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ScienceDirect
Forensic Science International: Genetics 1 (2007) 191–195
FSI GENETICS
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Short communication

High efficiency DNA extraction from bone by total demineralization ☆

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Received 24 January 2007; accepted 3 February 2007

Abstract

In historical cases, missing persons' identification, mass disasters, and ancient DNA investigations, bone and teeth samples are often the only, and almost always the best, biological material available for DNA typing. This is because of the physical and chemical barrier that the protein:mineral matrix of bone poses to environmental deterioration and biological attack. Most bone extraction protocols utilized in the forensic community involve an incubation period of bone powder in extraction buffer for proteinase digestion, followed by the collection of the supernatant, and the disposal of large quantities of undissolved bone powder. Here we present an extremely efficient protocol for recovery of DNA by complete demineralization, resulting in full physical dissolution of the bone sample. This is performed in a manner that retains and concentrates all the reagent volume, for complete DNA recovery.

For our study, we selected 14 challenging bone samples. The bones were extracted side-by-side with our new demineralization protocol and the standard extraction protocol in use at AFDIL. A real-time quantification assay based on the amplification of a 143 bp mtDNA fragment showed that this new demineralization protocol significantly enhances the quantity of DNA that can be extracted and amplified from degraded skeletal remains. We have used this technique to successfully recover authentic DNA sequences from extremely challenging samples that failed repeatedly using the standard protocol.

Published by Elsevier B.V.

Keywords: Mitochondrial DNA; Bone; DNA extraction; Demineralization; Forensic

1. Introduction

The ability to recover DNA sequence and STR data from bones and teeth exposed over time to a variety of environmental conditions has become a valuable tool for the identification of missing individuals and unknown remains [1]. Due to low levels of endogenous DNA, environmental, bacterial, and post-mortem DNA damage, as well as the potential presence of environment-born inhibitors that co-extract with DNA, the recovery of DNA data from degraded specimens can still pose a significant challenge.

Bone is a growing tissue made up mainly of collagen, a protein that provides a soft framework, and minerals that add strength and harden the framework. About 70% of bone consists of the inorganic mineral hydroxyapatite, which includes calcium phosphate, calcium carbonate, calcium fluoride, calcium hydroxide and citrate. Areas of extensive mineralization within the bone represent physical barriers to the extraction reagents and therefore prevent the release of DNA molecules. Most of the current DNA extraction protocols for bones and teeth are based on the incubation of powdered material in an ethylene diamine tetra-acetic acid (EDTA)-containing extraction buffer. The EDTA both demineralises the bone (to an extent dependent on the EDTA concentration and the volume of extraction buffer) and inactivates DNAses by chelating bivalent cations such as Mg++ or Ca++.

Bone and tooth extraction protocols often involve the incubation of powdered material in a lysis buffer, followed by the collection of the supernatant. When the supernatant is collected, undissolved powder which also contains unextracted DNA, is discarded. Alternative extraction protocols use demineralization steps that extensively wash/soak bone powder in large volumes of EDTA prior to separation of the powder for extraction [2]. Again, however, DNA is discarded—this time in the EDTA wash solutions.

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1872-4973/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.fsigen.2007.02.006

Here, we present a highly efficient protocol for the recovery of DNA by full demineralization, resulting in full physical dissolution of the bone powder and quantitative recovery of all DNA released by the complete demineralization procedure.

2. Materials and methods

DNA extraction, PCR set-up and work was performed in a dedicated ancient DNA laboratory according to generally accepted safety and ancient DNA guidelines: the personnel involved wore disposable masks, lab suits, and sterile latex gloves. All equipment for DNA extraction was cleaned with a 20% bleach solution and UV irradiated at 254 nm in a cross-linker for 20–45 min. Additionally, all real-time and STR PCR set-up was performed in a separate laboratory facility.

