Performance of the Human Quantifiler, the Investigator Quantiplex and the Investigator ESSplex Plus kit for quantification and nuclear DNA typing of old skeletal remains

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Abstract: Aim. We tested the performance of two real-time polymerase chain reaction (PCR) human quantification kits (Human Quantifiler (Applied Biosystems) and Investigator Quantiplex kit (Qiagen)) and forensic identification short tandem repeat (STR) Investigator ESSplex Plus (Qiagen) kit on approximately 70 years old skeletal remains.

Methods. We analysed 54 bones and teeth excavated from Second World War mass graves in Slovenia. Genomic DNA was obtained from 0.5 g of bone or tooth powder after total demineralization. The DNA was purified in a Biorobot EZ1 (Qiagen) device. The same extract was used for quantification and STR typing with all three kits using the amplification conditions recommended by the manufacturers.

Results. In almost two thirds of the samples the results of quantification were up to 6 times higher using the Human Quantifiler than using the Investigator Quantiplex kit, because of the degradation of DNA in old skeletal remains and amplification of shorter DNA fragment with the Human Quantifiler kit. The autosomal STR typing with the Investigator ESSplex Plus kit was successful in 52 out of the 54 samples, which represent a 96% success rate.

Conclusion. The commercially available Investigator ESSplex Plus kit can be used for STR typing of old skeletal remains with the DNA extraction method optimised in our laboratory and without any changes to the manufacturers’ PCR amplification protocols. The Human Quantifiler kit and Investigator Quantiplex kit together can be used for estimation of the degree of DNA degradation in compromised old bone samples.

Key Words: DNA quantification, autosomal STR typing, bones, teeth, Second World War.

In skeletonised human remains bones and teeth are the only accessible source of DNA which can be preserved for a long time. In them binding of DNA to hydroxyapatite provides stability of DNA and its preservation [1]. Old skeletal remains usually contain minute amounts of DNA, potential inhibitors of PCR reactions are present, DNA became compromised due to degradation, and the exceptional risk of contamination limits the success of DNA typing [2, 3].

In addition, the chemistry and methods used for DNA extraction and amplification may have a strong effect on the amplification success [4, 5]. As shown by different studies total demineralization is the best method of DNA extraction from old bone material [6-8]. Accordingly, for performing this study we changed our previous extraction protocol with partial demineralization [9] to total demineralization.

Correct DNA quantification is an essential part to obtain reliable STR typing results. Some important information (quantity and presence of PCR inhibitors) about compromised bone and tooth samples can be obtained using commercial real-time PCR quantification kits, the most often used for DNA quantification by forensic DNA analysts. Incorrect DNA quantification
due to the presence of PCR inhibitors may affect
experiment results and the CT values of an internal PCR
control (IPC) must be carefully checked [10]. According
to different length of DNA fragments amplified using
different quantification kits the degree of degradation
can be estimated. In order to determine the quantity of
nuclear DNA and presence of inhibitors of PCR reaction
and to estimate the degree of DNA degradation in old
bone and tooth samples the QuantifilerTM Human
DNA Quantification Kit (Applied Biosystems, Foster
City, CA, USA) and recently released the Investigator
Quantiplex kit (Qiagen, Hilden, Germany, EU) were
used for quantification. The Quantifiler amplifies 62 base
pairs (bp) ampiclon [11] and the Quantiplex kit amplifies
146 bp ampiclon [12] of nuclear DNA.
Analysis of autosomal STRs is invaluable in
identification of skeletal remains of missing persons
and disaster victims [13]. In order to obtain autosomal
STR profiles from old bones using commercial kits it is
important to test their performance on compromised
biological samples. Several commercial STR typing kits
have been released in the last 3 years. Among them there
is also the Investigator ESSplex Plus Kit (Qiagen) with
the extended European Standard Set (ESS) of loci. Some
concordance and population studies have been published
for the Investigator ESSplex kit (Qiagen) [14-20] and no
study was published for the Investigator ESSplex Plus
kit which is more sensitive and more robust than the
Investigator ESSplex Kit [21].
However, no study has been performed using the
ESSplex Plus Kit on old skeletal remains. Considering
the concept of new STR kits (increased tolerance to
common inhibitors and increased sensitivity to obtain
full profiles from low-level DNA samples) we wondered
if we could expect good performance even on skeletal
remains originating from Second World War. In order to
evaluate DNA typing performance of ESSplex Plus kit a
total of 54 bone and tooth samples from Slovenian WWII
graves were analysed.

