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# A Comparative Analysis of the AmpFlSTR Identifiler and PowerPlex 16 Autosomal Short Tandem Repeat (STR) Amplification Kits on the Skeletal Remains Excavated from Second World War Mass Graves in Slovenia

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**Abstract: Aim.** This paper describes a comparative study of two different amplification kits for autosomal STR typing of skeletal remains excavated from Second World War mass graves in Slovenia. We analyzed 92 bones and teeth and compared the genetic profiles obtained using the AmpFlSTR IdentifilerTM Amplification Kit (Applied Biosystems) and the PowerPlex 16 System (Promega).

**Methods.** We cleaned the bones and teeth, removed surface contamination and ground them into a powder using liquid nitrogen. Prior to deoksiribo nucleic acid (DNA) isolation with Biorobot EZ1 (Qiagen), 0.5g bone or tooth powder was decalcified. The nuclear DNA of the samples was quantified using the real-time polymerase chain reaction (PCR) method. The amplification protocols for both kits were optimised for old skeletal remains.

**Results.** We extracted 0.4 to 100 ng DNA/g of powder from the bones and teeth. Both amplification kits showed very similar efficiency on the 70 year old bones and teeth, since DNA typing was successful in 81 out of the 92 bones and teeth with both amplification kits, which represent an 88% success rate.

**Conclusions.** The comparative study indicated that the commercially available Identifiler and PowerPlex 16 PCR amplification kits are reliable for short tandem repeat (STR) typing of World War II skeletal remains with the DNA extraction method and PCR amplification conditions optimised in our laboratory, since very often complete STR profiles of autosomal DNA were obtained with both kits.

**Key Words:** STR typing, Bones, Teeth, Second World War, Mass grave victims, Slovenia.

Acquiring the autosomal short tandem repeat (STR) profiles from ancient skeletal remains is always a challenge in forensic deoksiribo nucleic acid (DNA) investigations. In old bones and teeth, small amount of endogenous DNA, the presence of polymerase chain reaction (PCR) inhibitors, the degradation of DNA and the exceptional risk of contamination often limit the success of DNA typing [1-3]. Mitochondrial DNA testing is regularly employed in the forensic identification of aged skeletal remains [4-6]. Our goal was to optimise and compare the commercially available amplification kits to acquire autosomal STR profiles of aged bones and teeth for the molecular genetic identification of victims in the Second World War mass graves in Slovenia.

The study covers 92 bones and teeth (77 femurs, one humerus, 12 tibias and two teeth) excavated from two Second World War mass graves in Slovenia where mass executions took place in the period during and after the Second World War (1945). The Commission on Concealed Mass Graves in Slovenia has recently registered almost 600 hidden mass graves from that period [7].

We analysed the bones from the Konfin I mass grave, located in a karst cave where 88 victims were killed. We also analysed bones and teeth from the mass grave found in the forest at Mount Storžič, where four

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massacre victims were excavated from the soil. For both mass graves we obtained the informed consent from the living relatives for molecular genetic typing of bones and teeth. The skeletal remains were excavated under the direction of local archaeologists and anthropologists.

Under a decree by the government of the Republic of Slovenia, the Commission on Concealed Mass Graves in Slovenia entrusted the identification of the victims in both mass graves to our institute. We identified three victims among the skeletal remains from the mass grave at Mount Storžič [8,9] and we identified 28 Slovenian victims among the skeletal remains from the Konfin I mass grave [10]. For this mass grave we matched four additional victims to living relatives from Croatia with a posterior probability higher than 99.9% as recommended by Biesecker *et al.* [11], Brenner and Weir [12], and Prinz *et al.* [13].

The STR typing of autosomal DNA was performed on the skeletal remains using the AmpFlSTR IdentifilerTM PCR Amplification Kit (Applied Biosystems) and the PowerPlex 16 System (Promega). The amplification protocols for both kits were optimised for old skeletal remains. For bones where these two kits did not reveal amplification products, mainly due to degraded DNA, we also performed miniSTR typing using the AmpFlSTR MiniFilerTM PCR Amplification Kit (Applied Biosystems). STR typing was also carried out for persons who were included in the elimination database. This report discusses the efficiency of the Identifiler and PowerPlex 16 amplification kits on 90 bone samples and two tooth samples from the 70 years old skeletal remains excavated from the WWII mass graves in Slovenia.

