APPLICATION NOTE

# Genetic Extraction and Analysis from Weathered 6 Year Old *Bos Taurus* Bones through Bead Ruptor Homogenization.

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## Introduction

DNA analysis of human and animal skeletal remains provide vast amounts of insight for evolutionary, archeological, medical, and forensic studies. Bone structure has proven successful in housing varying amounts of genomic and mitochondrial DNA, long after the soft tissues have degraded. [1] While osseous tissues retain DNA longer than the soft tissues of the body, nuclear DNA and mitochondrial DNA found in bone are both shown to degrade exponentially at fluctuating rates depending on their post-mortem time interval and the environmental conditions which they are exposed to. [2] This degradation pattern invokes urgency for processing samples upon their retrieval, in order to mitigate as much genetic degradation as possible once in the lab. The current methodologies for the extraction of genetic material from aged bones involve multiple degradation procedure options to expose the genetic material for extraction. These methods require exposing the material to aggressive demineralizing and denaturing chemical buffers such as EDTA and DTT for prolonged periods, with some requiring up to 80 hours of incubation in these chemical baths. [5] Other methodologies rely extensively on commercial based purification kits and multiple rounds of PCR prior to reaching a workable quantity of purified DNA. [2] Yet others, claim that multiple rounds of mechanical disruption of the sample in conjunction with demineralization treatments will yield high working quantities of extracted DNA. [5,6]

The method described herein uses a combination of chemical treatments and mechanical disruption to extract and amplify DNA from fossilized *Bos taurus* bone. The bone sample was obtained from the foothills of Northern Alabama as seen in Figure 1, where it sat exposed to the elements in natural conditions for approximately 6 years prior to excavation. This publication will attempt to address one of the major challenges facing post-mortem DNA extraction techniques; the limited yield of genetic recovery from aging bone, while maintaining an efficient timeline of recovery to prevent further degradation upon retrieval of the specimen. [2,3,4]

## Materials and Methods

After undergoing bone excavation and preparation the specimens were homogenized using the Omni Bead Ruptor 96 and underwent the DNA extraction via the protocols outlined below. Once DNA was successfully extracted from the bones, we ran PCR reactions in an attempt to amplify both nuclear and mitochondrial coded for genes.

- **Bead Ruptor 96 (CAT # 27-0001)**
- **50 mL Milling Jars (CAT # 27-006)**
- **25 mm Milling Balls (CAT # 27-203)**
- **Tissue DNA Purification Kit (CAT # 26-007)**

Tissue DNA Purification Kit
Cat# 26-007

25 mm Milling Balls
Cat# 27-206

50 mL Milling Jars
Cat# 27-006

Bead Ruptor 96
Cat#27-0001

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## Bone Preparation

Two bone types and some teeth were excavated from a cattle ranch in Northern Alabama and used in this experiment; four teeth, one rib, one humorous, and one thoracic vertebra. Figure 1 shows the location and state in which the bones were originally found. These four bones represent two common bone types, long (humorous and rib) and irregular (vertebra), as well as teeth. All were evaluated and based on its condition, the rib bone was most intact and was chosen for use in this study. The bone was cut transversely and massed out to approximately 4.5g of sample for each bone milling prep. Prior to physical crushing of the bone, the sample was treated with a 0.5% sodium hypochlorite solution and washed to remove any remaining organic debris. It was then dried for 1 hour under a heating UV light source. [5] The remaining spongy bone tissue was then removed from the sample, leaving only the compact bone to be used in the bone milling procedure. [5]

Figure 1. Photographs of the condition and environment from which the bone samples were collected.

## Bone Milling

The prepped sample of compact bone was placed into a 50 mL stainless steel milling jar (Omni CAT # 27-006) with a 25 mm milling ball (Omni CAT # 27-206). Once the milling jar was sealed, it was submerged entirely in liquid nitrogen for 3 minutes. Upon completion of freezing, the milling jars were placed in the Bead Ruptor 96 (Omni CAT # 27-0001) and processed at 25 Hz for 2.5 minutes. The resultant bone powder homogenate was then removed and stored at -20° C while awaiting use for DNA extraction.