2.1. Bone samples

Fourteen human bones in various states of preservation, ranging in age from 5 to 100 years *post-mortem*, were selected for study. Six of these bones had previously been extracted following the protocols of the Armed Forces DNA Identification Laboratory (AFDIL) and successfully sequenced [3].

2.2. Pre-treatment of samples

Each sample was extensively sanded using an aluminium oxide sanding stone attached to a dremel tool (Dremel: Racine, WI). The entire surface of the bones was completely sanded to remove potential exogenous DNA. Then the samples were sonicated in 20% bleach for 5 min. Following the bleach wash, the bones were rinsed in UV-irradiated water and then sonicated for five additional minutes in UV-irradiated water. A final sonication wash was performed using 100% ethanol, and the bone was placed in a sterilized fume hood to air-dry overnight.

2.3. Physical and chemical breakup

The next day the samples were equally divided and powdered using two different techniques. Five of the 10 samples were powdered in a cryogenic impact grinder (CertiPrep 6750 Freezer Mill, Spex/Mill, Spex, Metuchen, NJ) following the manufacturer's instructions. One sample was powdered in a sterilized Waring MC2 blender cup (Warring:-Torrington, CT) and 1.0/1.2-L laboratory blender mortar. The remaining four samples were powdered using both methods (Table 1).

For each sample, bone powder was extracted using the AFDIL casework protocol as described in Edson et al. [3]. Briefly, this protocol entails incubation of approximately 1–2 g of bone powder in 3 mL of an extraction buffer (10 mM Tris, pH 8; 100 mM NaCl; 50 mM EDTA, pH 8.0; 0.5% SDS) and 100 μL 20 mg/mL Proteinase K at 56 °C with a gentle agitation (overnight). The next day, an organic extraction procedure of the raw extracts was performed. Purification and concentration of the extracted DNA was carried out using multiple TE buffer (10 mM Tris; 1 mM EDTA, pH 7.5) washes in a Centricon 100 centrifugal filter unit (Millipore).

We have previously determined (data not shown) that for each gram of powder, 15 mL of EDTA 0.5 M was necessary to completely dissolve the bone powder. In this study, we compared the AFDIL casework and the total demineralization protocols, using between 0.6 and 1.21 g of finely ground bone powder (Table 1 (already described above)). For each sample extracted using the demineralization buffer, the bone powder was incubated overnight in 9–18 mL of extraction buffer (EDTA 0.5 M, 1% lauryl-sarcosinate) and 200 μL of 20 mg/mL Proteinase K, in a rotary shaker at 56 °C. DNA was extracted with an equivalent volume of phenol/chlororm/isoamyl alcohol (25:24:1). The supernatant was concentrated to a volume slightly less than 2 mL using Centrifugal Filter Units (30 kDa, Amicon Ultra-15, Centricon+20, or Centriplus from Millipore). The remaining 2 mL of DNA extract were transferred into a Centricon 30 centrifugal filter unit (Millipore) and washed three times with irradiated water (UltraPure™

Table 1
Treatments and relative quantities of mtDNA detected in samples extracted either by total demineralization or casework protocols

Samples	Freezer mill method			Blender cup method		
	Bone powder (g)	A, demineralization	C, casework	Bone powder (g)	B, demineralization	D, casework
1	1.02	54	6	1.0	33	6
2	1.0	79	7	0.8	56	NR
3	1.0	42	2	1.02	43	6
4	0.6	2.5	0.8			
5	0.9	34	0.9			
6	1.0	10,668	2379			
7	1.0	393	158			
8	1.02	125	42	1.21	400	36
9	1.0	191	52			
10				1.01	20	0.07

Rows A and C were processed using the freezer mill, bone powder quantity listed (in g). Rows B and D were powdered with the blender cup method, bone powder quantity listed (in g). See Section 2 for detailed description of the real-time PCR assay results.

DNase/RNase-Free Distilled Water, Invitrogen). The final volume of all extracts was 100 μL.

