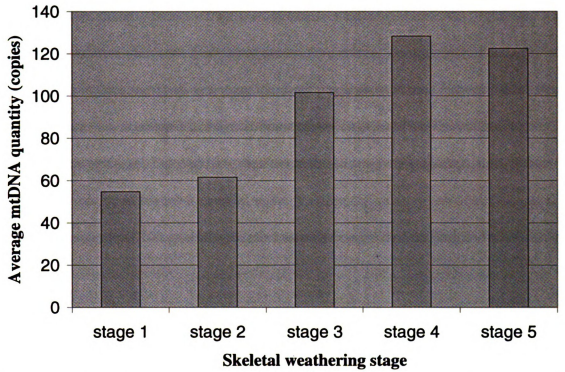
The mtDNA quantity calculated for each sample was categorized by the complete skeletal weathering stage, where a degradation value was given for a whole skeleton. Table 6 gives the average mtDNA quantity values obtained for each skeletal weathering stage. The general trend was that bones in the higher skeletal weathering stages had an increased quantity of mtDNA (Figure 10). The quantity of mtDNA decreased slightly between skeletal stage four and five.

Table 6. Average mtDNA quantity in each skeletal weathering stage

Skeletal weathering stage	Average mtDNA quantity (copies/μL)	Number of samples
Stage 1	54.7	4
Stage 2	61.6	20
Stage 3	101.6	14
Stage 4	128.3	9
Stage 5	122.5	8

The average mtDNA quantities (in copies/μL) found in each skeletal weathering stage. There were no stage zero bones available for analysis.

Figure 10. Average mtDNA quantity in each skeletal weathering stage.



The average mtDNA quantity (given in copies of DNA) is on the y-axis, and the skeletal weathering skeletal stages range from 1 (least weathered) to 5 (most weathered) along the x-axis.

ANOVA was performed to compare the amount of mtDNA found in each skeletal weathering stage. No statistical difference was found between the amount of mtDNA extracted and the skeletal weathering stage ($p=0.06$), but given the closeness to significance of these data, the possibility of a difference among weathering stages should not be completely discounted.

Skeletal weathering stage categorized by bone type

The mtDNA quantities obtained were further broken down by bone type (ribs, femurs, and pelves) in each stage for analysis. The average mtDNA quantities obtained for each are presented in Tables 7, 8, and 9. For all samples, the greatest quantity was

found in stages four or five, and the lowest quantity was found in either stage two or one. In ribs, mtDNA quantity increased from stage one to stage two, decreased slightly in stage three, and then increased from stage three to stage five. The quantity of mtDNA found in femurs decreased between stage one and two, then increased between stage two and stage four, then decreased in stage 5. The average amount of mtDNA in pelves decreased between stage one and two, then increased in stages two through five. These trends are portrayed in Figure 11.

Table 7. Average mtDNA quantity in rib samples categorized by skeletal weathering stage

Rib samples categorized by skeletal weathering stage	Average mtDNA quantity (copies/μL)	Number of samples
Stage 1	42.0	1
Stage 2	91.5	7
Stage 3	85.4	5
Stage 4	91.0	4
Stage 5	100.2	1

The average mtDNA quantity found in each stage for rib samples. Also shown is the number of samples in each stage.

Table 8. Average mtDNA quantity in femur samples categorized by skeletal weathering stage

Femur samples categorized by skeletal weathering stages	Average mtDNA quantity (copies/μL)	Number of samples
Stage 1	60.3	2
Stage 2	56.3	7
Stage 3	130.0	5
Stage 4	195.8	3
Stage 5	104.1	5

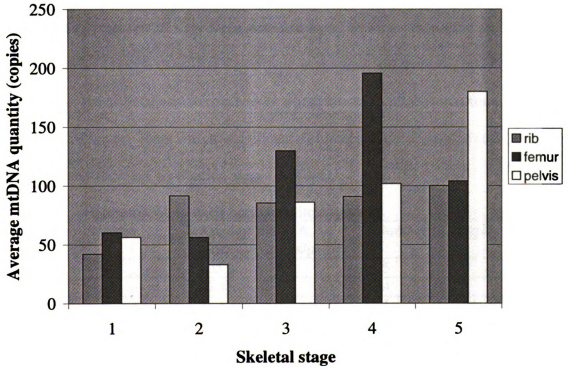
The columns are the same as Table 7, except the mtDNA values obtained are for femur samples.

Table 9. Average mtDNA quantity in pelvic samples categorized by skeletal weathering stage

Pelvic samples categorized by skeletal weathering stage	Average mtDNA quantity (copies/μL)	Number of samples
Stage 1	56.3	1
Stage 2	33.1	6
Stage 3	86.1	4
Stage 4	101.8	2
Stage 5	180.1	2

The columns are the same as Table 7, except the mtDNA quantities obtained are for pelvic samples.

Figure 11. Average quantity of mtDNA in each skeletal weathering stage categorized by bone type.



The average mtDNA quantity in each skeletal stage is given on the y-axis, and the skeletal stages range from 1 (least weathered) to 5 (most weathered) along the x-axis. In each stage, the left column portrays rib samples, the middle column femur samples, and the right column pelvic samples.

There appears to be a general upward trend in the relationship of mtDNA yields and skeletal degradation. However, there was no significant difference among the stages for ribs, femurs, or pelves ($p=0.95$, $p=0.13$, and $p=0.12$ respectively).

Individual bone weathering stage

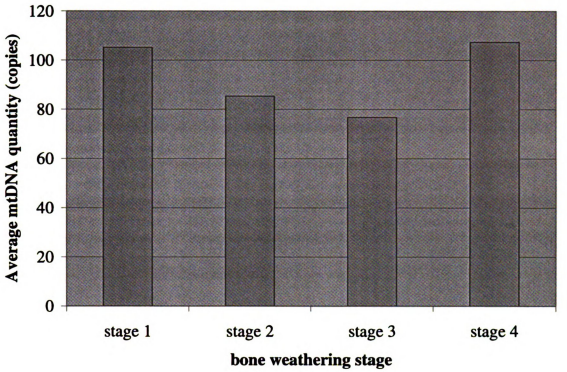
The mtDNA quantities were categorized by the bone weathering stages, where each individual bone was staged for analysis. Table 10 gives the average mtDNA quantity obtained for each bone weathering stage. Figure 12 shows mtDNA quantity decreased from stage one to stage three, then increased between stage three and stage four. The largest mtDNA quantities were found in the least and most degraded bones. This contrasts the skeletal weathering results where the most mtDNA was found in the more degraded bones. There was no significant difference in the amount of mtDNA extracted across the bone weathering stages ($p=0.71$).

Table 10. Average mtDNA in each bone weathering stage.