## MATERIALS AND METHODS

We followed the published recommendations [14-20] to ensure quality standards and prevent contamination in the molecular genetic laboratory. We created an elimination database that allowed traceability in the event of contamination. In the database we included everyone who had been in contact with the skeletal remains in any phase of the working process (excavation, storage, anthropological analysis or molecular genetic analysis). We included extraction-negative controls in every batch of extraction and PCR-negative controls in every amplification reaction to verify the purity of the extraction and amplification reagents.

We collected buccal swabs on sterile cotton swabs from the persons included in the elimination database. The bone and tooth samples for DNA analysis were collected, labelled and photo documented. For genetic investigations, a 5 to 10 cm fragment was taken from each bone and molars were removed from jawbones. Bone samples were cleaned mechanically and chemically while tooth samples were cleaned chemically and irradiated with UV light for 2 x 30 min with the tooth rotated 180° between each exposure prior to grinding into a powder.

The bone surface was decontaminated by the physical removal of the surface using a rotary sanding tool (Dremel) and liquid nitrogen. The bones and teeth were rinsed in 5% Alconox detergent (Sigma-Aldrich), water and 80% ethanol. Grinding in a TissueLyser (Retsch) homogenizer using liquid nitrogen followed. The whole procedure was carried out in room designed exclusively for processing old skeletal remains. Mechanical cleaning was performed in a closed citostatic C-(MaxPro)3-130 (Iskra Pio) safety cabinet. Genomic DNA was obtained from 0.5 g of bone or tooth powder according to Zupanič Pajnič *et al.* [10].

The DNA was purified in a Biorobot EZ1 (Qiagen) device using the EZ1 DNA Investigator Card and the EZ1 DNA Investigator Kit (Qiagen). Following the manufacturer's instructions [21] the Biorobot EZ1 was used to obtain genomic DNA from decalcified bone precipitate using the large-volume protocol, and from the elimination database samples using the "tip dance" protocol. The extraction-negative controls were included in the extraction process to verify the purity of the extraction reagents.

The DNA extracts from all the bone and tooth samples were quantified and levels of PCR inhibitors monitored using the QuantifilerTM Human DNA Quantification Kit (Applied Biosystems). The reactions were carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the SDS software, version 1.0 (Applied Biosystems) according to the manufacturer's instructions [22]. STR typing of the autosomal DNA was performed for the bones and teeth using two amplification kits: the AmpFlSTR IdentifilerTM PCR Amplification Kit (Applied Biosystems) and the PowerPlex 16 System (Promega). Additionally, for bones where these two kits did not reveal any amplification products, mainly due to degraded DNA samples, the AmpFlSTR MiniFilerTM PCR Amplification Kit (Applied Biosystems) was used.

The AmpFlSTR IdentifilerTM PCR Amplification Kit and the PowerPlex 16 System contain the same 13 core STR loci and amelogenin, whereas the AmpFlSTR IdentifilerTM PCR Amplification Kit also contains loci D2S1338 and D19S433. The PowerPlex 16 System also contains the loci Penta E and Penta D. The AmpFlSTR MiniFilerTM PCR Amplification Kit (Applied Biosystems) contains eight STR loci shared with the AmpFlSTR IdentifilerTM PCR Amplification Kit, but uses shorter amplicons, which makes them more likely to be successful on fragmented DNA.

The PCR protocols for all three amplification kits were optimised for old skeletal remains and differed for the bone or tooth samples with lower or higher amounts of DNA. For the Identifiler and MiniFiler amplification

kit, the limit between the lower and higher amounts of DNA was 50 pg/µl DNA, and for the PowerPlex 16 the limit was 28 pg/µl DNA.

The amplification protocols are described in Zupanič Pajnič *et al.* [10]. All the reactions were performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Simultaneously with the forensic samples, we amplified the positive control and negative PCR, as well as the extraction controls. The fluorescent-labelled products of the amplification kits were separated using an automatic ABI PRISMTM 3130 Genetic Analyzer (Applied Biosystems) using the 3130 Performance Optimized Polymer 4 (Applied Biosystems) and the GeneScan-500 LIZ (Applied Biosystems) internal size standard with the AmpFlSTR Identifiler PCR Amplification Kit (Applied Biosystems) and the AmpFlSTR MiniFiler (Applied Biosystems), and ILS 600 (Promega) internal size standard with the PowerPlex 16 System (Promega) kit. The genetic profiles were determined using the Data Collection v 3.0 and GeneMapper ID v 3.2 (Applied Biosystems) computer software. In persons from the elimination database, STR typing was performed using the AmpFlSTR IdentifilerTM PCR Amplification Kit (Applied Biosystems).

