

Research Article

Multiplexed SNP Typing of DNA after Demineralization and Organic Extraction from a Bone

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Abstract

Analysis of DNA from bones and teeth still suffers from low efficiency of extraction methods and poor reliability of results during forensic typing, because of co-extraction of microbiological contaminants and DNA degradation.

Here we tested 15 variants of DNA extraction from old human bone using silica columns, organic phenol-chloroform-isoamyl alcohol solution, and combination of those two, with additional decalcification of different duration time. Multiplex PCR and single nucleotide polymorphism genotyping were performed to estimate the efficiency of extraction process and the level of DNA degradation. In comparison with silica-based method, the organic extraction gave more complete profiles with shorter time of decalcification, also less allele drop-outs and higher fluorescence level, independently of the additional cleaning. The minimal duration of successful decalcification was 48h. Decalcification is an essential step prior to DNA extraction from a bone. Phenol-chloroform- isoamyl alcohol solution effectively extracts DNA which can be further used in forensic analyses.

Keywords: DNA extraction; Decalcification; Forensic science; Multiplex; Old bone; Single nucleotide polymorphism typin.

Introduction

Positive identification of human fossil remains is a crucial issue in a forensic work. For over the last two decades multiplex STR (short tandem repeats) profiling has become a routine procedure in cases of identifying victims of mass disasters, missing persons and paternity

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testing [1]. Another issues are cases of remains with no presumable relatives or museum specimens and historical personages [2]. In such cases SNP (single nucleotide polymorphism) profiling has become one of the most relevant techniques and is often used as a supplementary tool in externally visible characteristics (EVCs), ancestry or kinship analyses [3-5]. Based on multiplex reactions using a single nucleotide genotyping techniques, it is possible to estimate complex traits such as eye color (IrisPlex tool) [6], hair color (HIrisPlex tool) [4] mtDNA-based bio-geographic ancestry [7,8] or use genomic DNA-based 52-plex assay for identification purposes [9]. Rapid and sensitive SNP technology often gives positive results for extremely degraded samples when conventional STR typing fails. Still, it faces many problems, e.g. allele drop-outs and incomplete or no amplification due to the high degree of sample degradation. DNA extraction process can itself increase other problematic issues, such as further DNA degradation, presence of inhibitors or loss of DNA template. Therefore, an adequate sample preparation and extraction method are crucial steps for successful forensic typing. Despite many studies and techniques an efficient DNA extraction from solid tissues (bones and teeth) can still constitute a significant problem. Specific morphological structure of a bone as well as a variety of environmental conditions of bone preservation require peculiar handling with such a specimen [10]. There is usually a low level of endogenous DNA that is co-extracted with a large amount of exogenous microbiological DNA contamination from the surrounding of a bone, the bone itself or that is introduced during biological material collection and/or storage [11,12]. Moreover, DNA extracted from old samples is degraded and the chemical reagents used during extraction process can cause further damage. Another problem is the presence of contamination and inhibitors in samples which co-extract with DNA and can affect an efficient amplification in the downstream PCR, STR or SNP profiling [10,13,14]. However, sometimes bones are the only available specimen that can be the source of DNA. According to some authors, it is even better to extract DNA from bones or teeth than from poorly preserved soft tissue [13,15,16]. It is also important what is considered an "old" material, since environmental conditions seem affect DNA preservation much stronger than the passage of time [10]. Therefore, so far there is no universal protocol and distinct approaches are used depending on the stage of decomposition or time of the specimen's storage. Despite the fact that several commercial kits are designated to bones and teeth, still some authors choose more hazardous and complicated approaches or adapt kits designated to other tissues according to own lab experience and technical equipment.

Here we report the results of comparison of total of 15 different protocols for DNA extraction from an old bone. We used silica-based method, organic method using phenol- chloroform-isoamyl alcohol solution and combination of those two methods, with decalcification step of different duration prior to DNA extraction process. 6-SNP multiplex genotyping assay known as IrisPlex was used to verify the efficiency of DNA extraction process and estimate the level of DNA degradation and the possibility of successful amplification [6].