Bone weathering stage	Average mtDNA quantity (copies/μL)	Number of samples
Stage 1	105.2	15
Stage 2	85.4	24
Stage 3	76.8	11
Stage 4	107.4	5

The average mtDNA quantity (in copies/μL) for each bone weathering stage is given above. Additionally, the number of samples in each stage is portrayed.

Figure 12. Average mtDNA quantity for each bone weathering stage.



The average mtDNA quantity (in copies) is on the y-axis, and the bone weathering stages range from 1 (least weathered) to 4 (most weathered) along the x-axis.

Bone weathering stages categorized by bone type

The mtDNA quantities obtained for each bone stage were further categorized into each bone type for analysis (Tables 11, 12, and 13). As can be seen in Figure 13, there is no apparent common trend among the three bone types. However, there are observable trends within bone types. Ribs samples had the highest mtDNA quantity in the most degraded bones. Pelves showed the opposite trend with the greatest mtDNA quantity in the least degraded bones. In femurs the greatest quantity of mtDNA was found in the second stage followed by the first, then third.

Table 11. Average mtDNA quantity for rib samples categorized by bone weathering stage

Rib samples categorized by bone weathering stage	Average mtDNA quantity (copies/μL)	Number of samples
Stage 1	9.5	1
Stage 2	74.9	7
Stage 3	100.5	5
Stage 4	107.4	5

The average mtDNA quantity (in copies/μL) for rib samples in each bone stage is given above. The number of samples analyzed is also shown.

Table 12. Average mtDNA quantity for femur samples categorized by bone weathering stage.

Femur samples categorized by bone weathering stage	Average mtDNA quantity (copies/μL)	Number of samples
Stage 1	103.2	12
Stage 2	107.6	9
Stage 3	64.2	1

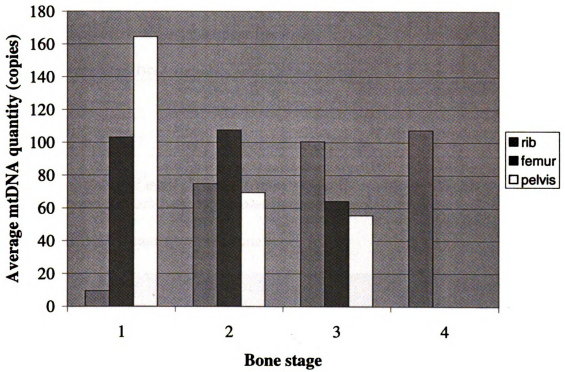
Columns are as in Table 11, with mtDNA quantity averages given for femur samples.

Table 13. Average mtDNA quantity for pelvic samples categorized by bone weathering stage.

Pelvic samples categorized by bone weathering stages	Average mtDNA quantity (copies/μL)	Number of samples
Stage 1	164.6	2
Stage 2	69.5	8
Stage 3	55.6	5

Columns are as in Table 11, with mtDNA quantity averages given for pelvic samples.

Figure 13. Average mtDNA quantity versus bone stage categorized by bone type



The average mtDNA quantity (in copies) in each bone stage is shown. The bone stages range from 1 (least weathered) to 4 (most weathered) along the x-axis.

There were no femurs or pelves classified in stage 4, therefore all stage 4 results are for rib samples only. When the bone stages were also sorted by bone type, there was no significant difference in the mtDNA quantity obtained from rib, femur, or pelvic samples ($p=0.36$, $p=0.90$, $p=0.15$ respectively). It should be noted that there was only one stage one rib, and only one stage three femur.

Type of bone analyzed

The mtDNA quantity values obtained were analyzed by the type of bone from which the DNA was extracted. Femurs were found to have the highest mtDNA quantity, followed by ribs, and the lowest quantity obtained in pelves. Table 14 gives the average

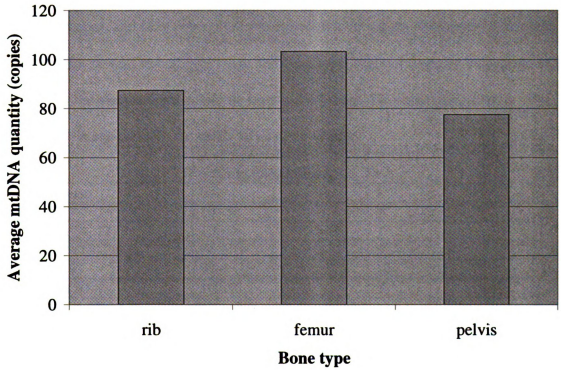
mtDNA quantity values for each type of bone analyzed, while Figure 14 presents these results visually. An ANOVA indicated there was no significant difference in the quantity of mtDNA obtained from each bone type (p= 0.55).

Table 14. Average mtDNA quantity in each bone type

Bone type	Average mtDNA quantity (copies/μL)	Number of samples
Ribs	87.4	18
Femurs	103.2	22
Pelves	77.6	15

The average quantity of mtDNA for each bone type is shown above. The number of samples analyzed for each bone type is also portrayed.

Figure 14. mtDNA quantity versus bone type



The average quantity of mtDNA in each bone type is given on the y-axis. The bone types (rib, femur, and pelvis (from left to right)) are on the x-axis.

Age of person at death

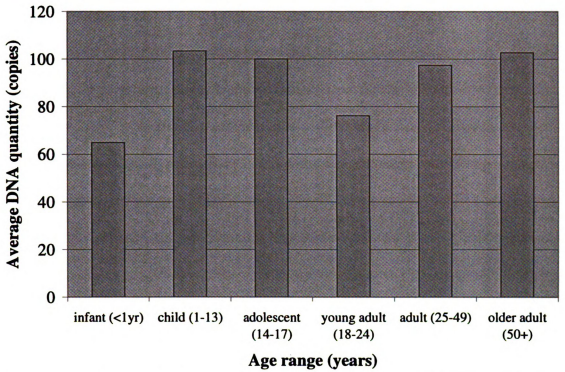
The ages at death were put into six categories for analysis: infant (less than 1 year old), child (1–13 years), adolescent (14–17 years), young adult (18–24 years), adult (25–49 years), and older adult (50+ years). The greatest mtDNA quantities were found in the children closely followed by the older adults, while the lowest were found in the young adults and infants. The average mtDNA quantity values for each age range are presented in Table 15. Figure 15 shows no obvious relationship between age and mtDNA quantity. There was no statistical difference between the amount of mtDNA obtained in each of the age ranges ($p = 0.98$). It should be noted that there was only one sample in the infant and older adult ranges, and only two samples in the adolescent range. No attempt to analyze these data with respect to both age range and bone type, skeletal stage, or bone stage was attempted as the sample sizes would be very small (or zero) in some categories.