The genetic profiles obtained from the bones and teeth were compared to the profiles of the people included in the elimination database to monitor possible contamination of the bone and tooth samples with modern DNA. We traced the success rate of the STR amplification using the Identifiler and the PowerPlex 16 PCR amplification kits for the 90 bones and two teeth and compared the amplification success rate for the femurs, tibias and humerus.

## RESULTS

The quantification of nuclear DNA resulted in the determination of over 4 pg DNA/µl of isolate in all the bones and teeth (Table 1).

We extracted 0.4 to 100 ng DNA/g of powder from the bones and teeth. The highest amount of DNA was obtained from one femur (1 ng DNA/µl) and the two teeth (about 0.5 ng DNA/µl). The typing of autosomal STR loci with the Identifiler and PowerPlex 16 amplification kits was successful in 81 of the 92 bones and teeth, which represents an 88% success rate. We obtained genetic profiles using both kits for all 81 bones and teeth. The typing of femurs proved to be much more successful than tibias and humerus.

We successfully typed 97% of the femurs (75 out of 77 femurs) and only 31% of the tibias and humerus (4 out of 12 tibias and one humerus). After some additional typing of 8 tibias, two femurs and one humerus using the miniSTR AmpFlSTR MiniFilerTM PCR Amplification Kit (Applied Biosystems), we obtained autosomal STR profiles for 6 tibias and managed to increase the amplification success rate for the tibias and humerus up to 77%. Complete Identifiler-STR genetic profiles (15 STR loci and amelogenin) were obtained from 74 bones and teeth (71 femurs, one tibia and two teeth), partial profiles with one STR loci missing were obtained from 6 bones (3 femurs and 3 tibias) and a partial profile with two STR loci missing was obtained from one femur (Table 1).

Primarily the longer loci D2S1338 and FGA were missing. In 7 bones with partial profiles, 8 loci were not amplified, in one bone D7S820 and FGA, in one bone CSF1PO, in one bone FGA and in 4 bones D2S1338. Complete PowerPlex 16-STR profiles (15 STR loci and amelogenin) were obtained from 73 bones and teeth (70 femurs, two tibia and two teeth), partial profiles with one STR loci missing were obtained from 3 bones (2 femurs and one tibia), partial profiles with two STR loci missing were obtained from 2 femurs, STR profiles with 11 loci amplified were obtained from one femur and one tibia and an STR profile with 6 loci amplified was obtained from one femur (Table 1).

Primarily the longer loci D18S51, Penta E, CSF1PO, FGA and Penta D were missing. In 8 bones with partial profiles, 27 loci were not amplified, in two bones Penta E, in one bone Penta D, in one bone Penta E and CSF1PO, in one bone Penta E and FGA, in one bone D18S51, Penta E, D16S539, CSF1PO and D8S1179, in one bone D21S11, D18S51, Penta E, D16S539 and CSF1PO, and in one bone TH01, D21S11, D18S51, Penta E, D13S317, D7S820, CSF1PO, Penta D, D8S1179 and FGA.

## DISCUSSION

Recently some studies on the efficiency of DNA extraction methods from bone samples have been published. Loreille *et al.* [23] described the significantly enhanced quantity of the extracted DNA after the total demineralization of the bone material. Lee *et al.* [1] presented a modified large-scale silica-based extraction combined with complete demineralization. We performed 72-hours of decalcification and precipitate with incompletely decalcified bone and tooth powder was usually obtained.

We used a Biorobot EZ1 (Qiagen) for DNA isolation from 70 year old bones and teeth and for purifying it. Nagy *et al.* [24] used an automated device, the BioRobot M48 (Qiagen), for isolating DNA from bones just a few years old. Montpetit *et al.* [25], Kishore *et al.* [26], and Valgren *et al.* [27] obtained DNA from various casework samples using an automated isolation and purification device, the Biorobot EZ1 (Qiagen), for which they found a high purification efficacy. DNA extraction proved effective from relatively small 0.5g bone or tooth samples.