MATERIALS AND METHODS
Cleaning and pulverizing of skeletal samples, DNA
extraction, quantification, and amplification setup
were conducted following published recommendations
[1, 22-27] to ensure quality standards and to prevent
contamination in the molecular genetic laboratory.
Extractions were performed with strict precautions
including protective clothing, equipment and surfaces
treated with bleach and irradiated with UV light. Skeletal
remains were processed in a closed citostatic
C-(MaxPro)3-130 (Iskra Pio, Šentjernej, Slovenia,
EU) safety cabinet in a room designed exclusively
for processing old skeletal remains. We also included
contamination monitoring in all steps using blank
controls. We processed 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 and plastics. We
also created an elimination database for each mass grave
containing all persons that had been in contact with the
skeletal remains at any phase of excavation, storage,
anthropological analysis, or molecular genetic analysis.
The elimination database is used to check for authenticity
of genetic profiles obtained from old skeletal remains and
allows traceability in the case of contamination.

Human bones and teeth around 70 years old
excavated from four Second World War mass graves
in Slovenia were used for the study. Fifty-four samples
were marked S1 - S54 (Table 1).
We analysed the bones and teeth from the Konfin
I, Konfin II, Storžič, and Bodovlje Gorge mass graves.
From the Konfin I mass grave, 21 femurs and 10 tibias
were analysed, and two femurs and one tooth (molar)
were analysed from the Storžič grave. Four femurs were
analysed from the Bodovlje Gorge mass grave, and 16
teeth (11 molars and 5 premolars) were analysed from
the Konfin II mass grave. A total of 37 bone samples (27
femurs and 10 tibias) and 17 tooth samples (12 molars
and 5 premolars) were evaluated in this study.
We collected buccal swabs on sterile cotton swabs
from the persons included in the elimination databases.
For genetic investigations, a 5 to 10 cm fragment was
taken from each femur and tibia. Twelve molars were
removed from 5 upper and 7 lower jawbones and five
premolars were removed from 3 upper and 2 lower
jawbones. The bone samples were cleaned mechanically
and chemically while the 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 cleaned
by the physical removal of the surface using a rotary
sanding tool (Dremel, Racine, WI, USA).
During drilling the bone was cooled down with
liquid nitrogen. The bones and teeth were rinsed in 5%
Alconox detergent (Sigma-Aldrich, St. Louis, MO,
USA), water and 80% ethanol. The samples were left
to dry overnight at room temperature. Grinding in a
TissueLyser (Retsch, Haan, Germany, EU) homogenizer
using liquid nitrogen followed. Every bone and tooth
was prepared separately using sterile, disposable tools
in a specially designed room. DNA was extracted from
powder as described by Zupanič Pajnič et al. [13] with
some modification. In brief, DNA was obtained from
0.5 g of bone or tooth powder incubated in 50 ml tube
in 8.5 ml of 0.5 M EDTA pH 8.0 (Promega, Madison,
WI, USA) overnight at 37 °C in a Thermomixer comfort
(Eppendorf, Hamburg, Germany, EU), shaken at 750
rpm. After decalcification, precipitate with completely
decalcified bone or tooth powder was obtained. The
liquid from 50 ml tube was equally divided into four 2
ml tubes.
After centrifugation at 13,400 rpm for 1.5 minutes in a MiniSpin (Eppendorf) centrifuge, the supernatant was discarded from all four tubes and 0.3 ml of ultrapure distilled water (Gibco, Carlsbad, CA, USA) was added. The liquid from all four tubes was combined into one 2 ml tube and centrifuged at 13,400 rpm for