2.4. Real-time DNA quantification and Inhibition monitoring

Each DNA extract was quantified using a real-time assay for relative quantification of a 143 bp fragment of mitochondrial DNA (modified after [4]). All samples were run in comparison to known quantities of 9947a DNA (Promega, Madison, WI). Reported values represent the quantity of mtDNA detected in the sample as compared to the quantity of mtDNA amplified from a known concentration (*total genomic and mtDNA*) of standard 9947a. For example, a result of 10 pg/μL indicates that the sample has the same quantity of mtDNA as a 9947a standard, whose total genomic and mtDNA concentration is 10 pg/μL. Thus, the quantities reported should be interpreted as relative values, not absolute values of mtDNA.

Internal positive controls (IPCs) were used for the detection of PCR inhibitors in our DNA extracts. Because inhibitors can skew the results of real-time data we diluted the extracts for quantitation and report quantification results only for assays where the IPC showed no sign of inhibition. Negative controls and reagent blanks were included in every step of the study and never showed any sign of contamination.

2.5. mtDNA and STR typing

mtDNA was sequenced as in [3]. Low copy number STR amplifications were conducted using the PowerPlex 16 system (Promega Corporation, Madison, WI) or the Yfiler system (Applied Biosystems, Foster City, CA). Thermal cycling temperatures and times were performed according to the manufacturer's recommendations. However, for each multiplex, twice the recommended Taq concentration and six additional PCR cycles were used (36 cycles). PCR products were separated on an Applied Biosystems 3100, and analyzed using Genescan software version 3.7. Genotyper version 3.7 was used to assign allele calls to electropherograms, using the allelic ladders provided in the respective kits as references.

3. Results

3.1. Total demineralization protocol versus standard protocol

Experiments were conducted to address the following questions. First and foremost, we were interested in comparing the DNA yield from a protocol focusing on complete demineralization of the bone powder to a standard protocol that involves the disposal of significant quantities of undissolved skeletal material (data not shown). In this particular experiment, we also investigated any potential benefits of using a freezer mill versus a blender cup. Since freezer mills tend to grind bone more finely than do blender cups, we were interested in determining whether or not this resulted in increased DNA yields. A total of 28 extractions were performed: for nine samples, the bones were ground with the freezer mill and the powder equally split for subsequent extraction with the total demineralization and standard extraction protocol. For five specimens, bones were ground in a blender cup and extracted with both protocols as outlined above. The results are summarized in Table 1, with relative quantities of mtDNA, according to real-time data, listed for each sample, grinding method and extraction method. Six samples with a known mtDNA haplotype from previous casework investigations showed concordant results with our total demineralization protocol. In every extraction using the demineralization protocol, we observed total dissolution of the bone powder. For each comparative extraction, the total demineralization procedure yielded higher amounts of DNA than the standard protocol: on average, approximately 4.6 times more DNA was recovered with the new protocol. However, for any given bone sample anywhere between 2.5 and 100+ times more DNA was recovered.

3.2. Freezer mill versus blender

For those samples extracted with all four treatments, DNA yields from freezer mill extractions did not consistently yield more DNA. In fact, on a per gram basis of bone powder (comparing both protocols), the average DNA yields from the blender cup were actually higher than those from the freezer mill (65 pg/μL compared to 44 pg/μL). On a sample by sample basis, however, the results were inconsistent. Four of the comparative extractions yielded more DNA with the freezer mill and four extractions yielded more with the blender cup. For each of these categories (freezer mill and blender cup) two of the extractions were conducted with the total demineralization protocol and two with the standard protocol. So, while it is clearly beneficial to use the total demineralization extraction, the benefits of one grinding method over the other appear minimal.

3.3. Reduction of sample material

Given the increased yields observed with the demineralization extraction protocol, another parameter we were interested in varying was the quantity of starting skeletal material.

