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Forensic DNA Typing of Old Skeletal Remains Using AmpFlSTR®Identifiler® PCR Amplification Kit

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Abstract

Background: In this study DNA typing of old skeletal remains was improved through AmpFlSTR®Identifiler®PCR amplification kit using different approaches.

Methodology: DNA extraction was carried out by silica columns based total demineralization extraction method.DNA quantification was carried out by Real Time PCR. DNA amplification was carried out by using AmpFlSTR®Identifiler® PCR amplification kit with modified conditions. Capillary electrophoresis and Data analysis were carried out by Genetic Analyzer 3130 (ABI) and GeneMapper ID software version 3.2.

Results: DNA was detected in 17 out of 24 skeletal remains. Among them, in 7 samples DNA was in the range of 1-10 pg/µL, in 4 samples it was in the range of 22-69 pg/µL and in 6 samples the DNA was in the range of >100 pg/µL. The C_T value (<30) of 40 cycles indicated that the PCR inhibitors were removed during DNA extraction method. Promising results were obtained by increasing the number of PCR cycles from standard 28 to 33 instead of 32 in PCR reaction. Finally it was observed that consensus approach produced reliable and reproducible DNA profiles from old skeletal remains.

Conclusions: Forensic DNA typing of old skeletal remains, through a multiplex AmpFlSTR®Identifiler® PCR amplification kit, is improved by using: a highly effective DNA extraction method, modified and optimized PCR conditions, increasing sensitivity of PCR amplification and consensus approach.

Keywords: Forensics; DNA typing; Old skeletal remains; AmpFlSTR®Identifiler® PCR amplification kit

Introduction

Problems occur during DNA typing of old skeletal remains with low quantities of template DNA. Low template DNA (LT DNA) or Low copy number DNA (LCN DNA) refer to any small amount of DNA (≤ 100-200 pg/µL) present in degraded DNA sample. More recently, LCN referred to any DNA sample or DNA profile where stochastic effects are present and/or where the alleles detected are below a laboratory defined stochastic threshold. Stochastic effects includes allele drop out, allelic drop-in, heterozygous peak height imbalance, locus drop-out and elevated stutter peaks [1,2]. Extraction and quantification of DNA for all DNA samples are very necessary. However, some time quantification results may be unreliable, particularly in case of degraded DNA samples, and results with nil/low quantity of DNA give full profiles and significant quantification results give nil/partial DNA profiles [3].

There are several approaches to analyze old skeletal remains with low quantity of DNA. These are: using highly effective DNA extraction method, increasing sensitivity of PCR amplification and consensus approach [3,4]. Consensus approach is more reliable and reproducible [5,6]. The aim of this study was to improve DNA typing of old skeletal remains, through a multiplex AmpFlSTR®Identifiler® PCR amplification kit, using a highly effective DNA extraction method, increasing sensitivity of PCR amplification and applying consensus approach.

Methodology

Collection of old skeletal remains

24 human old skeletal remains were collected from 200-500 years old mass graves of Pakistan for DNA typing. Approval for sample collection was obtained from the ethical review committee of the Centre of Excellence in Molecular Biology, University of the Punjab Lahore Pakistan. The samples were labeled, photo-documented and stored at -20°C till use.

Cleaning, pre-treatment and grinding of samples

Cleaning, pre-treatment and grinding of samples were carried out in forensic research laboratory, CEMB, University of the Punjab Lahore Pakistan. The samples were handled with gloved hands and forceps to avoid contamination by skin cells/sweat. The outer surfaces of bone samples were sanded with a motor drill and a dental bur to remove potential contamination. Each bone sample was broken into small fragments using a dental diamond disk and irradiated with ultraviolet light for 10-15 minutes. The bone fragments were treated mechanically and chemically with scalpel blades, dremel tool, 10 % bleach, distilled water and 95 % ethanol to remove the remaining contaminated soil, inhibitory substances and other dirt and debris, and placed in a sterilized fume hood to air-dry overnight. The samples were grinded into fine powder using liquid nitrogen and a SPEX 6750 Freezer/Mill (SPEX CertiPrep, Metuchen, NJ). The bone powder were transferred to a 15 mL falcon tube and stored at -20°C until DNA extraction.