Materials & Methods

Bone preparation

All steps of the preparation and extraction procedures were performed in the laminar flow cabinet. We used the diaphysis of ulna from human male, that was stored for over 50 years in The Department of Anatomy, University of Medical Sciences in Poznan, Poland, in moderate temperature and humidity conditions, in a wooden-glass cabinet. The bone was cut into several pieces, 4-cm long each. All pieces were subjected to mechanical and chemical cleaning. We used metal file to remove the outer layer of the bone and then immersed each bone piece in 10% sodium hypochlorite solution (NaOCl) (Sigma) for 15 min. Then, the bones were left to dry completely and washed in 70% ethanol and water, twice. After that, the bones were submerged in liquid nitrogen for about 10 min and crushed into small fragments. All fragments were grinded to a fine powder using a metal blender. If not used immediately, the bone powder was frozen and kept in -20°C until further analyses.

DNA extraction

We performed 15 different variants of DNA extraction protocols. (Table 1) contains characterization of each variant of DNA isolation from bone tested in this study. One- and two-step extractions were carried out. During one-step extraction the bone powder was designated for the lysis and isolation process right after the bone was grinded. During two-step extraction we firstly applied the decalcification step (for 48h, 72h, 96h and 120h) using 0,5M EDTA (ethylene diamine tetra-acetic acid) solution, pH=8,0. For DNA isolation we used the silica-based method (QIAamp DNA Investigator Kit, Qiagen) and organic method, e.g. the Phenol-Chloroform-Isoamyl Alcohol (PCIA, 25:24:1) (Ambion) method. Additionally, the organic protocol was modified by adding the step of cleaning, using silica columns (Qiagen).

Decalcification process

In order to decalcify the bone 5ml of 0.5M EDTA (Sigma) was used for every 1g of bone powder and the samples were incubated in conical tubes sealed with Para film, on a rotator mixer with slow rotation (10 rpm), at room temperature (RT), overnight. EDTA solution was changed every 24 hours by centrifugation (2000 g for 5 min), discarding the supernatant and adding the fresh EDTA. To test different variants, the bone powder was collected, respectively, after 48h, 72h, 96h and 120h of demineralization, and frozen in -20°C until the next step.

Silica based method

400mg of the bone powder of samples S11-S15 was incubated using the ATL lysis buffer from QIAamp DNA Investigator Kit and the protocol was followed according to the manufacturer's instructions.

Organic method

We used self-prepared lysis buffer, containing 1mM dithiothreitol (DTT), 10mM Tris, 50mM EDTA (pH=8,0), 100mM NaCl and 0,5% sodium dodecyl sulphate (SDS). 1ml of the lysis buffer was used for every 500mg of the bone powder with addition of 30µl of proteinase K (20mg/ml, EURx). The lysis buffer was freshly prepared and the lysis was performed at 56°C in a thermoshaker with shaking (850 rpm), for 16h. After 16h of incubation 0,5ml of PCIA solution was added to all samples (S1-S10) and mixed by vortexing for 10 sec. Then, the samples were centrifuged for 15 min at 12 000 g at RT, to separate the layers. The upper DNA-containing aqueous phase of samples: S1, S3, S5, S7 and S9 was transferred to the new tubes and an equal volume of chloroform was added to the aqueous phase, mixed briefly and centrifuged at 12 000 g for 10 min at RT. This step was in order to remove any residual phenol and was proceeded twice.

Sample ID	Time of decalcification	Method of DNA extraction	Details	Comments	DNA concentration [ng/µl]
S1	0 h	PCIA	Lysis buffer containing EDTA, DTT, SDS, NaCl; Precipitation performed with 3M Sodium Acetate and 99,8% ethanol.	After 16h of lysis the bone sediment is still visible.	30.7
S2	0 h	PCIA + Silica			29.4
S3	48 h	PCIA		After 16h of lysis the bone sediment is still visible, although the amount of the bone powder is smaller than in undecalcified samples.	29.3
S4	48 h	PCIA + Silica			28.7
S5	72 h	PCIA			28.8
S6	72 h	PCIA + Silica		After 16h of lysis the bone powder dissolves completely.	31.7
S7	96 h	PCIA			13.1
S8	96 h	PCIA + Silica			26.9
S9	120 h	PCIA			13.5
S10	120 h	PCIA + Silica			
S11	0 h	Silica	The manufacturer's ATL lysis buffer; Precipitation performed with 99,8% ethanol.	After 16h of lysis the bone sediment is still visible.	5
S12	48 h	Silica			46
S13	72 h	Silica		30.4	
S14	96 h	Silica		After 16h of lysis the bone powder dissolves completely.	3.2
S15	120 h	Silica			15.5