Table 15. Average mtDNA quantity in each age range.

Age ranges	Average mtDNA quantity (copies/μL)	Number of samples
Infant (<1 year)	64.9	1
Child (1–13)	103.4	3
Adolescent (14–17)	100.0	2
Young adult (18–24)	76.3	3
Adult (25–49)	97.4	13
Older adult (50+)	102.7	3

The average mtDNA quantity obtained in each age range is presented. The number of samples studied for each age range is also given.

Figure 15. Average mtDNA quantity in each age range

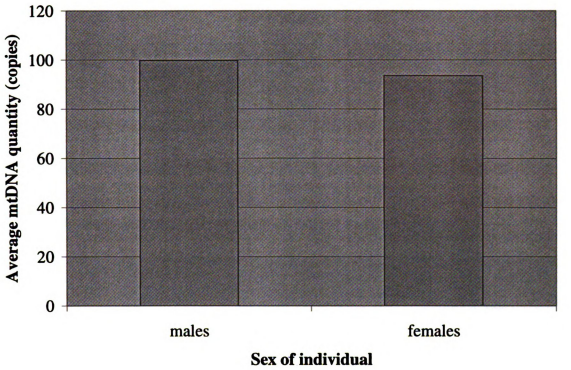


The average quantity of mtDNA for each age range is given. The age ranges (in years) are along the x-axis.

Sex estimates and average mtDNA quantities

The average quantity of DNA was compared to the individual's sex; samples where the sex was not determined were excluded from these analyses. The average quantity extracted from males (n=17) was 99.8copies/μL, the average from females (n=6) was 93.6copies/μL. Figure 16 is a visual representation of average mtDNA quantity for each sex. The quantity obtained in males was similar to that found in females, and there was not a statistical difference in mtDNA quantity between the sexes (p=0.81).

Figure 16. Average mtDNA quantity for each sex.



The average quantity of mtDNA found in males and females is shown.

Sex of individual and bone type

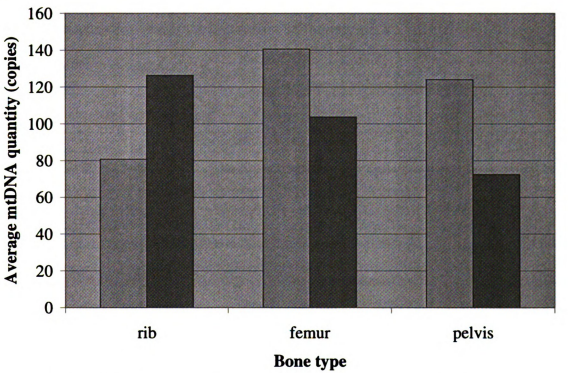
The mtDNA quantity results based on the type of bone from which the DNA was extracted in addition to the sex of the individual was analyzed. As with the comparisons based on sex alone, samples where the sex was not determined were excluded from these analyses. Figure 17 is a visual representation of the average mtDNA quantity in each bone type for each sex. No apparent trend common to all bone types was noted in these data. The male samples had greater quantities of mtDNA than female samples in the rib and pelvic bone samples, and both sexes had similar quantities in the femur bone samples.

Table 16. Average mtDNA quantities in each sex for each bone type

Bone type	Sex	Average mtDNA quantity (copies/μL)	Number of samples
Rib	Males	89.6	12
Rib	Females	60.7	4
Femur	Males	113.9	14
Femur	Females	114.0	5
Pelvis	Males	89.5	9
Pelvis	Females	72.5	4

The average mtDNA quantity (in copies/μL) for rib, femur, and pelvic samples for each sex is given above. The number of samples analyzed is also shown.

Figure 17. Average mtDNA quantity in each sex for each bone type.



The average quantity of mtDNA and type of bone is shown. The male samples are portrayed in the left columns and the female samples on the right.

There was no statistical difference noted between the sexes for the rib, femur, and pelvic samples ($p=0.36$, $p=0.72$ and $p=0.99$ respectively).

PCR inhibition results

In the experiments testing PCR inhibition, samples were spiked with known quantities of DNA. A chi-square test was utilized to determine if there was a statistical difference between the amount of DNA added to the sample, and the actual mtDNA quantity measured. For samples found to be inhibited (termed the "data subset", see Table 17), the effectiveness of adding BSA or Enhancer was tested. The sample was considered inhibited if at least two-thirds of the mtDNA values obtained fell below the quantity added for each DNA concentration. Paired t-tests were performed to examine the effectiveness of adding one of the two anti-inhibitory agents.

Table 17. Inhibited samples

Sample name	Number of values below the expected
111R	4
132F	6
132R	6
402P	6
539R	6
345P	6
409BR	6
448R	6
540P	6
167P	6
545P	6

The samples in the left column are the subset of samples used for the BSA and Enhancer experiments. The sample name consists of the burial number, sample letter (if given) and the bone type, R=rib, F=femur, and P=pelvis. The number of times where the sample's average mtDNA quantity fell below the amount of DNA added is shown in the right column.

Samples spiked with 6,000,000 copies

Addition of 6,000,000 copies of standard DNA generated the results shown in Table 18. As can be seen in Figure 18, the values obtained were greater with the addition

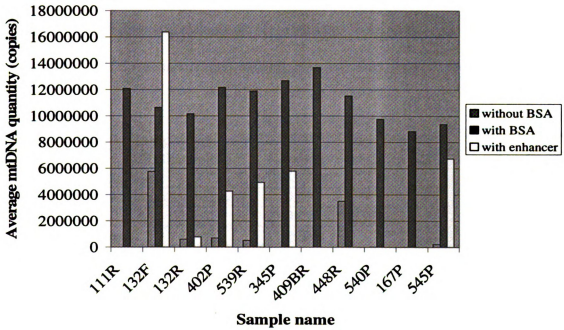
of BSA than without BSA for every sample. The quantity obtained was the greatest with the addition of the enhancer for one sample.

Table 18. Results for samples spiked with 6,000,000 copies of mtDNA

Samples spiked with 6,000,000 copies	Average quantity of mtDNA (copies/μL)
Samples with no additions	4,472,506
Samples with no additions (data subset)	1,029,195
Samples with BSA (data subset)	11,162,913
Samples with Enhancer (data subset)	3,536,583

The left column indicates the type of test performed. The average mtDNA quantity obtained in each series of experiments is given in the right column.

Figure 18. Samples spiked with 6,000,000 copies of control DNA



The average quantity of mtDNA for each sample spiked with 6,000,000 copies of standard DNA is portrayed on the y-axis. The columns represent the sample without any anti-inhibitory additions, with the addition of BSA, and with the addition of the enhancer (respectively). The sample name is composed of the burial number and the type of bone, R=rib, F=femur, P=pelvis.

