Highly Effective DNA Extraction Method for Nuclear Short Tandem Repeat Testing of Skeletal Remains from Mass Graves

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Aim To quantitatively compare a silica extraction method with a commonly used phenol/chloroform extraction method for DNA analysis of specimens exhumed from mass graves.

Methods DNA was extracted from twenty randomly chosen femur samples, using the International Commission on Missing Persons (ICMP) silica method, based on Qiagen Blood Maxi Kit, and compared with the DNA extracted by the standard phenol/chloroform-based method. The efficacy of extraction methods was compared by real time polymerase chain reaction (PCR) to measure DNA quantity and the presence of inhibitors and by amplification with the PowerPlex 16 (PP16) multiplex nuclear short tandem repeat (STR) kit.

Results DNA quantification results showed that the silica-based method extracted on average 1.94 ng of DNA per gram of bone (range 0.25-9.58 ng/g), compared with only 0.68 ng/g by the organic method extracted (range 0.0016-4.4880 ng/g). Inhibition tests showed that there were on average significantly lower levels of PCR inhibitors in DNA isolated by the organic method. When amplified with PP16, all samples extracted by silica-based method produced 16 full loci profiles, while only 75% of the DNA extracts obtained by organic technique amplified 16 loci profiles.

Conclusions The silica-based extraction method showed better results in nuclear STR typing from degraded bone samples than a commonly used phenol/chloroform method.

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Analysis by nuclear short tandem repeats (STRs) has been proven invaluable for identifications in mass fatality incidents such as plane crashes, terrorist attacks, natural disasters, armed conflict, or any other case where traditional methods of identification are insufficient (1-4). DNA-STR testing often provides the strongest evidence of identity in cases of high degradation of human remains.

Bone and teeth are excellent sources of DNA for human identity testing which uses STRs or mitochondrial DNA (mtDNA) (5-7), and in cases of extreme degradation may be the only suitable material available for successful typing. However, relatively specialized techniques are required for the extraction of DNA from bone, particularly when the bones have been exposed to adverse environmental conditions and DNA is degraded and/or present in low amounts. The physical and chemical barriers in bone that protect the DNA from environment and microbial assault also hinder the access of reagents in the extraction process (8,9). Another major difficulty is co-extraction of compounds inhibitory to the polymerase chain reaction (PCR) (10-13), particularly with skeletal samples exposed to soil or other environmental contaminants.

It is beyond the scope of this article to review the wide variety of DNA extraction methods that have been reported for bone, but two of the major approaches, with innumerable variations, are 1) organic extraction methods involving phenol/chloroform (7,14,15) and 2) silica-binding methods where guanidinium-based chaotropic salts are used both to disrupt proteins, as well as mediate highly specific binding of DNA to silica particles via ionic salt bridges (12,16,17). Because of the high specificity of DNA binding to silica, inhibition is often less of a problem with this method, and silica binding purifications are sometimes used as secondary clean-up steps after organic extractions to remove inhibitors (15).

Nuclear STR profiling has been found highly successful by the International Commission on Missing Persons (ICMP) in their large-scale effort to identify skeletal remains from mass graves in the former Yugoslavia (5,6). In this work, the ICMP has used a silica-binding method based on substantial protocol modifications of a commercially available DNA extraction kit. This DNA extraction method was also used very successfully on more than 1800 bone and tooth samples from the 2004 Asian tsunami that were processed by the ICMP (our unpublished data).

In this article, we compared the quantitative performance of the ICMP-developed silica extraction method with a standard organic phenol/chloroform extraction method on the same set of femur samples. Also, we evaluated the levels of PCR inhibitors present in the extract, as assessed by quantitative PCR with internal controls and the amplification obtained with multiplex nuclear STR testing. This comparative study will hopefully assist other groups in adopting simple and effective protocols for DNA isolation from degraded bone in casework relating to mass disasters, terrorist attacks, or mass graves.