Alonso *et al.* [14] and Vanek *et al.* [20] described a method where one or two grams of bone powder

**Table 1.** Nuclear DNA quantity (Quantifiler™ Human DNA Quantification Kit, Applied Biosystems) expressed in pg DNA/µl of isolate and the efficiency of autosomal STR typing (the AmpF*l*STR Identifiler™ PCR Amplification Kit, Applied Biosystems, and the PowerPlex 16 System, Promega), expressed as the number of successfully typed autosomal STRs, in the bones and teeth found in the Slovenian Second World War mass graves.

| Bone/tooth sample | Quantity (pg/µl) | Identifiler STR loci | PP 16 STR loci | Bone/tooth sample | Quantity (pg/µl) | Identifiler STR loci | PP 16 STR loci |
|---|---|---|---|---|---|---|---|
| tooth 1 | 550 | 16/16 | 16/16 | femur 32 | 111 | 16/16 | 16/16 |
| tooth 2 | 510 | 16/16 | 16/16 | femur 33 | 130 | 16/16 | 16/16 |
| tibia 1 | 69 | 15/16 | 16/16 | femur 34 | 75 | 16/16 | 16/16 |
| tibia 2 | 50 | 15/16 | 11/16 | femur 35 | 36 | 16/16 | 16/16 |
| tibia 3 | 300 | 15/16 | 15/16 | femur 36 | 150 | 16/16 | 16/16 |
| tibia 4 | 67 | 16/16 | 16/16 | femur 37 | 41 | 16/16 | 16/16 |
| tibia 5 | 35 | 0/16 | 0/16 | femur 38 | 64 | 16/16 | 16/16 |
| tibia 6 | 26 | 0/16 | 0/16 | femur 39 | 42 | 16/16 | 16/16 |
| tibia 7 | 47 | 0/16 | 0/16 | femur 40 | 100 | 16/16 | 16/16 |
| tibia 8 | 100 | 0/16 | 0/16 | femur 41 | 64 | 16/16 | 16/16 |
| tibia 9 | 14 | 0/16 | 0/16 | femur 42 | 77 | 15/16 | 15/16 |
| tibia 10 | 32 | 0/16 | 0/16 | femur 43 | 280 | 16/16 | 16/16 |
| tibia 11 | 39 | 0/16 | 0/16 | femur 44 | 130 | 16/16 | 16/16 |
| tibia 12 | 23 | 0/16 | 0/16 | femur 45 | 150 | 16/16 | 16/16 |
| humerus 1 | 5 | 0/16 | 0/16 | femur 46 | 30 | 16/16 | 15/16 |
| femur 1 | 221 | 16/16 | 16/16 | femur 47 | 50 | 16/16 | 16/16 |
| femur 2 | 60 | 16/16 | 14/16 | femur 48 | 43 | 16/16 | 16/16 |
| femur 3 | 37 | 16/16 | 16/16 | femur 49 | 8 | 16/16 | 16/16 |
| femur 4 | 70 | 16/16 | 16/16 | femur 50 | 26 | 15/16 | 16/16 |
| femur 5 | 50 | 16/16 | 16/16 | femur 51 | 57 | 16/16 | 16/16 |
| femur 6 | 40 | 16/16 | 14/16 | femur 52 | 47 | 16/16 | 16/16 |
| femur 7 | 106 | 16/16 | 16/16 | femur 53 | 77 | 16/16 | 16/16 |
| femur 8 | 26 | 16/16 | 11/16 | femur 54 | 100 | 16/16 | 16/16 |
| femur 9 | 237 | 16/16 | 16/16 | femur 55 | 101 | 16/16 | 16/16 |
| femur 10 | 26 | 16/16 | 16/16 | femur 56 | 90 | 16/16 | 16/16 |
| femur 11 | 39 | 16/16 | 16/16 | femur 57 | 47 | 16/16 | 16/16 |
| femur 12 | 116 | 16/16 | 16/16 | femur 58 | 18 | 16/16 | 16/16 |
| femur 13 | 23 | 16/16 | 6/16 | femur 59 | 91 | 16/16 | 16/16 |
| femur 14 | 56 | 16/16 | 16/16 | femur 60 | 14 | 16/16 | 16/16 |
| femur 15 | 79 | 16/16 | 16/16 | femur 61 | 120 | 16/16 | 16/16 |
| femur 16 | 62 | 16/16 | 16/16 | femur 62 | 109 | 16/16 | 16/16 |
| femur 17 | 43 | 16/16 | 16/16 | femur 63 | 73 | 16/16 | 16/16 |
| femur 18 | 10 | 16/16 | 16/16 | femur 64 | 48 | 16/16 | 16/16 |
| femur 19 | 24 | 16/16 | 16/16 | femur 65 | 47 | 16/16 | 16/16 |
| femur 20 | 69 | 14/16 | 16/16 | femur 66 | 24 | 16/16 | 16/16 |
| femur 21 | 17 | 16/16 | 16/16 | femur 67 | 250 | 16/16 | 16/16 |
| femur 22 | 46 | 16/16 | 16/16 | femur 68 | 33 | 16/16 | 16/16 |
| femur 23 | 114 | 16/16 | 16/16 | femur 69 | 36 | 16/16 | 16/16 |
| femur 24 | 120 | 16/16 | 16/16 | femur 70 | 34 | 16/16 | 16/16 |
| femur 25 | 35 | 16/16 | 16/16 | femur 71 | 93 | 16/16 | 16/16 |
| femur 26 | 100 | 16/16 | 16/16 | femur 72 | 15 | 16/16 | 16/16 |
| femur 27 | 51 | 16/16 | 16/16 | femur 73 | 43 | 15/16 | 16/16 |
| femur 28 | 110 | 16/16 | 16/16 | femur 74 | 86 | 16/16 | 16/16 |
| femur 29 | 1000 | 16/16 | 16/16 | femur 75 | 59 | 16/16 | 16/16 |
| femur 30 | 270 | 16/16 | 16/16 | femur 76 | 4 | 0/16 | 0/16 |
| femur 31 | 142 | 16/16 | 16/16 | femur 77 | 5 | 0/16 | 0/16 |