## DNA Extraction

0.5g of homogenized bone powder was massed out into a sterile 15 mL conical tube and incubated in 2.5 ml of EDTA extraction buffer A (0.3 M sodium acetate, 10 mM Tris-HCl, 1 mM EDTA, 1% SDS, pH 7.8) and 20 µL proteinase K overnight in 56° C water bath. [3,4] The sample was vortexed and inverted to ensure total saturation of the powder by the extraction solution.
The samples were then vortexed and inverted again to resuspend any pellet that may have formed during digestion, prior to being centrifuged for 2 mins at 10,000 rpm. The supernatant was pipetted off and placed in 700 µL aliquots in new 2 mL tubes. 700 µL of phenol: chloroform (pH 6.7:8.0) reagent was added to each tube and they were centrifuged at 10,000 rpm for 1 min. The top layer containing the DNA was removed and placed in a clean 2 mL tube. The washing step was repeated twice, only adding 250 µL of phenol: chloroform to the sample prior to centrifugation. [4]

The DNA containing supernatant was then treated with chloroform: isoamyl alcohol (24:1, pH 8.0) in a ratio of 1:1 and centrifuged at 10,000 rpm for 1 min. The supernatant was extracted from each of the tubes and recombined into a clean 2 mL tube. 800 µL of this solution was used in a QIAprep® Spin Miniprep Kit, while the remaining was stored in -20° C. [4]
Prior to adding DNA to the QIAprep column, 500 µL of Buffer PB was washed through the column and it was spun dry for 30 sec at 13,000 rpm with the flow through discarded. 800 µL of sample was then loaded into the column and centrifuged

for 1 min. The flow through was discarded and a second Buffer PB wash was performed to help remove any contaminating nuclease or carbohydrate contents. 750 µL of Buffer PE was added and the column spun for 30 sec at 13,000 rpm discarding the flow through. The column was spun again for 1 min to dry and remove any residual wash buffers. After drying, the column was placed into a clean 2 mL tube. 50 µL of eluting buffer was added and the DNA was eluted by centrifugation at 13,000 rpm for 1 min. The extracted DNA was then quantified on a Thermo Fisher Scientific NanoDrop 2000 Spectrophotometer (Figure 3).

## DNA Amplification

The extracted DNA was amplified via PCR with primers targeting two *Bos taurus* genes as seen in Table 1. Gen Bank sequences were used to develop the primers following the *GH1* sequence AC_000176.1 and the *mtCOX1* sequence KT827244.1. PCR was conducted using 2 ng of extracted DNA and the following combination of primers and enzymes; 2 µL of template DNA (diluted to 1 ng/ µL), 5.5 µL of 2 µM forward primer, 5.5 µL of 2 µM reverse primer, and 12 µL Hot Start Taq 2X Master Mix (New England BioLabs # M0496S). The PCR was run using a BioRad T100 Thermo Cycler, completing a total of 40 reaction cycles on the 25 µL mixture. The PCR cycling was run as follows; 95° C for 3:00 min, 95° C for 0:30 min, 55° C for 0:30 min, 68° C for 1:00 min, cycled through 40X and then cooled to 68° C for 5:00 prior to holding at 4° C for an infinite amount of time until samples were retrieved.

| ***B. taurus* PCR Target Genes** | | | |
|---|---|---|---|
| **Gene** | **Product** | **Product Size** | **Primers (5' – 3')** |
| *GH1* | growth hormone 1 | 223 | Fw: CCC TCC AGG GAC TGA GAA CAT |
| | | | Rv: AGT TCA CCA GAC GAC TCA GG |
| *mtCOX1* | mitochondrial cytochrome oxidase subunit 1 | 243 | Fw: AAC AGG CTG AAC CGT GTA CC |
| | | | Rv: GCT GCT AAT TAC AGG GAG CGA |

Table 1. *B. taurus* genes targeted for PCR amplification with the primer sequenced used for each amplification.