Materials and methods

ICMP laboratory setup

The bone STR testing laboratory is divided into six areas as follows: physical cleaning, chemical cleaning, grinding, DNA extraction, PCR setup, and PCR amplification/fragment analysis. All work is done in plexiglas hoods, except for the sequencer loading. All areas were cleaned daily with 0.5% sodium hypochlorite and equipment is not mixed from the different areas. All staff wore laboratory coats, facemasks, hairnets, nitrile gloves, and separate labcoats are used for each area. All staff regularly bleached their gloves to prevent cross contamination. All consumbles that came in contact with DNA extracts were purchased as DNA-free and further UV irradiated (>1.0 J/cm) for at least 15 minutes.

Bone samples

Twenty femur samples were randomly selected for this study, as it was supposed that this number would be high enough to detect significant trends in the performance of the two methods and would permit a reasonably representative range of sample preservation. The femur samples were from people killed in the Balkans during the armed conflicts between 1992 and 1995. The initial weight of the samples was between 12 and 22 g. The remains were exhumed from mass graves.

Cleaning of bone samples

The surface materials such as bone marrow, adhering tissue, and dirt were cleaned from the bones using a rotary sanding tool (Dremel, Racine, WI, USA). Following the removal of surface material, an additional 2-3 mm of the bone was ground away to remove other contaminants. The remaining bone was then placed in a 50 mL tube and further cleaned by inversion for 30 seconds in 30 mL of distilled water, inversion for 30 seconds in 10% commercial bleach (0.5% sodium hypochloride), and inversion for 30 seconds in 96% ethanol. Following the chemical cleaning, bones were dried at 50°C for two hours.

Grinding of bones

Chemically cleaned bones were ground, in a sterile hood, into a fine powder using a blender apparatus (Waring, Torrington, CT, USA). The bone powder was then divided equally into two 50 mL tubes, each containing between 5.6 and 9.8 g of the sample.

Silica-based DNA extraction

The procedure is based on Qiagen's Blood Maxi Kit (Qiagen, Hilden, Germany), with substantial protocol modifications. The ground bone powder was incubated in 15 mL of ATL extraction buffer with 10 mg of proteinase K (20 Units/μg) (Invitrogen, Carlsbad, CA, USA) and 300 μL of 1 M DTT (Invitrogen) and incubated for 18 hours at 56°C in a shaking water bath. A second digestion was performed by addition of 14 mL of buffer AL (Qiagen), inversion for 30 seconds, and incubation at 70°C for one hour in a water shaking bath. The remaining bone material was removed by centrifugation (Eppendorf, Hamburg, Germany) at ~1000×g for 5 minutes and the supernatant transferred to another 50 mL tube. Twenty two milliliters of 96% ethanol was added and the samples were mixed by inversion for 15 seconds. The DNA was bound to Qiagen Blood Maxi columns by adding 15 mL of the extraction mix three times, centrifugation at 2000×g for 3 minutes in a swinging bucket rotor, and discarding the flow through. The columns with bound DNA were then washed with 10 mL of AW1 buffer (Qiagen), centrifuged at 2000×g for 3 minutes, and the flow through was discarded. A second wash was performed by addition of 10 mL AW2 buffer (Qiagen), centrifugation at 2000×g for 3 minutes, and discarding the flow through. The remaining AW2 buffer was removed by centrifugation for 10 minutes at 2000×g. The DNA was eluted by the addition of 3 mL of AE buffer (Qiagen) preheated to 72°C and centrifugation at 2000×g for 3 minutes. A second elution was performed just as the first one. The 6 mL of eluted DNA was concentrated to approximately 0.5 mL by addition to 15 mL Centriplus YM-100 columns (Millipore, Billerica, MA, USA) and centrifugation at 2000×g for 3 minutes in a swinging bucket rotor. The remaining 0.5 mL of retentate was then removed and added to a Centricon YM-100 column (Millipore), and spun for 15 minutes in a 38° fixed angle rotor until the retentate reached approximately 50 μL. The retentate was washed twice by addition of 2 mL of water (ultra pure) followed by centrifugation for 15 minutes in a 38° fixed angle rotor until the retentate reached approximately 50 μL. The retentate was then removed to a 1.5 mL tube. The Centricon membrane was washed with 100 μL of water and that wash solution was then added to the 1.5 mL tube with the concentrated DNA.