There was a significant difference at α=0.001 between the amount of DNA measured and the copies added to the reaction, as well as between the amount of DNA quantified with and without BSA (p=0.0000000911). There was not a significant

difference between the amount of DNA quantified with and without the enhancer (p=0.067), however, it did cause an improvement in yield (as compared to samples without any anti-inhibitory additions) for six samples.

Samples spiked with 600,000 copies

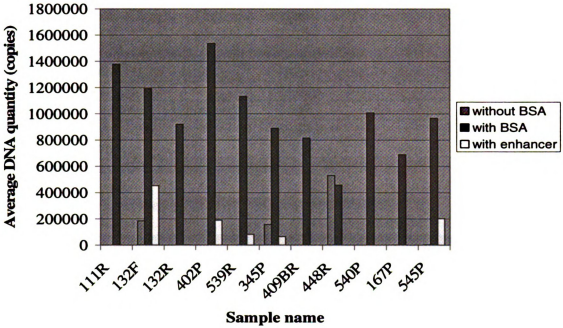
The addition of 600,000 copies of standard DNA resulted in the values presented in Table 19. As shown in Figure 19, the mtDNA quantities obtained were higher with the addition of BSA for ten out of the eleven samples. One sample produced higher results without any anti-inhibitory additions. As some samples had a higher quantity of mtDNA with the addition of the enhancer than without, it appeared to cause an improved yield in certain samples.

Table 19. Results for samples spiked with 600,000 copies of mtDNA

Samples spiked with 600,000 copies	Average quantity of mtDNA (copies/μL)
Samples with no additions	469,365
Samples with no additions (data subset)	79,632
Samples with BSA	998,761
Samples with Enhancer	90,569

Columns are presented in the same format as Table 18.

Figure 19. Samples spiked with 600,000 copies of control DNA



The information is presented as in Figure 18.

The results of the chi-square showed that the amount of DNA obtained at this level was statistically different than the amount of DNA with which the sample was spiked (α=0.001). Additionally, there was a significant difference between the amount of DNA quantified with and without BSA (p=0.0000256). There was not a significant difference between the amount of DNA quantified with and without the enhancer (p=0.86).

Samples spiked with 60,000 copies

Addition of 60,000 copies of standard DNA generated the results given in Table 20. Figure 20 shows higher quantity results were obtained with the addition of BSA for

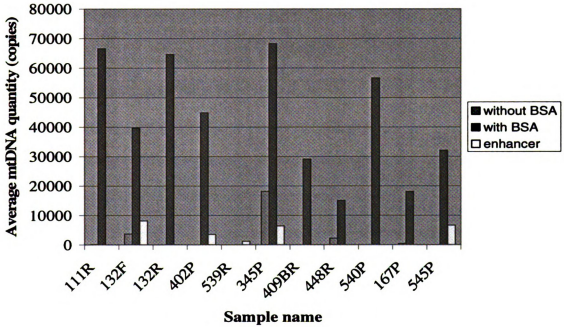
ten out of the eleven samples. One sample was only quantifiable in the presence of the enhancer.

Table 20. Samples spiked with 60,000 copies of mtDNA

Samples spiked with 60,000 copies	Average quantity of mtDNA (copies/μL)
Samples with no additions	42,639
Samples with no additions (data subset)	2,247
Samples with BSA	39,542
Samples with Enhancer	2,365

Columns are presented in the same format as Table 18.

Figure 20. Samples spiked with 60,000 copies of control DNA



The information is presented in the same format as Figure 18.

There was a significant difference at α=0.001 between the quantity of mtDNA obtained and the 60,000 copies added, as there was between the amount of DNA quantified with and without BSA (p=0.000191). There was not a significant difference between the amount of DNA quantified with and without the enhancer (p=0.935).

Samples spiked with 6,000 copies

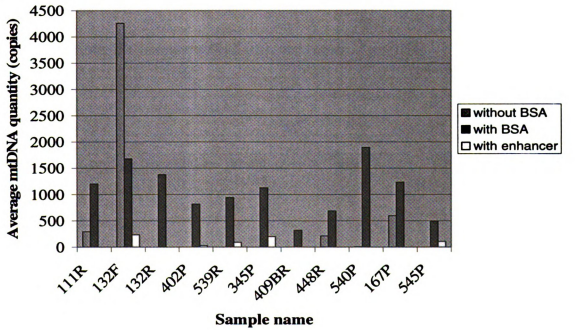
Table 21 gives the quantity results for each sample spiked with 6,000 copies of standard DNA. Figure 21 shows ten samples gave increased results with the addition of BSA. For one sample the highest mtDNA quantity was obtained without addition of either BSA or the enhancer. The quantity of mtDNA obtained with the addition of the enhancer was increased in four samples.

Table 21. Results for samples spiked with 6,000 copies of mtDNA

Samples spiked with 6,000 copies	Average quantity of mtDNA (copies/μL)
Samples with no additions	1,907
Samples with no additions (data subset)	489
Samples with BSA	1,072
Samples with Enhancer	60.7

Columns are presented as in Table 18.

Figure 21. Samples spiked with 6,000 copies of control DNA



The average quantity of mtDNA for each sample spiked with 6,000 copies of standard DNA is portrayed as in Figure 18.

There was a significant difference at α=0.001 between the amount of DNA measured and the 6,000 copies added, while there was not between the amount of DNA quantified with and without BSA (p=0.121). There was not a significant difference between the amount of DNA quantified with and without the enhancer (p=0.300).

Samples spiked with 600 copies

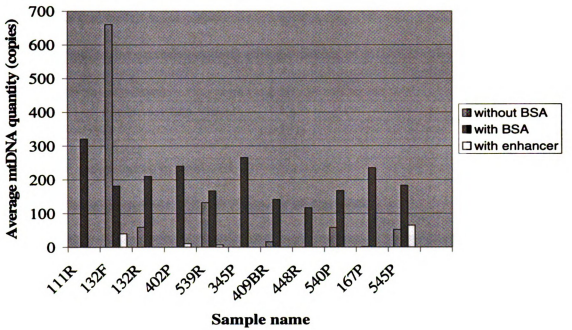
The quantity results for samples spiked with 600 copies of mtDNA are portrayed in Table 22. As can be seen in Figure 22, ten samples had increased quantities with the addition of BSA, while one sample had higher results without any additions (sample 132F). Interestingly, this was the same sample that had higher results when spiked with 6,000 copies of DNA. The addition of the enhancer seemed to cause little improvement at this level.

Table 22. Results for samples spiked with 600 copies of mtDNA

Samples spiked with 600 copies	Average quantity of mtDNA (copies/µL)
Samples with no additions	193
Samples with no additions (data subset)	89
Samples with BSA	203
Samples with Enhancer	11.5

The mtDNA quantities obtained are presented as in Table 18.