## Analysis of PCR Products

The PCR products were quantified on a Thermo Fisher Scientific Nanadrop 2000 Spectrophotometer as seen in Table 2. Additionally, the products were electrophoretically separated on a 2% agarose gel and stained with ethidium bromide for visualization as seen in Figure 2.

| Sample ID | Extraction DNA (ng) | GH1 PCR Product (ng) | mtCOX1 PCR Product (ng) |
|---|---|---|---|
| *B. taurus* | 390 | 0 | 11,072 |
| Commercial Beef Prep Method 1 | 147.5 | 7,807 | 7,010 |
| Commercial Beef Prep Method 2 | 1,455 | 5,217 | 8,562 |

Table 2. Comparison of extracted DNA contents and the PCR products of nuclear gene *GH1* and mitochondrial gene *mtCOX1* in a 25 µL solution.

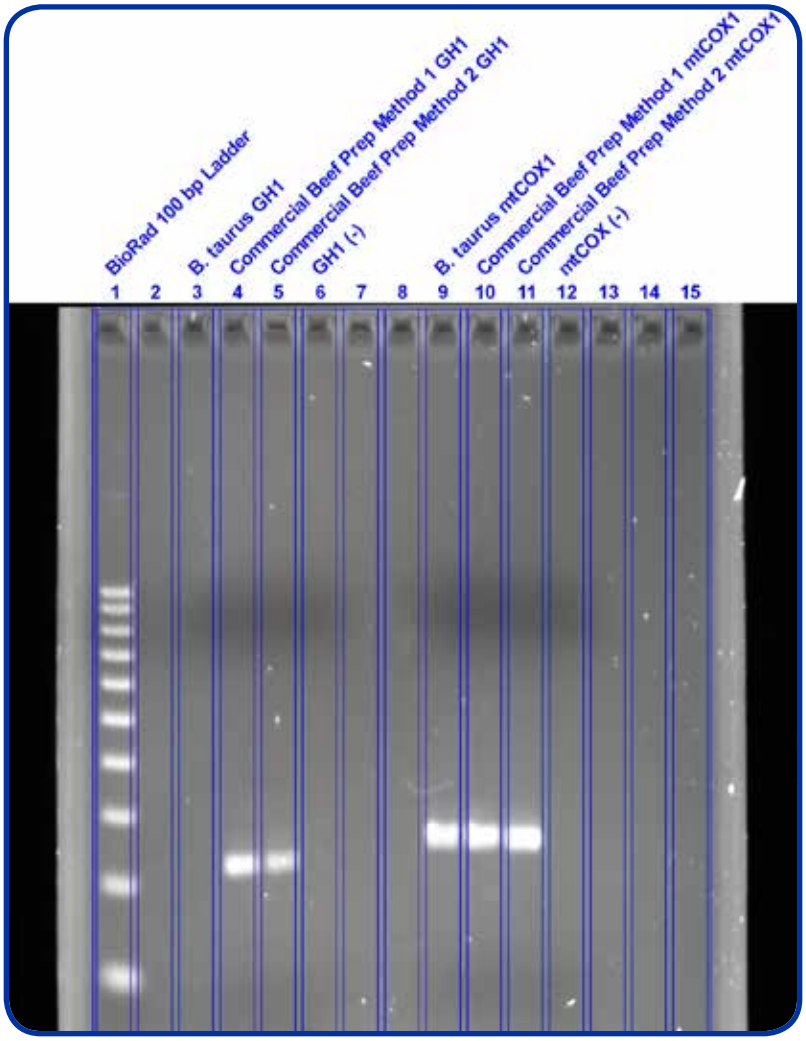


Figure 2. PCR agarose gel visualization of nuclear DNA gene *GH1* and mitochondrial DNA gene *mtCOX1* examined using the *B. taurus* bone preparation and two different extraction methods on commercial beef products. The lanes of the gel are as follows; (1) BioRad 100bp Ladder, (2) Empty, (3) *B. taurus GH1*, (4) Commercial Beef Prep Method 1 *GH1*, (5) Commercial Beef Prep Method 2 *GH1*, (6) *GH1* Negative Control, (7) Empty, (8) Empty, (9) *B. taurus mtCOX1*, (10) Commercial Beef Prep Method 1 *mtCOX1*, (11) Commercial Beef Prep Method 2 *mtCOX1*, (12) *mtCOX1* Negative