Extraction of DNA from old skeletal remains

DNA extraction was carried out twice using silica columns after total

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demineralization extraction method. 0.5 g bone powder of each sample was dissolved in 15 mL of extraction buffer (0.5 M EDTA and 0.5% SDS) with 150 µL of 20 mg/mL Proteinase K and incubated at 56°C for 48 hours. After first incubation, it was treated with an additional 150 µL of 20 mg/mL Proteinase K (Sigma, Saint Louis, MO) and incubated at 56°C for 1 hour. 7.5 mL of the supernatant and 38 mL of PB buffer from the QIAquick PCR purification kit (Qiagen) were added to a new 50 mL falcon tube. Mixed well and centrifuged at 3000 × g for 5 minutes. The mixture was passed through a QIAampBlood Maxi column (Qiagen, Hilden, Germany) using QIAvac 24 Plus and QIAvac Connecting System (Qiagen, Hilden, Germany). The column was washed with 15 mL of PE buffer from the QIAquick PCR purification kit (Qiagen), placed in a 50 mL collection tube and centrifuged at 3000 × g for 5 minutes to remove the remaining PE buffer. The tube was discarded and the QIAamp Maxi column was kept in a new 50 mL falcon tube. 1 mL of nuclease-free double distilled water (ddH_2O) was added to QIAamp Maxi column, closed the cap, kept at room temperature for 5 minutes and centrifuged at 3000 × g for 5 minutes. This step was repeated to obtain 2 mL of eluted DNA. 10 mL of the PB buffer was added to the eluted DNA and mixed well. The mixture was passed through the QIAamp Mini spin column (Qiagen) using QIAvac 24 Plus and QIAvac Connecting System (Qiagen, Hilden, Germany). The column was washed with 750 µL of PE buffer (QIAquick PCR purification kit, Qiagen), kept in a 2 mL collection tube and centrifuged at 13200 rpm for 3 minutes to dry the membrane completely. QIAamp Mini column was placed in a clean 1.5 mL tube and the 2 mL collection tube was discarded. 100 µL of nuclease-free double distilled water (ddH2O) was added to QIAamp Mini column. Incubated at room temperature for 5 minutes and centrifuged at 8000 rpm for 1 minute. The QIAamp Mini column was discarded and eluted DNA was stored at -20°C till use. All extractions were accompanied by negative controls (nuclease free water was used instead of bone powder).

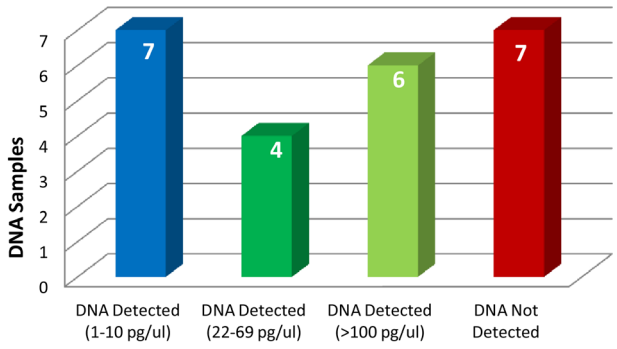


Figure 1: Quantification of DNA.

S.No	Sample ID	Type of Bone	Quantity of DNA (pg/µL)	IPC (C_T)
1	FRL 1	Humerus	112.5 ± 17.68	28.7 ± 0.13
2	FRL 2	Tibia	5.5 ± 2.12	29.3 ± 0.62
3	FRL 3	Ulna	5.5 ± 0.71	29.3 ± 0.84
4	FRL 4	Metacarpal	ND	29.3 ± 0.6
5	FRL 5	Tibia	69.5 ± 20.51	29.7 ± 0.25
6	FRL 6	Ulna	104 ± 5.66	29.1 ± 1.06
7	FRL 7	Ulna	22.5 ± 6.36	29.4 ± 0.56
8	FRL 8	Radius	38.5 ± 9.19	29.2 ± 0.88
9	FRL 9	Radius	2.5 ± 2.12	29.8 ± 0.04
10	FRL 10	Skull	117.5 ± 3.54	29.5 ± 0.41
11	FRL 11	Tibia	140.5 ± 7.78	29.2 ± 0.71
12	FRL 12	Femur	171 ± 5.66	29 ± 0.88
13	FRL 13	Ulna	109 ± 12.73	29 ± 0.67
14	FRL 14	Ulna	3.5 ± 2.12	27.8 ± 2.46
15	FRL 15	Radius	ND	28.4 ± 1.53
16	FRL 16	Femur	ND	29.5 ± 0.13
17	FRL 17	Tibia	4.5 ± 0.71	29.1 ± 0.55
18	FRL 18	Radius	2 ± 1.41	29.7 ± 0.17
19	FRL19	Femur	ND	29.8 ± 0.1
20	FRL20	Humerus	ND	29.8 ± 0.28
21	FRL21	Metacarpal	22 ± 7.07	28.9 ± 0.77
22	FRL22	Fibula	ND	29.3 ± 0.22
23	FRL23	Radius	ND	28.5 ± 1.11
24	FRL24	Metacarpal	6.5 ± 6.36	29 ± 0.28