Table 1: Characterization of organic/silica-based methods of DNA extraction from a bone performed in this study. We tested an organic methods (using phenol-chloroform-isoamyl alcohol in ratio 25:24:1, respectively), silica-based method, and a combination of those two, with or without an additional pre-treatment step with 0,5M EDTA as a decalcifying agent for a specified period of time. Different chemical reagents were used for lysis of a bone powder and for DNA precipitation, as indicated. DNA yields were estimated using Nano Drop measurements. DTT-dithiothreitol; EDTA-ethylene diamine tetra-acetic acid; PCIA-phenol-chloroform-isoamyl alcohol; SDS- sodium dodecyl sulphate.

After the second centrifugation the upper phase was transferred to the new tubes and the samples were treated with 2,5µl of RNAse A (EURx) and incubated for 5 min at RT. The DNA was precipitated using 10% of the volume of 3M Sodium Acetate, pH=5,2-7,0, and one volume of ice cold 99.8% ethanol. The samples were centrifuged at full speed for 15 min at RT to pellet the DNA and washed with 200µl of ice cold 75% ethanol. The DNA was suspended in 50µl of nuclease-free water and left at 4°C overnight to dissolve.

Modified organic method

Samples S2, S4, S6, S8 and S10 were proceeded to the additional cleaning using silica columns (Qiagen). For this purpose, the lysis and centrifugation steps were performed as in the classic organic protocol (see above). Next, the upper aqueous layer was transferred to the new tubes, treated with 2.5µl of RNAse A and incubated for 5 min at RT. Then, the samples were proceeded to the Qiagen silica columns protocol. The DNA concentration was estimated by the Nano-drop measurements. The reagent blank controls were prepared for each variant of extraction protocols.

DNA Analysis

All samples were genotyped using IrisPlex assay, which includes 6 SNPs in 6 genes. We performed multiplex PCR of those 6 variants followed by a minisequencing multiplex SNP typing using SNaPshot system. PCR and SBE (single base extension) primers were adopted from [17] and the SBE primers longer than 30bp were additionally HPLC purified (Oligo.pl). The first step of our analysis was the amplification of 6 fragments in one multiplex reaction. It was performed using 3µl of DNA extract, 1U of FastTaq polymerase (Roche), 200µM dNTP and defined concentration of each primer (Table 2), in a final volume of 10µl. Cycling conditions were as follows: 95 °C for 10min, 33cycles of 95°C for 13sec, 60 °C for 30sec, 72 °C for 30sec, and final extension 72 °C for 15min. PCR products were treated with Exo/SAP mix containing 20U/µl of Exonuclease I and 1 U/µl of Shrimp Alkaline Phosphatase (New England BioLabs) to remove unincorporated dNTPs and primers. The conditions were 37 °C for 60 min followed by 80 °C for 15 min.

The second step was a single base extension reaction performed in a total volume of 5µl using 2µl of ABI SNaPshot ready reaction mix (Applied Biosystems), 2µl of PCR product and defined concentration of each SBE primer under the following conditions: 95 °C for 2min and 25 cycles of 95 °C for 10sec, 50 °C for 5sec and 60 °C for 30sec. The products were cleaned using 1U/µl of Shrimp Alkaline Phosphatase (New England BioLabs), under conditions: 37 °C for 60min followed by 80 °C for 15min. 2µl of cleaned product was added to 7.75µl mix of formamide (Applied Biosystems) and 0.25µl of size standard (GeneScan 120 LIZ Size Standard, Applied Biosystems) and analyzed by the capillary electrophoresis on the ABI 3130 Genetic Analyzer (Applied Biosystems) using 36-cm length capillary and POP-7 polymer. Run parameters were as follows: injection voltage of 2.5 kV for 10sec and electrophoresis for 20min at 60 °C. The results were analyzed with GeneMapper v4.0 (Figure 1).

Each sample was run total of three times. The reference samples were the buccal swab DNA from brown-eyed and blue-eyed Caucasian individuals (Figure1a, Figure1b). The amplicons for each SNP variant from 6-plex tested in this study were run in separate reactions to adjust the PCR conditions, primers' concentrations and to evaluate the mobility of the SBE products in our genetic analyzer (not shown).