Figure 22. Samples spiked with 600 copies of control DNA



The mtDNA quantity values are presented as in Figure 18.

There was a significant difference at α=0.001 between the amount of DNA measured and the 600 copies added. Femur sample 47 gave inconsistent results and was excluded from these results. With the addition of BSA, there was a significant difference at this level (p=0.0389). There was not a significant difference between the amount of DNA quantified with and without the enhancer (p=0.223).

Samples spiked with 60 copies

Addition of 60 copies of standard DNA generated the results shown in table 23. Figure 23 shows that for nine samples, increased quantities were obtained with the addition of BSA. In two samples, the highest quantities were obtained with no anti-inhibitory additions. One of these samples, (132F) also produced higher quantities without any anti-inhibitory additions with the addition of 6,000 copies, and 600 copies. It

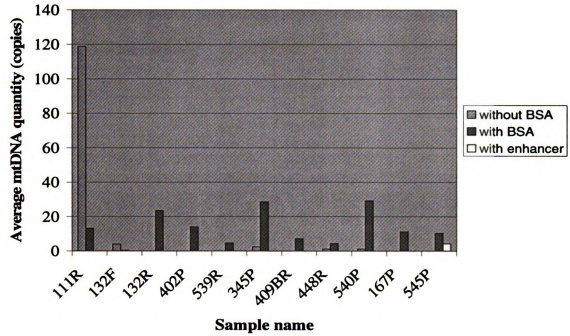
is of note that the average quantity of mtDNA obtained in the presence of the enhancer was less than one at this level.

Table 23. Results for samples spiked with 60 copies of mtDNA

Samples spiked with 60 copies	Average quantity of mtDNA (copies/μL)
Samples with no additions	101.9
Samples with no additions(data subset)	12.8
Samples with BSA	13.5
Samples with Enhancer	0.43

Columns are presented as in Table 18.

Figure 23. Samples spiked with 60 copies of control DNA



The mtDNA quantities obtained are presented as in Figure 18.

There was a significant difference at α=0.001 between the amount of DNA measured and the amount of DNA added. There was not a significant difference between the amount of DNA quantified with and without BSA (p=0.346), nor between the amount of DNA quantified with and without the enhancer (p=0.297). Pelvic sample 167 gave inconsistent results and was excluded from these results.

Percent inhibition

The average amount of mtDNA measured by the SDS software was divided by the concentration of the DNA added to the sample and multiplied by one hundred to give the percent of DNA obtained. One hundred minus the percent of DNA obtained was considered the percent inhibition. Table 24 gives the average percent inhibition for each DNA concentration analyzed for the broad inhibition study. The percent inhibition was similar for the 6,000,000 through 60,000 copy level, along with the 60 copy level. The 6,000 and 600 copy levels also have inhibition percentages that were close to each other.

Table 24. Percent inhibition.

Number of copies added	Average percent inhibition
6,000,000	25.5%
600,000	22.8%
60,000	28.9%
6,000	68.0%
600	67.8%
60	30.2%

The average percentage of inhibition for each number of DNA copies added is in the right column.

The amount of inhibition was also examined for the subset of samples that were previously shown to be inhibited. For this group, the percent inhibition appears to be relatively constant among all DNA concentrations added. The percent inhibition results are shown in Table 25.

Table 25. Percent inhibition for the sample subset.

Number of copies added	Average percent inhibition
6,000,000	82.8%
600,000	86.7%
60,000	96.3%
6,000	91.9%
600	85.2%
60	78.7%

The amount of DNA added to the sample is given in the left column. The average percentage of inhibition for each DNA concentration is in the right column.

The amount of inhibition with the addition of BSA was examined for the subset of samples previously shown to be inhibited (Table 26). The percentage of inhibition increased with decreasing DNA concentrations from 6,000,000 copies to 6,000 copies. From 6,000 copies down to 60 copies the percentage of inhibition remained somewhat constant.

Table 26. Percent inhibition for samples treated with BSA.

Number of copies added	Average percent inhibition
6,000,000	-86.0%
600,000	-66.5%
60,000	34.0%
6,000	82.1%
600	66.2%
60	78.0%

The average percentage of inhibition for samples treated with BSA is shown above. Negative inhibition values indicate that the value obtained is larger than the amount of mtDNA added to the reaction.

The amount of inhibition was also examined for the subset of samples that were treated with the enhancer and were previously shown to be inhibited. The results, presented in Table 27, show that the percent inhibition stayed similar among all DNA ranges except for the 6,000,000 copy level.

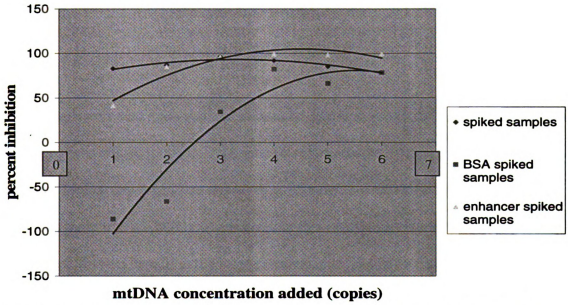
Table 27. Percent inhibition for samples treated with Enhancer.

Number of copies added	Average percent inhibition
6,000,000	41.4%
600,000	84.9%
60,000	96.1%
6,000	99.0%
600	98.2%
60	99.3%

The average percentage of inhibition for samples treated with Enhancer is portrayed above.

Figure 24 is a graph of the percentage of inhibition against the amount of DNA added to the sample for the following three conditions: without any anti-inhibitory additions, with BSA, and with the enhancer. The spiked samples in the graph, without any anti-inhibitory additions, were the same samples that were run with BSA and the enhancer. The percentage of inhibition for the samples without any anti-inhibitory additions, and the enhancer appear similar. Inhibition in samples that contained BSA did not seem to act in the same manner as those that contained Enhancer, or contained no inhibition treatment.

Figure 24. Percent inhibition versus DNA quantity



The percentage of inhibition for each amount of mtDNA added is on the y-axis. The DNA concentration (from 6,000,000 – 60) is on the x-axis. Each point has a corresponding mtDNA concentration added. The DNA concentration decreases as the x-value increases. From left to right, the first points (at x=1) correspond to 6,000,000 mtDNA copies added. At x=2, 600,000 copies were added. At x=3, 60,000 copies were added. At x=4, 6,000 copies were added. At x=5, 600 copies were added. 60 mtDNA copies were added at x=6.