## Commercial Beef Preparation Method 1

30 mg of stew beef purchased from a local supermarket was prepared using the Omni Tissue DNA Purification Kit (CAT # 26-007). No deviations from the kit provided protocol were made.

## Commercial Beef Preparation Method 2

For this preparation method, 3g stew beef purchased from the local supermarket was flash frozen in liquid nitrogen and disrupted on the Bead Ruptor 96 at 25 Hz for 3:00 min. After being powdered, it was shaken and incubated in 0.5 M EDTA for 80 hours, centrifuged at 4,000 rpm for 15 mins. [6] DNA was then extracted on the resulting supernatant using the Omega Bio-Tek E.Z.N.A. Plasmid DNA Midi Kit (Omega Bio-Tek CAT # D6904-03). [6] No deviations were made from the manufactures instructions when using the kit.

## Results

Using bead milling in conjunction with chemical degradation and extensive purification procedures, we were able to successfully isolate mitochondrial DNA from aged bone as seen in Figure 2 and Table 2. However, when using the aforementioned methodologies we were unable to procure any appreciable amounts of nuclear DNA from the same sample. Figure 3 and Table 2 show the relationship between average extraction quantities and average amplicon production quantities from the various preparation methods and samples examined. This illustrates that the 15.6 ng/µL of DNA extracted from the fossilized bone was almost entirely mitochondrial DNA, due to the fact that no *GH1* amplicons were produced when the extracted DNA was mixed with the same PCR mixtures as the two commercial beef preparations.