Results and Discussion

The aim of our work was to determine the best method of DNA extraction from an old bone for the highest recovery of un degraded and amplifiable DNA. Most authors describe the efficiency of forensic DNA purification in the reference to the successful STR profiling. Here, we describe several variants of DNA extraction protocol from the old human bone to be tested in a multiplex single nucleotide polymorphism genotyping, commonly applied in routine forensic analyses. Based on the SNP profiles from all samples we assessed a consensus profile of tested DNA which includes all possible alleles that showed up in the three replicates in at least one sample. We discussed the efficiency of amplification in the reference to the presence or absence of expected alleles, as well as the fluorescence intensity measured as RFU (relative fluorescence unit) level. According to the standard STR typing, the level of 50RFU was taken as a threshold [17, 18].

SNP ID	Chromosome position	Gene	PCR primers (5' -> 3')	PCR product	Primer conc	SBEprimer (5' -> 3')	Primer direction	Total primer length	SBE primer conc	Alleles detected
rs1800407	15:25903913	OCA2	TGAAAGGCTGCCTCTGTCT	127 bp	0,4 µM	ttttttGCATACCGGCTCTCC	Forward	23 bp	0,1 µM	G/A
			CGATGAGACAGAGCATGATGA							
rs16891982	5:33987450	SLC45A2	TCCAAGTTGTGCTAGACCAGA	128 bp	0,4 µM	tttttttttAAACACGGAGTTGA	Forward	29 bp	1 µM	G/C
			CGAAAGAGGAGTCGAGGTTG			TGCA				
rs12203592	6:341321	IRF4	ACAGGGCAGCTGATCTCTTC	115 bp	0,4 µM	ttttttttttttttTTGGTGGGTA	Forward	35 bp	0,3 µM	C/T
			GCTAAACCTGGCACCAAAAAG			AAGAAGG				
rs12913832	15:26039213	HERC2	TGGCTCTCTGTGCTGATCC	87 bp	0,4 µM	ttttttttttttttttttttGCGTGCAG	Reverse	41 bp	1,2 µM	C/T
			GGCCCTGATGATGATAGC			AACTTGACA				
rs1393350	11:88650694	TYR	TTCCTCAGTCCCTCTCTGC	80 bp	0,4 µM	ttttttttttttttttttttTTTGTA	Reverse	47 bp	1,1 µM	C/T
			GGAAGGTGAATGATAACACG			AGACCACACAGATT				
rs12896399	14:91843416	SLC24A4	CTGGCGATCCAATCTTTGT	104 bp	0,4 µM	ttttttttttttttttttttTCTTT	Forward	53 bp	1,125 µM	G/T
			CTTAGCCCTGGGTCTTGATG			AGGTCAGTATATTTGGG				

Table 2: Characteristics of 6 SNP markers used in the IrisPlex assay, including PCR and SBE primer sequences and concentrations, PCR-polymerase chain reaction; SBE-single base extension

First of all, a rough mechanical cleaning of the outer surface of the bone, followed by a chemical cleaning, were performed. It has been previously shown that removing the outer surface of the bone is a critical step as it greatly reduces the amount of bacterial and/or fungal contamination [11,19-21]. Furthermore, sodium hypochlorite is a strong oxidant and, if used before isolation, it removes 99% of bacterial contamination as well as many inhibitors of downstream PCR analyses, leaving the endogenous DNA intact [11,13,19,20]. Kemp & Smith [22] showed that immersion of a bone in at least 6% sodium hypochlorite for 15 minutes prior to DNA extraction was both the most practical and cost-effective approach for removal of exogenous contamination from ancient bones. In this study, the DNA concentration measurements were obtained using the Nano Drop, therefore the concentration value might constitute for both endogenous DNA as well as bacterial contamination. We performed series of amplification of different DNA extract volumes to determine the minimal DNA input for PCR (not shown). Only the 3µl or higher volume of DNA extract gave visible bands on the electrophoresis and that amount was used in further analyses in this study.