Discussion

Real time PCR challenges

As stated in the Introduction, the ABI 7700 SDS calculates DNA quantity based on an extrapolation from a standard curve (Figure 8). Due to this, the quantity calculated is often not a whole number. Additionally, if the detected fluorescence crossed the threshold, regardless of cycle number, a quantity value will be calculated. This value can be far below the lowest standard (in this study, 6 copies) allowing for results to be less than one copy of DNA. Additionally, the quantity results obtained are only accurate if the standards fit the standard curve well. Therefore it is crucial to minimize errors, both when diluting the standard DNA and when adding it into the reaction plate.

Goals

There were two main goals of this study. The first was to determine if the level of weathering was related to the mtDNA quantity that could be extracted from the bone. It may seem logical that bones that appear in good condition would be better sources of DNA than those that are highly weathered, but this relationship has never been shown. It would be of obvious benefit to the forensic scientist to be able to determine if enough DNA can be extracted from a bone for analysis merely by determining visually the level of degradation of the bone. The second goal was to determine if there was a relationship between bone type and mtDNA quantity in degraded samples. If a forensic scientist knew the bone type that yielded the most DNA, time would not be wasted attempting to extract DNA from bones shown to be poor sources.

There are many variables that could confound an attempt to correlate bone type or bone/skeletal weathering with the ability to obtain DNA. Therefore, the skeletal material obtained from the Voegtly cemetery was ideal for this study. This site was utilized for a known amount of time, and the bones were subjected to the same environment. This allowed for study of bone type and degradation, while controlling other variables. Further, the possible effects of age and sex on DNA quantity were also examined. A final goal was to address PCR inhibition, which is often found in aged bones (Hagelberg, 1991), in a systematic way. Inhibition directly influences the quantification process utilized in this study, and therefore directly relates to the main goals of the research undertaken here.

Skeletal weathering and mtDNA quantity

The relationship between the appearance of a skeleton and how much DNA could be extracted from it was examined. The skeletons were graded by the guidelines put forth by Behrensmeyer (1978), where a complete skeleton is put into one of six stages. In this study, there were no stage zero skeletons available for analysis, and only a limited number of stage one skeletons. Somewhat surprisingly, no significant difference was found between the amount of DNA extracted and how weathered the skeleton appeared at an α=0.05 significance level. However, the p-value obtained (p=0.06) was not far from being significant, thus it is possible that there was a real difference among the different skeletal stages. One potential reason for these results, given that there appeared to be a trend (with the exception of stage 4) (Figure 10) which approached statistical significance, is that the degradation process occurring in the bone makes the DNA more

accessible, and therefore easier to extract. Another possible influencing factor was the pH of the soil. Research by another graduate student in the laboratory has shown a correlation (r^2 =0.21) between the pH of the soil found in the burials and the skeletal weathering stages (unpublished), with a lower pH resulting in more weathered bone. A possible explanation for this is that at a neutral pH microorganisms are unhindered and can destroy DNA, while affecting bone less. When subjected to a weakly acidic environment, which is known to harm bone, microorganism numbers may decrease, although the pH is not low enough to harm DNA.

Previous research on the same samples found stage five bones had the highest amplification success, followed by stage two, one, and four, while stage three had the lowest (Misner, 2004). The quantification results presented here, when compared to Misner (2004), are interesting in that there does not appear to be a parallel between mtDNA quantity and amplification success, other than that no strong relationship between bone weathering and DNA was found. Alonso *et al.* (2004) observed an apparent relationship between the quantity and quality of mtDNA in ancient samples. However, in that study there were only nine samples analyzed. These samples were between 500 and 1500 years old, were not from the same environment, and were not graded as to their levels of degradation. Due to these factors, the study may be misleading regarding a relationship between DNA quality and quantity in aged bones.

Skeletal weathering stages categorized by bone type

It is also possible that certain bones types have an influence on the quantity of mtDNA that can be recovered from weathered skeletal remains. Therefore, each skeletal

stage was further subdivided by bone type. Using an ANOVA, there was no significant difference found among the stages when classified by rib, femur, or pelvis. However, there may be a trend in the mtDNA quantity found in the skeletal stages when classified by bone type (Figure 11). Stage four or five skeletons had the highest average quantity of DNA for ribs, femurs, and pelves, while the lowest average quantity was found in either stage one or two skeletons. This is consistent with what was found by Misner (2004), in that there was no significant difference in amplification success found when the skeletal weathering stages were further divided by bone type. A potential reason for these findings is the same as described above, that is, degraded bones may have DNA that is more easily extracted.

Bone weathering and mtDNA quantity

An important consideration for the results discussed above is that the original weathering stages were based on the skeleton as a whole. However, certain bones within the skeleton may be more or less degraded than others, and it is feasible that a highly degraded rib sample from a stage four skeleton may give a different result than a femur from that same skeleton. To account for this, a new classification system was created by Misner (2004). This system placed all bones into one of four categories based on the level of visual degradation. The goal was to determine if classifying the bones in this manner would bring to light findings that were potentially masked when the bones were classified based on the whole skeleton. When analyzed in this way, the highest quantity of mtDNA was found in stage four bones, followed by stage one, stage two and stage three (Figure 12). It is important to remember that the sample sizes were not equal among the stages;

stage four did not contain any femurs or pelves, therefore stage four results were based on the rib sample's quantity values alone. There was no significant difference detected between the quantities of mtDNA found in the individual bone stages. This was consistent with the findings of Misner (2004), where no relationship between amplification success and individual bone weathering was observed. These findings indicate that classifying bone degradation in this manner may be of minimal use to a forensic scientist.

No correlation was found between the pH of the soil and the bone stages (unpublished). A possible reason for this may lie in the experimental design. Soil was not collected from around each bone when the skeletal material was originally collected. The soil used for the study was therefore collected from wherever it could be found. This leaves open the possibility that different bones within the same burial plot may have been subjected to different conditions (i.e. soil pH), explaining why no relationship could be elucidated.

Bone weathering and bone type

The bone stages were also subdivided by their bone type for analysis (Figure 13). The highest mtDNA average quantity for ribs was found in stage four, followed by stage three, stage two, and the lowest quantity in stage one. It should be mentioned that there was only one sample in stage one. Noting this, an apparent trend appeared in rib samples (Figure 13), increased average mtDNA quantity associated with increased degradation. However, there was no statistical difference in rib samples divided by weathering stages and mtDNA quantity ($p=0.36$). Additionally, Misner (2004) found no statistical

difference between amplification success and the rib samples divided by stages. The highest amplification success was found in stage two, followed by stage three, stage four, and the lowest in stage one. The amplification success did not correlate with quantification ranks in these rib samples.

There were no femurs in stage four, and there was not a significant difference among femurs in stages one through three ($p=0.90$) (Figure 13). It should be noted that there was only one stage three bone. The highest average quantity of DNA was in stage two, followed closely by stage one and stage three. Misner (2004) found the same pattern with the greatest amplification success in stage two femurs, followed by stage one and stage three, and no significant difference across the stages.