Table 1: Concentration of DNA and C_T (Threshold cycle) values of Internal PCR Control (IPC).

Quantification of DNA using real time PCR

DNA quantification was carried out in duplicate using the Quantifiler® Duo Human DNA Quantification kit (Applied Biosystems) and the ABI Prism® 7500 Real Time PCR System (Applied Biosystems) with slightly modified reduced volume reaction. The quantification reaction was carried out in a total volume of 12 µL containing 1 µL DNA extract, 5.0 µL Quantifiler human primer mix and 6.0 µL Quantifiler PCR reaction mix. The set of eight dilutions of standard DNA was used as the DNA quantification standard according to the manufacturer's recommendation. All samples were analyzed in duplicate. Data analysis was performed using 7500 SDS software v 2.0.5 (Applied Biosystems) and Data collection was accompanied at a threshold of 0.2 and a baseline of 3-15. Following the Quantifiler manual, the level of PCR inhibitors was estimated by the C_T value of internal PCR control (IPC).

PCR amplification

PCR amplification was conducted twice using the AmpFlSTR®Identifiler® kit (Applied Biosystems) with reduced volume reaction mixtures consisting of 1.7 µL dH2O, 2.0 µL of Primer Mix, 3.8 µL of PCR Reaction Mix, 0.5 µL AmpliTaq Gold DNA Polymerase (5.0 U/µL) and 2 µL template DNA (≤100pg/µL) in a final reaction volume of 10 µL. Thermal cycling was conducted on PTC-200 (Peltier Thermal Cycler) DNA engine under the following conditions: 95°C for 11 min, 94°C for 1 min, 59°C for 1 min, 72°C for 1 min and a final extension at 60°C for 1 hour. The number of PCR cycles was kept 28, 32 and 33 during all experiments. Two separate PCR amplifications were performed for DNA extracts of each bone sample. All amplification reactions were accompanied by negative controls (nuclease free water).