Apart from the contamination, specific composition of bone matrix, where DNA is bounded by hydroxyapatite to form aggregates, constitutes a problem during an extraction process and might require previous dissociation of DNA from this structure. It is still argued whether this step is necessary prior to DNA extraction. Some authors recommend incubation of the bone with 0.5M EDTA solution [10,20,23]. EDTA is used not only for decalcification, but also inactivates

DNases by chelating bivalent ions such as Mg²⁺ or Ca²⁺ [13,24]. However, the same authors have indicated that EDTA may cause further degradation of DNA. Moreover, Jakubowska et al. [19] it suggests that EDTA can itself act as an inhibitor in the downstream PCR. In our study, decalcification turned out to be an essential step for both silica-based and organic methods. We obtained a partial SNP profile for undecalcified sample (S11) in silica-based method, with high background and very low level of RFU under the threshold (Figure 1m) and two other samples that were decalcified for 48h (S12) and 72h (S13) failed to amplify (Figure 1n, Figure 1o). Interestingly, those two samples gave the highest yields for silica-based method and no amplification could be the result of bacterial contamination of the samples or/and the presence of other inhibitors. Only the samples that were decalcified for 96h (S14) and 120h (S15) gave almost full profiles with only minor drop-outs: drop-out of the T allele in rs12203592 for sample S14 and drop-outs of the T alleles in rs12203592, rs1393350 and rs12896399 for sample S15 (Figure 1p, Figure 1q). The T alleles of rs12203592 and rs1393350 were missing in most sample profiles in this study. Allelic drop outs are often encountered in forensic studies and are due to an under-representation of one of a pair of heterozygous alleles and preferential amplification of the other allele. This could be the result of DNA degradation or very small amount of endogenous DNA in the sample [25]. (Table 3) shows schematic combination of alleles in 6-plex profile tested in this study for all the samples.

consensus profile:	G		G		C	T	C	T	C	T	G	T
marker ID:	rs1800407		rs16891982		rs12203592		rs12913832		rs1393350		rs12896399	
expected alleles:	G	A	G	C	C	T	C	T	C	T	G	T
samples:												
S1	do	-	+	-	do	do	+	+	do	do	+	do
S2	do	-	+	-	+	do	+	+	+	do	+	+
S3	do	-	+	-	+	do	+	+	+	+	+	+
S4	+	-	+	-	+	do	+	+	+	do	+	+
S5	do	-	do	-	+	+	+	+	do	+	+	+
S6	do	-	+	-	+	do	+	+	+	do	+	+
S7	sample failed to amplify											
S8	do	-	+	-	+	do	+	+	+	do	+	+
S9	+	-	+	-	+	do	+	+	do	do	+	+
S10	do	-	+	-	+	do	+	+	+	do	+	+
S11	do	-	+	-	+	do	+	+	do	do	do	do
S12	sample failed to amplify											
S13	sample failed to amplify											
S14	+	-	+	-	+	do	+	+	+	+	+	+
S15	+	-	do	-	+	do	+	+	+	do	+	do

Table 3: Schematic combination of alleles present and absent from 6-plex profile tested in this study indicated for each sample variant of DNA extraction.

“+” equates to an allele present in the profile; “-” equates to an allele absent from the profile; “do” equates to an allele drop out;

Similarly to the silica-based samples, the undecalcified sample (S1) in the organic method gave very poor profile with several drop outs, high background and RFU level mostly under the threshold (Figure 1c). However, the profile from the same sample, only with additional cleaning using the silica column (S2), turned out to be more complete with significantly higher fluorescence signal and almost no background noise (Figure 1d). This profile was similar to other samples in PCIA method, regardless of the time of decalcification (Figure 1e, Figure 1g, Figure 1k). Only one sample (S7) failed to amplify (Figure 1i). Interestingly, cleaning the samples with silica columns in most cases gave the profile of similar alleles, only with relatively higher RFU and lower or no background noise (Figure 1f, Figure 1h, Figure 1j, Figure 1l). Therefore, the application of decalcification seemed more crucial for the DNA recovery in our study, than the time of incubation with EDTA. Thus, we strongly recommend decalcification of a bone powder prior to DNA extraction for at least 48h. Likewise, Capelli & Tschentscher [26] suggested that decalcification of a bone is a necessary step with minimal duration of 48h, or longer.