There were also no pelves in stage four, and no significant difference in mtDNA quantity among stages one through three ($p=0.15$). Stage one pelves had the highest average, followed by stage two and stage three. Along the same lines, Misner (2004) found a decrease in amplification success from stage one to stage three, but there was no significant difference in amplification success. This again shows a similarity between quality results and quantity results.

The indication when observing all the bone types across the bone stages is that there is no obvious quantitative benefit to choosing one of the three bone types over another.

Skeletal weathering versus bone weathering

The skeletal and bone weathering classifications are not interchangeable. When broken down into bone types, classification of individual bones gave different results

than classification of the skeleton as a whole. For example, the pelvic bones showed an increase in mtDNA quantity with increasing degradation when classified by skeletal weathering stage, but showed decreasing mtDNA yields with increasing bone weathering. This indicates that the pelvic bone tended to be degraded to a different extent than the skeleton as a whole. This differed from what was observed for rib samples. In both weathering staging system the ribs showed the greatest mtDNA quantity in the most degraded bones, and the smallest quantity in the least degraded. The specific reasons for these observations are not clear, but do show a difference in the two staging systems.

Bone type and mtDNA quantity

It was hypothesized that the dissimilarities in bone structure and surface area among femurs, ribs, and pelves would result in differences in mtDNA quantity. While the mtDNA quantity differences among bone types were not found to be significant, femurs contained the greatest quantity of mtDNA, followed by ribs, and pelves (Figure 14). Additionally, Misner (2004) had the highest amplification success for femurs, followed by ribs, and pelves. While there was no statistical difference in either DNA quantity or amplification success and bone type, both studies found among the three bone types that the femur was the best option. This information may be useful as a guide for forensic scientists when they have multiple bone types from a skeleton available for analysis.

mtDNA quantity and the age of a person at death

Some theorize that bones in the very young and the very old could have characteristics that affect the amount of DNA that can be extracted from them (see

Introduction). The results of this study showed no significant difference between the quantity of DNA and the age range to which a person belonged at their death (Figure 15). There did not appear to be a trend in the data either. However, there was only one infant sample, and the adolescent and older adult ranges each only contained 2 samples. It is possible that one or more of these samples was not representative of its age range, which would skew results. Similarly, Misner (2004) found no relationship between amplification success and age. The general indication is that the age of a person at their death has no bearing on the quantity of mtDNA that can later be extracted from their bones.

Sex and mtDNA quantity

At the start of this study it was theorized that there may be differences in the amount of mtDNA between the bones of males and females. Therefore, mtDNA quantity values obtained in this study were compared between the sexes (Figure 16). However, no significant difference between the amount of DNA extracted from males and females was found. Although males had a slightly higher average amount of mtDNA, the quantities obtained were very similar between the sexes. Misner (2004) found a significant difference between amplification success in males and females, with amplification occurring more often in males. The reason for the dissimilarity in amplification success and quantification is not clear.

The sexing of the bones was based on features of the pelvis and skull. In some cases these bones were not available, or were in poor condition. In these instances sex determination was based on other skeletal features, such as overall robustness. This can

be misleading because robust female bones could be typed as a male. Sex was also occasionally determined by objects found with the body, such as jewelry, or physical attributes, such as facial hair. It is possible that for some skeletons, sex was misjudged. If a skeleton was classified inaccurately, this could impact the results obtained.

Sex and mtDNA quantity by bone type

As previously stated, it is possible that certain bone types have more DNA than others. In order to eliminate the potential that this could skew the mtDNA sex averages, the sexed samples were further classified by bone type. Males were found to have a higher average quantity of mtDNA than females in rib and pelvic samples, while male and female femurs had virtually the same mtDNA quantity. However, none of the differences were significant. This is paralleled by the findings of Misner (2004) who found no significant difference in amplification success between the sexes when classified by bone type. These findings, when taken together, indicate there is no one bone type that is overly influencing the mtDNA quantity averages. There is no apparent advantage in choosing any given bone from a male as opposed to a female.

PCR Inhibition effects

PCR Inhibition was examined for three main reasons. One was to determine to what extent the level of inhibition present in a DNA sample might skew DNA quantification results. A second was to see if the amount of inhibition present in a sample was dependent on the concentration of DNA. The third was to determine if the addition of an anti-inhibitory agent could alleviate the inhibitory effect.

The Voegtly samples chosen for inhibition experiments (see Methods), were spiked with known concentrations of mtDNA from 6,000,000 copies down to 60 copies. The Voegtly samples themselves contained some mtDNA, of course, however for this study the estimates of DNA quantity were not adjusted for the original amount of DNA in the sample. This may be important at lower concentrations where the endogenous DNA may have an impact on the amount of DNA calculated. Since the samples typically contained less than 200 copies of mtDNA, at high DNA concentrations the affect of the endogenous DNA should be negligible.

The average quantity of measured mtDNA was lower than expected (the amount of known DNA added to the sample) for every concentration level, indicating inhibition throughout (Tables 18–23). Additionally, a chi-square test showed a significant difference (at α=0.001) between the amount of mtDNA added to the sample and the amount of mtDNA calculated by the real time instrument at all DNA concentration levels. The percentage of inhibition was not the same at all concentrations (see Table 24) however; it appears that there was more inhibition at the 6,000 and 600 copy level. With high concentrations of DNA (60,000 copies and above), the ratio of inhibitor to DNA is potentially smaller than with less DNA, and therefore the effect of inhibition could be lessened. At low DNA quantities (such as 60 copies), the amount of polymerase per DNA molecule will be increased (as opposed to higher DNA concentrations). This could allow for increased amplification in the presence of inhibitors. One could speculate that this is why it was at the 6,000 and 600 copy levels that the effect of inhibition was the greatest; these concentrations may represent a tradeoff between DNA quantity overcoming

inhibition, and relative polymerase levels being high enough to overcome it at the other extreme.

For all copy levels except 6,000 and 60, there was a significant difference between mtDNA quantities measured with and without the addition of BSA (Figures 18, 19, 20). While not significant, the mean mtDNA quantity obtained with the addition of BSA for the 6,000 copy level was over two times that found without the addition of BSA. At the 60 copy level, the mtDNA quantities obtained with and without the addition of BSA were very similar.

A t-test does not indicate if the addition of BSA increases or decreases inhibition, just if there is a significant difference between the values. However, examination of the DNA quantity values showed a decrease in inhibition upon addition of BSA. When 6,000,000 copies of DNA were added, all eleven bones analyzed had higher average mtDNA quantities with BSA than without. With the addition of 600,000 copies down to 600 copies, the mtDNA quantities obtained were higher with BSA than without for ten out of the eleven bones analyzed. When 60 copies were added, the average amount of mtDNA measured for samples with BSA was higher than without in nine out of eleven bones analyzed. This indicates that even when there is no statistical difference in the average quantity of DNA obtained, the addition of BSA consistently gives increased mtDNA yields on a per sample basis.