Capillary electrophoresis (CE) and data analysis

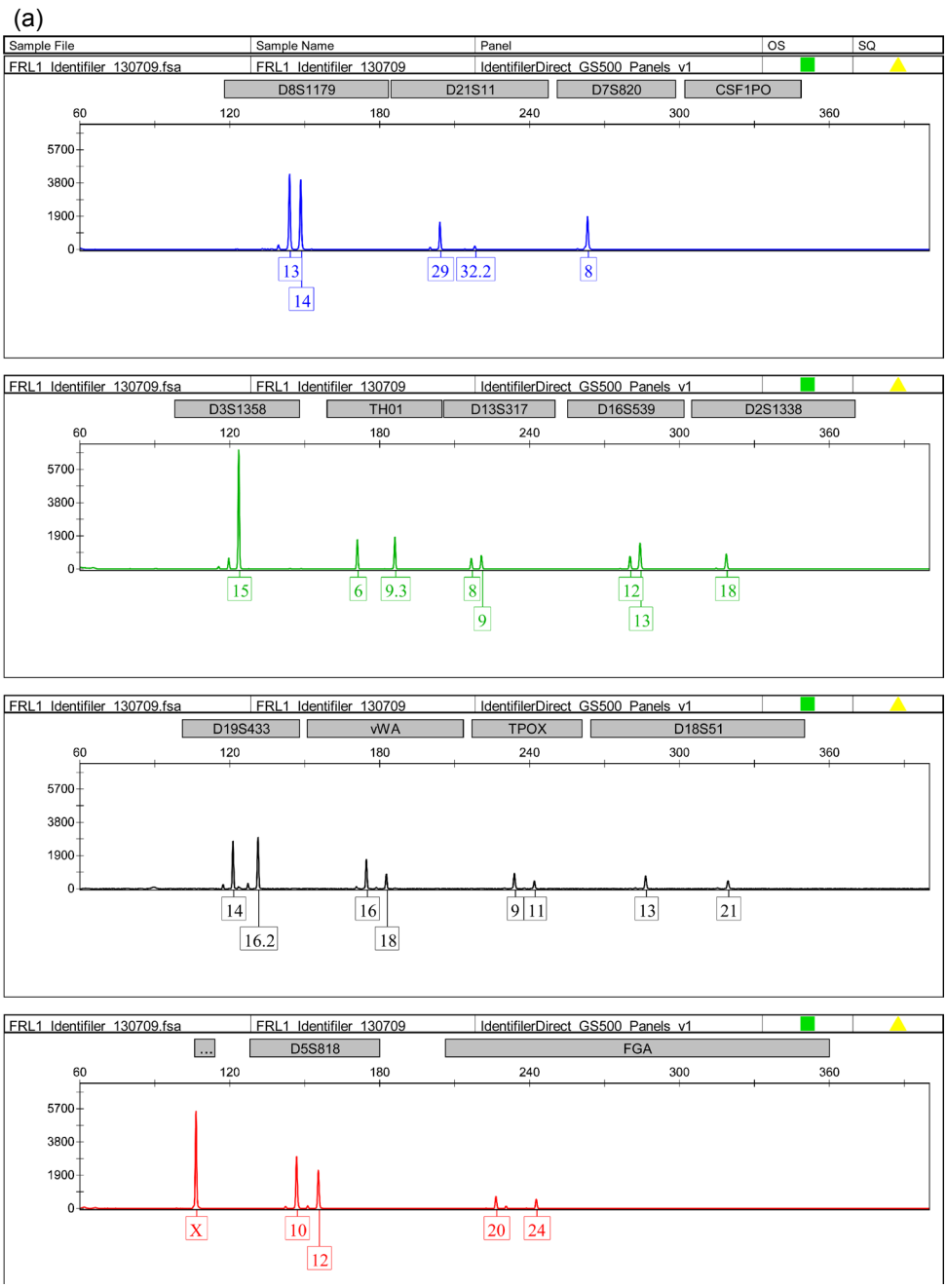
The PCR products were analyzed by capillary electrophoresis using ABI 3130 Genetic Analyzer (Applied Biosystems). Injection mixtures (consisted of 10 µL of Hi-Di formamide (ABI), 0.2 µL GeneScan® 500-LIZ™ size standard and 1.0 µL of PCR product for each sample in a final reaction volume of 11.2 µL) were loaded to a 96-well genotyping plate and covered with the rubber septa. The samples were heated at 95°C for 5 min to denature DNA into single stranded DNA and immediately placed on crushed ice for 3 minutes to stop DNA from renaturation. After cooling, samples were injected on the ABI Prism® 3130 Genetic analyzer (ABI). Data were analyzed by GeneMapper ID software version 3.2 (Life Technologies) with allelic ladders provided by the manufacturer. The STR results were presented as the number of amplified loci. Only the loci showing reliable results were counted. Allele with peak height above the interpretational threshold of 100 RFU (relative fluorescence units) was scored, and alleles with 15% or less peak height of the highest allele were not scored in each locus.

Consensus profiles were generated for each sample where an allele is observed in common from two replicate reactions [7].