Table 2
Treatments and relative mtDNA quantities of samples extracted using the total demineralization protocol vs. the standard protocol

Sample	Extraction	Bone powder	Real-time data
A	Demin.	0.2	2.83
	Casework	1.96	0.16
		1.65	0.16
B	Demin.	0.2	28.56
	Casework	2.02	0.34
		1.29	1.33
C	Demin.	0.2	5.95
	Casework	1.93	0.18
		2.16	0.33
D	Demin.	0.2	31.42
	Casework	2.04	1.19

See Section 2 for detailed description of the real-time PCR assay results.

Table 3
STR profiles obtained from LCN STR analysis

Markers	Sample 3		Sample 8				Sample 9	
	Demineralization	Casework	Demineralization (FM)	Demineralization (BC)	Casework (FM)	Casework (BC)	Demineralization	Casework
D3S1358	16, 17	16, 17	15, 18	15, 18	–	–	14, 15	–
TH01	7, 8	8, –	9.3, –	9.3, –	–	–	9, 9.3	9, –
D21S11	24.2, 29		29, –	29, –	–	–	30, –	–
D18S51	–	–	–	–	–	–	–	–
Penta E	–	–	–	–	–	–	–	–
D5S818	11, 13	11, 13	11, 12	11, –	–	–	11, 12	12, –
D13S317	10, 12	10, 12	–	8, 13	–	–	10, 12	–
D7S820	8, 9	–	–	–	–	–	11, 12	–
D16S539	–	–	–	–	–	–	9, 12	–
CSF1PO	–	–	9, –	–	–	–	9, 11	–
Penta D	–	–	10, –	–	–	–	–	–
Amelo.	*X*, *Y*	*X*, *Y*	*X*, *Y*	*Y*, –	*X*, –	*X*, –	*X*, *Y*	*X*, –
vWA	16, 18	16, 18	17, –	17, –	–	–	18, –	18, –
D8S1179	15, –	15, –	14, –	–	–	–	13, –	–
TPOX	–	8, –	–	8, –	–	–	9, –	–
FGA	–	–	22, –	–	–	–	21, –	–

Sample 8 was extracted with both, total demineralization and casework protocol, using either the freezer mill (FM) and a blender cup (BC) method.

Because the standard protocol requires between 1 and 2 g of bone powder, in many cases smaller elements go untested because the likelihood of successful DNA profiling is small. If the demineralization protocol produces sufficient and amplifiable quantities of DNA from small bone fragments, then the spectrum of skeletal elements that qualify for DNA testing broadens. In order to address this question, we tested four different bone fragments. Each bone was extracted with the standard protocol, using 1–2 g of bone powder, and the modified protocol, using 0.2 g of bone powder. In all samples tested, DNA yields from the total demineralization protocol (0.2 g of bone powder) were greater than the yields from 5 to 10 times more bone powder using the standard extraction (Table 2). On the basis of DNA-yield per gram bone powder, the total demineralization protocol using 0.2 g of bone powder resulted in an average of 228 times more DNA than the standard protocol using 1–2 g. Comparing this to the 4.6-fold improvement seen when the same amount of bone powder used for both extraction methods, our data suggest that extraction efficiency is much higher for smaller samples amounts. These observations are derived from a limited set of experiments that should be expanded prior to drawing definite, generalizable conclusions. Toward this goal, additional experiments currently underway further support these observations (data not reported).

Another benefit of the reduction of sample material for DNA extraction appeared to be the reduction of inhibition problems as assessed by shifts in the Ct of the internal positive control of the real-time quantitative PCR assay. Our findings (data not shown) confirmed the observation that when using more bone powder, the quantity of co-extracted inhibitors also increased. This problem can be overcome either by adding an additional purification step (e.g. the Cleamix kit from Talent (Trieste, Italy) or the PCR purification kit from Qiagen) or by using more Taq Polymerase during the amplification. However, by simply reducing the amount of input bone powder, inhibition can be minimized without additional reagents and/or steps that may also increase the risk of contamination.