The 150 µL of DNA was further concentrated to approximately 100 µL in a vacuum concentrator (Eppendorf).

Organic based DNA extraction

The ground bone powder was incubated with shaking at 55°C for 18-24 hours in 20 mL of organic extraction buffer (50 mM Tris-HCl, 100 mM NaCl, 50 mM ethylenediamine tetraacetic acid [EDTA], 0.5% SDS, pH = 8), and 20 mg of proteinase K(20 Units/µg) (Invitrogen). Following the incubation, 20 mL of phenol/chloroform/isoamylalcohol (25:24:1) (Invitrogen) was added, the tube was inverted for 30 seconds, and spun for 20 minutes at 2000 × g. The supernatant was transferred to another tube containing 20 mL of chloroform/isoamylalcohol (24:1), inverted for 30 seconds, and spun for 20 minutes at 2000 × g. One additional chloroform/isoamylalcohol extraction was performed exactly as the first one. The dilute DNA solution that remained after the three organic extractions was concentrated to 0.5 mL using a Centriplus YM-100 concentrator. The remaining 0.5 mL of retentate was then removed from the top of the Centriplus column, added to a Centricon YM-100 column (Millipore), and spun for 15 minutes in a 38° fixed angle rotor until the retentate reached approximately 50 µL. The retentate was washed twice by addition of 2 mL of water (ultra pure), followed by centrifugation for 15 minutes in a 38° fixed angle rotor until the retentate reached approximately 50 µL. The retentate was then removed to a 1.5 mL tube. The Centricon membrane was washed with 100 µL of water and the wash solution was then added to the 1.5 mL tube with the concentrated DNA. The 150 µL of DNA was further concentrated to approximately 100 µL in a vacuum concentrator (Eppendorf).

Amplification and fragment analysis

Samples from both extraction methods were PCR amplified using 10 µL of the final extract. Amplification was performed on an Applied Biosystems (ABI, Foster City, CA, USA) 9700 thermocycler using the Promega (Madison, WI, USA) PowerPlex 16 (PP16) kit. The recommended amplification conditions were followed, with only one modification. The extension time at 72°C was doubled from 30 seconds to one minute for the first 10 cycles, and from 45 seconds to 90 seconds for the next 22 cycles. Amplified STR fragments were analyzed on an ABI 3100 DNA sequencer using the exact directions listed in the manual provided by Promega. The raw data files were analyzed using ABI GeneScan and Genotyper (versions 3.7) software. The PowerTyper Macro V.2 (Applied Biosystems) was used to genotype the samples. Reportable loci were those that resulted from duplicate amplifications that are free from ambiguities between each other.

DNA quantification and assessment of PCR inhibitors present in the extracts

All DNA extracts were quantified in duplicate, by real time PCR, using the Applied Biosystems total Human Quantifiler® system and the Applied Biosystems 7700 Sequence Detection System. This quantification system involves a duplex PCR reaction with two independent sets of PCR primers and TaqMan probes. One primer set and the 6-FAM-labeled TaqMan probe is specific to human DNA, while the other primer set and VIC-labeled Taqman probe targets a synthetic sequence that is spiked into each amplification reaction as an internal positive control (IPC). The 6-FAM signal is monitored by an ABI sequence detection system and quantity can be calculated on the basis of the characteristics of the amplification signal. The amplification plot of the VIC-labeled probe is used to determine if any PCR inhibitors are present in the DNA extracts. The presence of PCR inhibitors retards the onset of exponential amplification in the PCR, and therefore can be detected by an increased Ct value for the IPC (which

should remain at a constant value in the absence of inhibitors).