Results and Discussion

Extraction and quantification of DNA

In DNA typing, extraction and quantification of DNA for all DNA samples is very necessary. Sometime quantification results of degraded samples may be unreliable, because samples having low/nil quantity of DNA giving full/partial DNA profiles and significant quantification results giving partial/nil DNA profiles [3]. DNA was extracted from old skeletal remains by silica columns based total demineralization extraction method. Real-time PCR quantification showed that the DNA was detected in almost 17 samples and not detected in 7 samples. It might be due to the reason that the samples were old and highly degraded. Majority of the degraded old samples produced <10 pg/µl DNA from 0.5 g of bone powder. In 7 samples, DNA was in the range of 1-10 pg/µL, in 4 samples it was in the range of 22-69 pg/µL and in 6 samples the DNA was in the range of >100 pg/µL (Figure 1). The internal PCR control (IPC) assay showed that PCR inhibitors were successfully removed from all of the extracted DNAs during qPCR, showing C_T values of <30 (Table 1). It might be due to the fact that using a highly effective DNA extraction method improved DNA quantification and removal of PCR inhibitors [8].



(b)

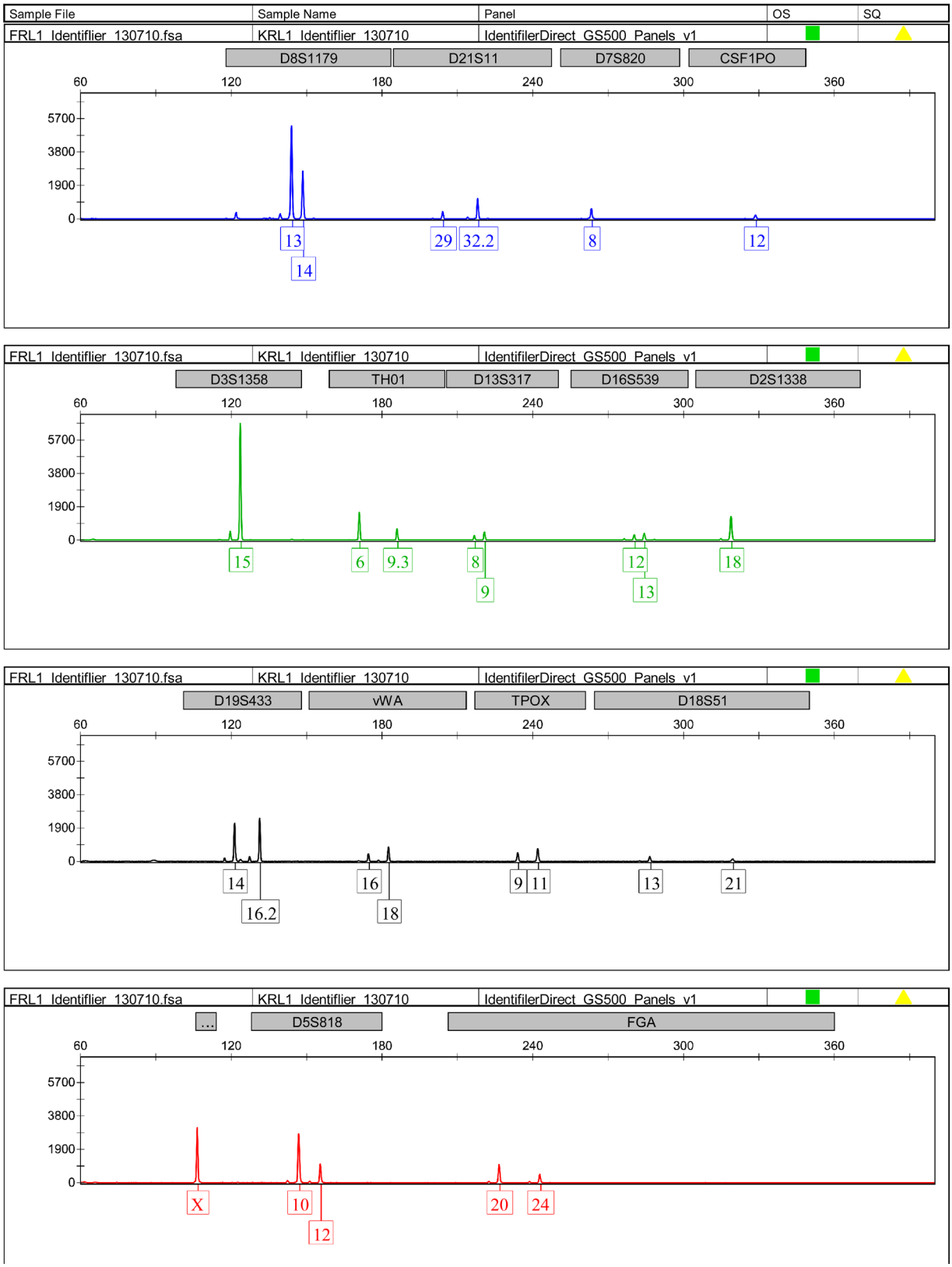


Figure 2: Increasing sensitivity of PCR amplification. [Figure 2(a) DNA profile with 32 number of PCR cycles]. [Figure 2(b) DNA profile with 33 number of PCR cycles].