Loreille et al. [13] it also performed a total demineralization of a bone using 15 ml of EDTA for every 1g of bone powder but the study lacks information about how long demineralization took place. What is more, the volume of EDTA exceeded 3 times the volume used in our study. Still, the authors obtained increased yields in comparison with the standard protocol, *i.e.* using EDTA only in lysis buffer overnight prior to PCIA extraction. On the contrary, other experiment [16] showed no improvement of DNA extraction using total demineralization and DNA yields were even smaller when additional incubation with EDTA was preceded. It is hard to compare the influence of EDTA as decalcifying agent on DNA between the silica-based and organic methods tested in this study, as we did not obtain extension products for several samples (S7, S12, S13). Nevertheless, the fluorescence signal was higher for the sample profiles in PCIA method when compared to the silica-based method and the RFU values for the PCIA variants without additional cleaning were still higher than those for the silica columns only. Also, the yields in our study differed more between the extraction methods than between decalcified and undecalcified samples within one method. The exception were two samples (S12 and S13) that gave the higher yields for silica-based protocol but failed to give SBE products, which could indicate bacterial or other exogenous contamination or the presence of inhibitors in those samples.

It is worth mentioning, that the lysis buffer that we used for the organic protocol contained EDTA and that the bone powder was exposed to it for 16h during overnight incubation, even in case of samples that were not decalcified with pure EDTA solution. The exact composition of the manufacturer's lysis buffer used in the silica-based method is not known, thus other than EDTA decalcifying agents might have been used. Another crucial problem, apart from the maximum DNA recovery, is potential presence of inhibitors that co-extract with DNA [10,13]. When more bone powder is used the amount of inhibitors also increases, therefore it is more favorable to use less bone powder with more accurate and trusted extraction method [13]. One approach is to use additional cleaning during the extraction process. Here, we applied the variant with purification the aqueous phase using the silica columns. It improved yields for 40% (2/5) of cleaned samples and diminished the background noise in all five (100%) SNP profiles.

Although the undeniable advantage of using the silica columns is the simplicity of technical handling and the safety of used reagents, the most efficient and reliable method in our study turned out to be the PCIA method. Similarly, some authors [16] considered the phenol-chloroform method with pre-treatment with EDTA to be the best out of 3 methods tested, while others [18] found both the silica and PCIA methods to be the finest for old bone out of 5 different methods tested. Moreover, the latter study related to bones dated to be about 750 years b.p. and the former considered bone as control sample for soft tissue DNA from cadaver of different stage of decomposition and the oldest sample was about 90 days old. What is even more interesting Salamon et al. [20], demonstrated that the structural organization of crystallites and the overall lamellar structure is similar between modern and fossil bones, unless they are exposed to some detrimental environment conditions [27].

On the contrary, Jakubowska et al. [19] found the phenol-chloroform method, accompanied with total demineralization, not to be suitable for degraded specimens and for STR profiling, while others applied this protocol with successful STR typing, though with no decalcification step [24]. One possible reason why the PCIA method worked best in our experiment is that we used the mixture of phenol-chloroform with isoamyl alcohol. Although phenol is commonly used for effective deproteinization, mixing it with chloroform and isoamyl alcohol in 25:24:1 ratio (respectively) prevents from preferential loss of some types of DNA [28]. The other reason in favor of PCIA in this study is the fact that, apart from ethanol, we used 3M sodium acetate (NaOAc) for DNA precipitation. Sodium acetate is used for removal of non-nucleic acid particles and making the DNA less soluble in a water phase, before precipitation with ethanol [29,30]. Indeed, here, the NaOAc solution was used only in PCIA and not in silica-based protocol. Another interesting issue concerns using only the supernatant and/or the bone powder for extraction. Höss and Pääbo [31] it extracted supernatant only [31], whereas Loreille et al. [13] suggested that there is still some DNA left in the bone powder after the lysis. What is important, in our experiment, the samples that were exposed to EDTA incubation for 48h and 72h, were still in solid form after the lysis process, while samples decalcified for 96h and 120h dissolved completely during the lysis, regardless of the buffer (commercial or self-made). Thus, longer demineralization greatly facilitates the technical aspects of extraction especially using silica method, as even the small fragments of the bone powder can clog the column and prevent effective purification.

Conclusion

In conclusion, our experiment proves the PCIA method to be the most effective and reliable for DNA extraction from an old bone specimen, with the minimal of 48h of demineralization. Therefore, it can be preceded in a relatively short time of three working days and the DNA can be further used in downstream forensic analyses.

Acknowledgment

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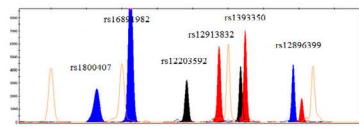


Figure 1a. Reference brown-eyed Caucasian individual.