For the 6,000–60 copy levels, higher mtDNA quantities were obtained without addition of BSA for femur sample 132 (Figures 21, 22, 23), but at the higher copy levels increased values were obtained for the samples with the addition of BSA. The reason for this is not clear. Additionally, at the 60 copy level rib sample 111 without the addition of

BSA had a mtDNA quantity that was almost nine times the quantity obtained with BSA. This may be an artifact, in that the mtDNA may not have been uniformly suspended in solution when it was collected, leaving the possibility that a non-representative amount of mtDNA was collected from rib sample 111 at the 60 copy level. That would explain why the quantity obtained without BSA was higher only at the 60 copy level. Time did not permit rerunning femur sample 132 and rib sample 111 to see if the results were reproducible. Since the sample size in this experiment was small (11) it is possible that femur sample 132 and rib sample 111 could have skewed the results found for the 6,000–60 copy levels.

With the addition of 6,000,000 copies and 600,000 copies of known DNA to the sample, the mtDNA quantity measured with the addition of BSA was actually higher than the amount of DNA added to the sample. The reasons for this are not clear. BSA was added to the standards as well as the unknowns, so they were run under the same conditions. There is the possibility that this was a random finding, in that PCR is so sensitive that a one cycle disparity can result in a two-fold DNA quantity difference. Additionally, only eleven samples were examined. Further work may need to be performed to resolve this effect.

A t-test was also used to ascertain a possible difference between the amount of DNA measured with and without the addition of the TaqMaster Enhancer. There were no significant differences between the quantity of DNA obtained with and without the enhancer for any level of DNA added (from 6,000,000 copies down to 60 copies). This indicates that the enhancer does not affect PCR inhibition. However, for some samples the amount of mtDNA measured was increased when it contained the enhancer, while it

was rarely less with the enhancer addition (Figures 19, 20, 21, 22, 23). However, the enhancer has not been tested for use with quantitative PCR (personal correspondence, Dave Dasko, Eppendorf representative). Its ingredients are proprietary, but it is possible that it may contain quenching properties that interfere with the real time detection. Therefore, it is possible that the enhancer alleviates inhibition, but the enhancer should not be used in real time PCR until this has been studied further.

Examination of the mtDNA quantity values obtained when BSA was added gave little indication as to whether or not inhibition was dependent on DNA concentration. For this reason the percentage of inhibition at each DNA concentration was determined (Tables 25, 26, 27). When percent inhibition was graphed against DNA added (Figure 24), an apparent pattern emerged. The percentage of inhibition for the samples without any anti-inhibitory additions, and the samples with the enhancer added, stayed relatively constant, independent of the concentration of DNA that was in the sample. An exception was the percentage of inhibition found with the addition of the enhancer for the 6,000,000 copy level, as it showed less inhibition than the other DNA copy levels. This could result from femur sample 132, where the sample containing Enhancer had a mtDNA yield that was much larger than the other values obtained at that copy level (Figure 18). As mentioned previously, this may be a random finding that is not reproducible.

The percentage inhibition of BSA treated samples did change with varying DNA concentrations (Figure 24). BSA appeared to be more effective in samples with greater quantities of DNA. The type of inhibition present in this study was not known, which makes an explanation as to why BSA appeared to be more effective at higher DNA concentrations difficult. It is possible that there were at least two kinds of inhibitor

present; one influenced by DNA concentration, and another by BSA addition. Concentration dependent inhibitors likely work by binding directly to the DNA. The more DNA there is, the more inhibitor there needs to be to cause the inhibitory effects. At high concentrations the sheer amount of DNA can, in a sense, swamp out the effect of DNA concentration dependent inhibitors, and inhibitory effects would be more extreme at lower DNA concentrations. BSA may not help alleviate this type of inhibition. Another type of inhibition, one where the inhibitor affects the polymerase, would not be dependent on the amount of DNA present. BSA may disrupt the ability of the inhibitor to bind to the polymerase, thus interrupting the inhibitor's mode of action. This would explain why, in samples not treated with BSA, inhibition was still seen at the higher DNA concentrations. Given the results seen in this study, it seems possible both types of inhibitors were present in the samples examined. BSA lessened the effects of the polymerase mediated inhibition at all DNA concentrations, while at higher DNA concentrations DNA binding inhibitors were swamped out.

The percentage inhibition obtained for the subset of samples known to be inhibited (the "data subset" previously described) was not the same as those found in the larger inhibition study (Tables 23, 24). This could result from the experimental design. Not all samples analyzed for the broad study were known to be inhibited, therefore, it is not surprising that there was a lower percentage of inhibition found in these samples when compared to the samples in the subset that were known to be inhibited. Additionally, the factors that may have caused the differing percentages among copy levels (DNA to inhibitor ratio, or DNA to polymerase ratio) may not have had as great an impact with more heavily inhibited samples. For example, it is possible that an increase

in the polymerase to DNA ratio does not impact amplification when there is a lot of inhibitor present.

Conclusions

Neither the age of a person at their death nor their sex appears to be related to the amount of mtDNA that could be extracted from the skeletal material. In general, when bones were classified based on the skeleton as a whole, more degraded skeletons contained more mtDNA. However, this trend was not observed when all bones were grouped by the visual amount of bone degradation. The results indicate little potential value to choosing a less degraded bone over a more degraded one when mtDNA analysis is desired.

Femurs were found to have the greatest mtDNA quantity, followed by ribs and pelves. This corresponded with the results obtained in a previous study of amplification success (Misner, 2004), using the same bone samples. While the mtDNA quantity differences among the bone types were not statistically different, the similarity between mtDNA quantity and amplification success indicates that femurs are the best choice among the three bone types examined.

Additionally, the effects of PCR inhibition on the Voegtly samples were studied. The results indicate that an accurate quantification measurement, using real time PCR, will not be obtained if inhibitors are present. In an attempt to counteract inhibitory effects, two types of anti-inhibitory agents were studied, BSA and TaqMaster Enhancer (a commercial product). The enhancer was not detrimental to PCR, nor was it of any obvious benefit. The addition of BSA was effective in alleviating inhibition at all DNA

concentrations examined, but appeared to be more effective in samples containing high DNA concentrations. The results indicate there may be more than one type of inhibitory action present, impacting these findings. For inhibited samples not treated with BSA, the percentage of inhibition did not appear dependent on the concentration of DNA contained within the sample.

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