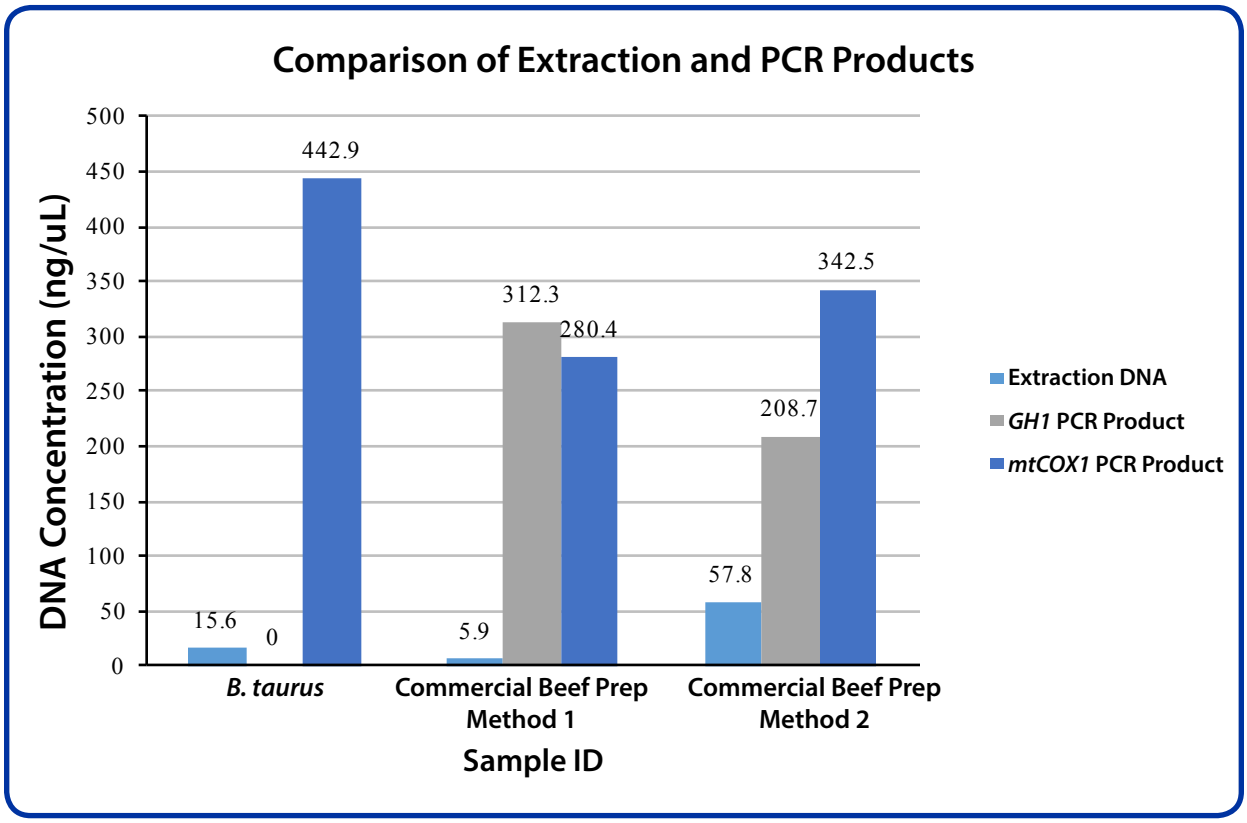


Figure 3. Comparison of average DNA amplification and extraction based on samples and preparation methods.

Out of concern that the EDTA buffer was exposing the genetic material to potentially damaging chemicals, we ran a sample of fresh commercially purchased beef which we had already extracted DNA from through an extended incubation period with the EDTA buffer (as described in Commercial Beef Preparation Method 2). [6] This preparation method was meant to overexpose a known viable nuclear and mitochondrial DNA source to EDTA in efforts to rule out the buffer causing damage to the desired genetic material during extraction. As seen in Figure 3 and Table 2, this prolonged exposure to EDTA did not damage the DNA from either cellular location, illustrated by the high concentration of PCR product for both the nuclear *GH1* and mitochondrial *mtCOX1* genes.

Additionally, when comparing the amplicon yield of the two different commercial beef sample preparations, we observed notable differences in the amplicon quantities based on the source type (nuclear or mitochondrial) of the template material and the extraction column used. As seen in Figure 3 and Table 2, the tissue DNA purification column produced an average of 312.3 ng/µL of nuclear DNA product, while producing 280.4 ng/µL of mitochondrial DNA product. These results were flipped when using the plasmid midi column prep, producing a higher 342.5 ng/µL of mtDNA, and lower average yields of 208.7 ng/µL

of nuclear DNA products. ANOVA analysis were run to determine that this was a significant difference between the amplicon yields on the different column types. This comparison allows us to draw two conclusions, first both columns can pick up nuclear and mitochondrial sourced DNA, second the type of column used will affect the extraction and resulting amplicon yields of each product based off of it genomic source.

## Conclusion

We successfully extracted and analyzed mitochondrial DNA from aging *B. taurus* remains using our extraction procedure described above. This experiment proved that using the Bead Ruptor 96 in conjunction with chemical digestion of aging bone samples can yield pure and amplifiable concentration of extracted mitochondrial DNA, but is unsuccessful at targeting nuclear DNA. We view the failure to recover viable nuclear DNA from the samples not as a failure of methodology, but more so as an indication of absence with regards to intact nuclear DNA in the sample to begin with. Examination of the literature states that it is possible to extract nuclear DNA from aged samples, however it is more frequently degraded than its mitochondrial counterpart. [2,4,5,6] It is possible that for the section of bone we sampled, there was not enough intact nuclear DNA to withstand purification and PCR.

Additionally, we observed notable differences in the DNA extracted from control samples based on the column type used. Omega's Midi columns proved to be more efficient at extracting mtDNA, where Omni's tissue purification columns were more efficient at retrieving nuclear DNA from the same sample of commercially processed beef. However, both columns were able to extract genes from both mitochondrial and nuclear sources of similar sizes for amplification.

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