Results

Assessment of inhibition

The results of the inhibition study (Table 1) showed that the IPC for the standard DNA samples required on average 26 cycles in order to reach the early log phase of the PCR reaction. DNA samples that were extracted by ICMP-developed silica extraction method required on average 27 cycles and the samples extracted by the organic method required on average 30 cycles to reach the same phase of the amplification. With the organic method, the samples 9100986 and 9100990 required over 30 cycles to reach the early log phase of the amplification and the samples 9102654, 9102658, and 9102659 required over 33 cycles. The corresponding silica extracts showed only minor levels of inhibition.

Quantification by real time PCR

Real time PCR quantification showed that ICMP silica-based extraction technique produced on average three times more DNA than the organic-based method (Table 2). The amount of DNA extracted per gram of bone powder by silica method ranged from 0.25 ng/g to 9.58 ng/g (median: 1.49), while the results of the organic method ranged from 0.0016 ng/g to 4.48 ng/g (median: 0.27).

STR results

All of the 20 samples extracted with the silica method produced 16 full loci profiles. However, 6 of the organic extracts failed to provide full profiles (Table 2). Not surprisingly, these were from bones that gave lower amounts of DNA with the organic method. All of these samples gave higher amounts of DNA with the silica method and, in fact, were not always among the lowest yielding samples. However, the bone samples that gave relatively higher amounts of DNA with the silica method also gave relatively higher amounts with the organic method.

Table 1. The presence of inhibitors in the DNA samples extracted by the International Commission on Missing Persons (ICMP) silica or standard organic methods

	Threshold cycle for log phase amplification with method*	
Sample	silica	organic
9100822	28.3	28.3
9100828	26.5	27.8
9100830	26.5	27.6
9100835	26.4	27.4
9100838	26.6	27.3
9100850	27.5	29.1
9100854	26.7	27.4
9100856	27.0	27.1
9100880	27.0	28.5
9100986	27.3	30.7
9100990	26.8	30.1
9100993	26.7	28.8
9101158	27.6	28.7
9101338	26.6	27.0
9102612	26.9	29.5
9102654	27.4	33.5
9102658	27.0	33.2
9102659	28.0	33.5
9102665	27.2	27.2
9102913	27.3	27.4

*Values are the average of two quantitation runs.

Table 2. Quantity of DNA isolated by International Commission on Missing Persons (ICMP) silica or standard organic method

		Method			
		silica		organic	
Sample	Mass (g)	concentration (pg/μL)	bone (ng/g)	concentration (pg/μL)	bone (ng/g)
9100990	9.8	147	1.50	0.16*	0.0016*
9100856	8.8	82	0.94	0.22*	0.0025*
9101158	7.3	197	2.70	6*	0.0777*
9100880	7.6	135	1.78	6*	0.0751*
9100850	5.7	164	2.88	7	0.1160
9102659	8.9	23	0.25	9	0.0965
9100830	8.1	199	2.45	11	0.1339
9100986	7.1	48	0.68	11	0.1566
9100835	5.6	53	0.95	11*	0.2017*
9102658	8.1	39	0.48	15*	0.1887*
9100993	5.9	92	1.56	20	0.3380
9102665	8.0	44	0.55	27	0.3391
9100828	8.4	125	1.48	31	0.3662
9100854	5.7	50	0.88	39	0.6888
9100822	8.7	92	1.06	63	0.7246
9102654	5.6	186	3.31	68	1.2116
9102913	7.0	174	2.49	85	1.2192
9100838	8.0	152	1.91	121	1.5149
9102612	8.1	108	1.33	141	1.7440
9101338	5.7	546	9.58	256	4.4880

*Fewer than 16 loci with reportable alleles.