3.4. STR analysis

In order to assess the practical implications of increased DNA yields by total demineralization versus our standard protocol in a casework setting, four samples from the first side-by-side experiments (equal quantities of bone powder) that showed relatively high mtDNA quantities (>100 pg/μL—Table 1) were subjected to STR testing using low copy number (LCN) analysis [5]. Samples 6 and 7 gave either full or partial profiles using either casework or total demineralization protocol amplified with the PowerPlex® 16 kit (Promega, Madison, WI). As expected, the total demineralization protocol proved to be most beneficial in those cases in which the DNA yields from the standard extraction protocol were poor. For example, the LCN profile recovered from Sample 8, extracted with total demineralization (powdered either with the freezer mill or the blender), gave a partial profile employing LCN techniques (Table 3). In contrast, this sample, when extracted with the standard AFDIL protocol, produced no STR results beyond a partial Amelogenin profile. Sample 9 gave a partial profile for both extraction methods. However, 17 more alleles at 9 additional loci were obtained with the total demineralization technique (Table 3). This was also the case, though the results were not quite as compelling, for a sample of relatively low mtDNA quantity (Sample 3) tested with LCN STR analysis. For all samples analyzed in this study, the extracts from the total demineralization protocol outperformed those from the standard protocol.

4. Conclusions

We have demonstrated that an extraction protocol that includes complete demineralization of the bone/tooth powder

significantly increases DNA yields and, therefore, improves DNA typing results from degraded skeletal elements. Furthermore, we have shown that adequate quantities of DNA can now be recovered from comparably small quantities of starting material. Hence, our demineralization protocol provides a significant advantage for successful DNA extraction, especially for challenging specimens previously discounted in forensic DNA typing.

The benefits of total demineralization were first demonstrated by Hagelberg and Clegg [2], who demineralized the bone powder in EDTA washes, but likely discarded DNA with each wash step (we have demonstrated that this occurs by concentrating such EDTA wash solutions and extracting significant quantities of DNA, not shown). One of the primary benefits of the protocol we describe is that the EDTA is a component of the lysis buffer and thus no DNA is lost in EDTA washes. A similar approach has been described in a study using dialysis against EDTA as a means for demineralizing bone and teeth, while fully retaining the liberated and purified DNA [6]. We have also experimented with dialysis in a number of formats, and while effective, the approach requires manipulation of the solutions which could be more difficult to perform from the standpoint of contamination avoidance. We prefer the method reported here as the manipulations are minimal and simple—however, when larger bone fragments are used, the method does involve concentration of relatively large volumes of solution, which also is not without some concern for increased contamination risk. In practice, though, we have not observed increased contamination.

In many cases, a standard extraction protocol that does not involve complete dissolution of the bone powder may be adequate for the recovery and typing of mtDNA fragments. However, we have encountered several situations in which mtDNA amplicons were only recovered when the total demineralization technique was applied. Moreover, even considering those cases for which demineralization may not be considered a necessity, the modified demineralization technique improves the number and variety of DNA tests that can be applied to any particular extract, by increasing the yield of extracted DNA and/or reducing the required sample material (more DNA, more options). Additionally, we have shown that the modified total demineralization protocol clearly increases the quality of STR profiles recovered from severely degraded specimens. While the improved quality of recovered DNA observed in this study is certainly a result of increased DNA quantity, our findings may also result from the recovery of higher quality templates. Complete dissolution of the bone powder likely permits access to larger, high-quality fragments of endogenous DNA that are harboured in exceptionally dense crystal aggregates of the bone matrix [7].

Acknowledgments

We would like to thank Suzanne Barritt-Ross, Christine Boyer, Suni Edson, Sarah Bettinger and Darren Haliniewski (AFDIL) for performing DNA extractions. Dr. Mark Leney and Dr. Alec Christensen (JPAC) for allowing us to use their material. We also thank James Canik, Kevin Carroll, Brion Smith, Dr. Louis Finelli and James Ross (AFDIL) for logistical, administrative, and computer support.

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