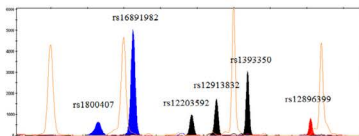


Figure 1b. Reference blue-eyed Caucasian individual.

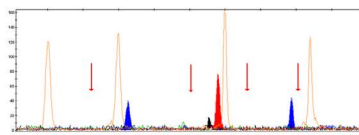


Figure 1c. Sample S1: undecalcified, PCIA.

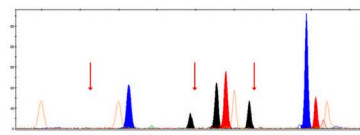


Figure 1d. Sample S2: undecalcified, PCIA with silica column cleaning.

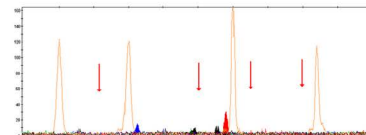


Figure 1m. Sample S11: undecalcified, silica column purification.

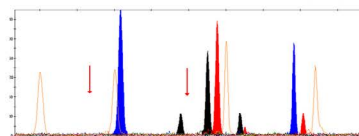


Figure 1e. Sample S3: decalcification for 48h, PCIA.

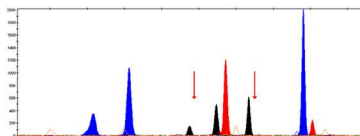


Figure 1f. Sample S4: decalcification for 48h, PCIA with silica column cleaning.

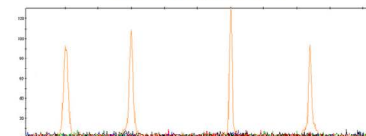


Figure 1n. Sample S12: decalcification for 48h, silica column purification.

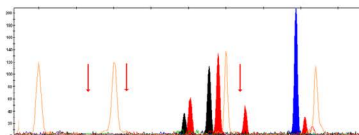


Figure 1g. Sample S5: decalcification for 72h, PCIA.

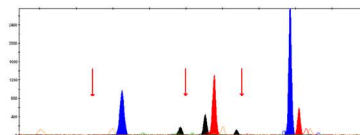


Figure 1h. Sample S6: decalcification for 72h, PCIA with silica column cleaning.

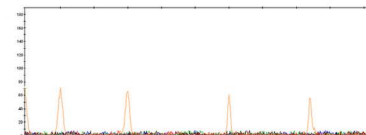


Figure 1o. Sample S13: decalcification for 72h, silica column purification.

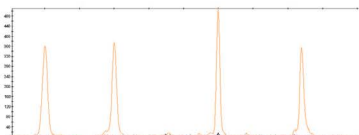


Figure 1i. Sample S7: decalcification for 96h, PCIA.

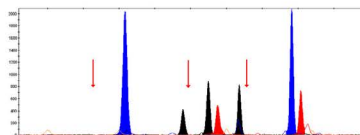


Figure 1j. Sample S8: decalcification for 96h, PCIA with silica column cleaning.

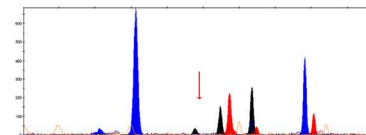


Figure 1p. Sample S14: decalcification for 96h, silica column purification.

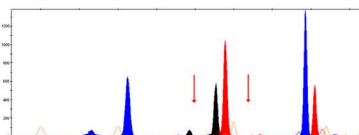


Figure 1k. Sample S9: decalcification for 120h, PCIA.

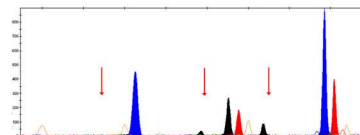


Figure 1l. Sample S10: decalcification for 120h, PCIA with silica column cleaning.

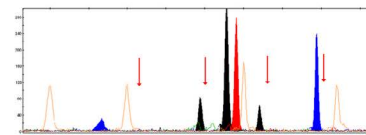


Figure 1q. Sample S15: decalcification for 120h, silica column purification.

Figure 1: An assessment of IrisPlex assay's profiles from 15 samples differing in the method of DNA extraction from a bone, from one individual.

Red arrow indicates allele drop outs in the reference to the consensus sequence in this study; PCIA-phenol-chloroform-isoamyl alcohol.

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