Discussion

The silica-binding DNA extraction method examined in this study was proven extremely effec-

tive in providing PP16 STR profiles from bones and teeth that are 3-15 years postmortem and that were exposed to a wide variety of detrimental environmental conditions (5,6). Most often these have been in contact with the soil and the other decomposing bodies in mass graves. Additionally, this method, combined with STR typing, gave a 95.4% success rate on 1823 bone or tooth samples from the 2004 Asian tsunami that were typed by the ICMP (our unpublished data). The success rate of this method is substantially higher than that of initial trials on samples from former Yugoslavia mass graves extracted using standard organic methods by the ICMP (our unpublished data) or outside laboratories (18). The current study was performed on the same bone samples using both methods to verify and expand this comparative analysis. The silica method yielded significantly higher amounts of DNA with lower levels of inhibition than the organic method.

The samples chosen for this experiment were femur samples that contain the highest amount of DNA per gram of all skeletal elements, except teeth (6). Larger quantity of DNA isolated by the silica-based extraction technique may suggest that the digestion buffers ATL and AL are more efficient than the Tris/NaCl/SDS/50 mM EDTA organic extraction buffer at releasing DNA from bone samples. EDTA is also present in the proprietary Qiagen buffers, although the concentration is not publicly known. It has been established that full demineralization of bone powder by high EDTA concentration has a very beneficial effect on DNA recovery (9); however, the presence of substantial quantities of bone powder after the ICMP silica extraction indicates that demineralization is not complete. Another possible reason for the larger final amount of DNA could be that the Qiagen silica membrane is more efficient in recovering the DNA than the phase separation process of the organic extraction technique.

The real time PCR quantification system Quantifiler was chosen to quantify the DNA extracted from the bone samples because it is accurate over a wide range of DNA concentrations (0.023 ng/μL to >50 ng/μL) and it is also capable of assessing the levels of PCR inhibitory compounds in a DNA extract. The silica-based DNA extraction technique described here has been shown to isolate on average three times more DNA than the organic extraction method. The samples extracted by the ICMP silica method were all quantified at 23 pg/μL or higher, while only 45% of the samples extracted by the organic method were above this level. On the whole, organic extractions also showed substantially higher levels of inhibition, with 65% of the extracts showing a one-cycle or greater increase in the Quantifiler Ct values for the internal positive controls.

It is well known that when the amount of input DNA in a PCR reaction is reduced below 250 pg, the results can show stochastic effects, such as peak imbalance and allele dropout (19). All samples in this study that had more than 150 pg added to the PCR reaction gave successful amplification of all 16 STR loci; this included all of the silica method extracts. On the other hand, 10 organic extracts had from 60-150 pg added to the PCR reaction (in a template volume of 10 μL); 4 of these gave successful amplification for all 16 loci, 4 for at least 13 loci (but fewer than 16), and 2 gave seriously deficient partial profiles. Samples 9100990 and 9102658 did not give full profiles with the organic method, but since they were substantially inhibited (with Cts elevated by three and five cycles, respectively, compared to the silica method), it is not known if low DNA, inhibition, or both contributed to the lack of full results.

In conclusion, our experience with large numbers of bone samples showed that it was difficult to predict DNA yield or profiling success from the appearance or characteristics of bones. Consistent with this, the samples that gave low-

er amounts of DNA or partial profiles showed no characteristics with regard to morphology or environmental context that would permit speculation as to why they may have failed, or why the correlation between yields of the two methods varied among samples. However, given a representative sampling of bones, it is clear that the higher level of DNA recovery with the silica method in some cases is essential to recovering a full DNA profile. The ability to successfully recover nuclear STR profiles from degraded skeletal remains has huge applicability to forensic identification efforts in mass disasters, terrorist attacks, or mass graves. It is important to compare and document the characteristics and efficiency of various DNA extraction methods in order to provide the basis for the selection of the most successful techniques and to establish the capabilities and limitations of methods applied. The silica extraction method developed by the ICMP demonstrated a high success rate on a wide variety of challenging samples. This method performed substantially better in terms of DNA yield, absence of inhibitors, and success in STR profiling than a standard organic extraction approach.

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