Loci	D8S1179	D21S11	D7820	CSF1PO	D3S1358	THO1	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA
Replication #1	14	30, 31.2	8, 11	12	15, 17	8, 9	8	11	23	13, 14	16, 18	8, 11	13, 17	X, Y	12, 13	21, 22
Replication #2	14	30, 30.2, 32.2	8, 11	12	15, 17	8, 9	8	11	23, 25	13, 14	16, 18	8, 11	17	X, Y	9, 12, 13	20, 21, 22
Consensus Profile	14	30	8, 11	12	15, 17	8, 9	8	11	23	13, 14	16, 18	8, 11	17	X, Y	12, 13	21, 22

Table 2: Construction of Consensus Profile [Digits below each locus are alleles].

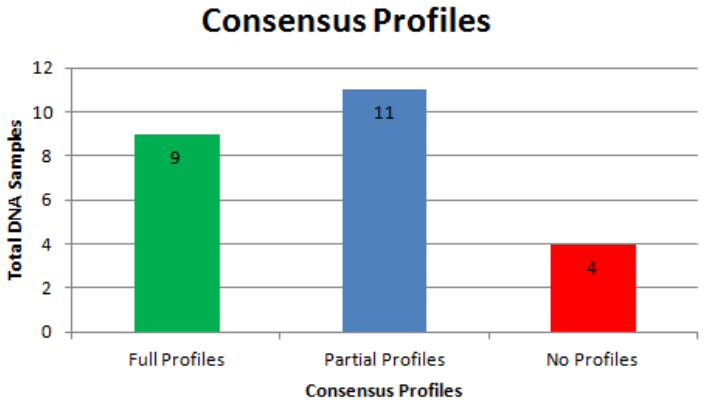


Figure 3: Outcome of DNA Typing of old skeletal remains.

Increasing sensitivity of PCR amplification

During this study, the extracted DNA was highly degraded, therefore standard PCR protocol of 28 cycles was failed to produce most informative DNA profiles from highly degraded human old skeletal remains. The sensitivity of PCR amplification was increased by extending the number of PCR cycles from 28 to 32 and then 33. During validation studies, it was showed that amplification of degraded DNA with AmpFlSTR®Identifiler® PCR kit offered promising results by increasing the number of PCR cycles from standard 28 to 33 instead of 32 in PCR reaction as shown in Figures 2a and 2b. It might be due to the fact that increase in the sensitivity of DNA profiling method permits DNA profiles to be obtained from old and highly degraded DNA samples [9]. No allele/locus drop-in occurred in negative control.

Consensus approach

The interpretations of DNA profiles become very difficult when analyzing old skeletal remains with low template (≤100-200 pg/μL) or highly degraded DNA [7]. In fact, by simply extending the number of PCR cycles, the quantity of amplified product increases, but the stochastic effects (allele drop-in, drop-out, high stutter, peak height imbalance etc.) in the resulting DNA profiles increase as well [10]. According to DNA interpretation rules, consensus approach was used for producing more reliable and reproducible DNA profiles [11]. For each of the degraded old skeletal sample, two replicates were produced independently. Consensus DNA profiles were created with an allele observed in common from both replicate reactions of each sample (Table 2). After the analysis of 24 highly degraded old skeletal remains, 9 full DNA profiles, 11 partial profiles and only 4 samples with no profile were obtained as shown in Figure 3.

Conclusions

Forensic DNA typing of old skeletal remains, through a multiplex AmpFlSTR®Identifiler® PCR amplification kit, is improved by using: a highly effective DNA extraction method, modified and optimized PCR conditions, increasing sensitivity of PCR amplification and consensus approach.

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