were used for DNA extraction, and Davoren *et al.* [17] published a DNA extraction method efficacious on five grams of bone powder. Lee *et al.* [1] obtained genomic DNA from 0.4 to 0.5 g of bone powder, which is similar to our method. We extracted 0.4 to 100 ng DNA/g of powder from the WWII bones and teeth. Marjanovič *et al.* [28] obtained up to 10 ng DNA/g of bone powder from bones of the same age. Yamamoto *et al.* [29] extracted 5 ng DNA/g of femur from 16 year old skeletal remains of a newborn, Tahir *et al.* [30] obtained 8 ng DNA/g of bone from 27 years old exhumed skeletal remains, and Lee *et al.* [1] obtained up to 85 ng DNA/g of powder from bones from the Korean War.

The comparative study indicated that the AmpFlSTR IdentifilerTM PCR Amplification Kit (Applied Biosystems) and the PowerPlex 16 System (Promega) are reliable for STR typing of 70 years old skeletal remains using a DNA extraction method and PCR amplification conditions optimised in our laboratory [10].

In our laboratory, in addition to increasing the number of cycles and extending the elongation time, BSA was added, double amount of AmpliTaq Gold DNA Polymerase was used, the amount of Primer Pair Mix was increased, and final extension step was prolonged in comparison to the PowerPlex 16 amplification protocol used by Marjanović *et al.* [28] for identifying World War II victims. Irwin *et al.* used six additional PCR cycles and twice the recommended Taq concentration in the PowerPlex 16 System for identifying the 50-year-old skeletal remains of pilot James B. McGovern [31] and missing U.S. service members from World War I, World War II and the Vietnam War [32].

Lee *et al.* [1] used the Identifiler PCR amplification kit with reduced reaction volume, five additional PCR cycles, and a double amount of AmpliTaq Gold DNA Polymerase for STR typing 55-year-old skeletal remains from Korean War. We increased the number of cycles only for samples with lower amount of DNA by 3 in the Identifiler and 2 in the PowerPlex 16 amplification kit. Among the long bones, femurs performed the highest rate of successful autosomal STR typing, followed by tibias and humerus. These findings are in concordance with those reported by Miloš *et al.* from the International Commission on Missing Persons [33].

## CONCLUSIONS

We managed to obtain nuclear DNA from bones and teeth that were 70 years old for successful autosomal STR typing with the commercially available Identifiler and PowerPlex 16 PCR amplification kits. DNA extraction proved effective using relatively small 0.5g bone or tooth samples, which enabled us to acquire autosomal genetic information from low quantity samples.

The methods of DNA extraction and amplification used for the molecular genetic identification of Second World War mass grave victims in Slovenia have proved highly efficient because we obtained 0.4 to 100 ng DNA/g from the bones or teeth and very often complete STR profiles of autosomal DNA. The Identifiler and PowerPlex 16 amplification kits showed a very similar degree of efficiency on old bones and teeth. With both kits, we obtained genetic profiles for 81 bones and teeth, which represents an 88% success rate. In the process of STR typing skeletal remains from the Second World War mass graves, we minimized the possibility of contamination during genetic investigations.

The authenticity of the genetic profiles of bones and teeth was confirmed by clean isolation and amplification-negative controls for nuclear DNA, identical genetic profiles obtained using the Identifiler and PowerPlex 16 amplification kits, the mismatch of the genetic profiles of bones and teeth with persons from the elimination database, and the positive identification of mass graves victims [8-10].

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