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass grave</th>
<th>Sample type</th>
<th>Investigator Quantiplex kit Quantity (ng/µl)</th>
<th>Human Quantifier kit Quantity (ng/µl)</th>
<th>ESSplex Plus STR loci</th>
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<tr>
<td>S1</td>
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<td>tibia</td>
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<td>15/15</td>
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<td>0.04</td>
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<tr>
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<td>0.07</td>
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<td>0.05</td>
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<td>0.07</td>
<td>15/15</td>
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<td>0.07</td>
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<td>0.02</td>
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<td>0.01</td>
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<td>0.04</td>
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<td>0.03</td>
<td>15/15</td>
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<td>0.08</td>
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<td>0.15</td>
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<td>0</td>
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<td>15/15</td>
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<tr>
<td>S32</td>
<td>Storžič</td>
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<td>0.01</td>
<td>0.01</td>
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<tr>
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<tr>
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<td>0.10</td>
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<td>0.01</td>
<td>12/15</td>
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<tr>
<td>S44</td>
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<td>0.13</td>
<td>0.08</td>
<td>15/15</td>
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<tr>
<td>S45</td>
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<td>0.04</td>
<td>0.03</td>
<td>15/15</td>
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<td>15/15</td>
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<td>tooth LM2-maxilla</td>
<td>0.66</td>
<td>0.94</td>
<td>15/15</td>
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</tbody>
</table>
1.5 minutes, and the supernatant was discarded. Then 20 μl of 1 M DTT (Sigma-Aldrich), 100 μl of G2 buffer and 60 μl of proteinase K (both EZ1 DNA Investigator Kit, Qiagen) was added to the precipitate and it was incubated overnight at 56 °C in a Thermomixer comfort (Eppendorf), shaken at 950 rpm. This was followed by centrifugation at 1.900 rpm for 1.5 minutes in a MiniSpin (Eppendorf) centrifuge.

Up to 400 μl of supernatant was transferred to the sample tube and 1 μl of cRNA (EZ1 DNA Investigator Kit, Qiagen) was added. The DNA was purified in a Biorobot EZ1 (Qiagen) device using the EZ1 DNA Investigator Card and EZ1 DNA Investigator Kit (Qiagen). Following the manufacturer’s instructions [28] the Biorobot EZ1 was used to obtain genomic DNA from decalcified bone or tooth precipitate using the trace protocol and from elimination database buccal swab samples using the “tip dance” protocol. The final volume of bone and tooth extracts was 50 μl, of which 2 μl was used for quantification with the Quantiplex and Quantifiler kit, and up to 15 μl was used for amplification of the autosomal STRs with the ESSplex Plus kit.

This kit amplifies 15 polymorphic STR markers (TH01, D3S1358, vWA, D21S11, D16S539, D1S1656, D19S433, D8S1179, D2S1338, D10S1248, D22S1045, D12S391, FGA, D2S441, and D18S51) and gender-specific amelogenin simultaneously in a single PCR. Real-time quantification reactions were carried out in a 7500 Real Time PCR System (Applied Biosystems), using the HID Real-Time PCR Analysis Software, version 1.1 (Applied Biosystems) according to the manufacturer’s instructions [11, 12].

DNA typing of autosomal STR loci was performed according to the manufacturer’s instructions [29] without any additional optimization of PCR conditions. Maximum of 1 ng of DNA was used for amplification of samples. All the bones and teeth used in this study were previously typed with at least one of the following kits: Identifiler (Applied Biosystems), PowerPlex 16 System (Promega), PowerPlex ESX 17 System (Promega) or NGM kit (Applied Biosystems), and their autosomal profiles were already known and used for confirmation of genetic profiles obtained with the ESSplex Plus kit [13, 20, 30].

STR typing was also carried out for persons who were included in the elimination databases using the Identifiler or NGM kit (Applied Biosystems). Their genetic profiles were compared with those obtained from the bones and teeth to monitor possible contamination of the bone and teeth samples with contemporary DNA. The fluorescent-labelled products were separated with capillary electrophoresis on an automatic ABI PRISM™ 3130 Genetic Analyzer (Applied Biosystems) using the 3130 Performance Optimized Polymer 4 (Applied Biosystems) and the DNA size standard 550 BTO (Qiagen). The genetic profiles were determined using the Data Collection v 3.0 and GeneMapper ID v 3.2 (Applied Biosystems) computer software. Peaks with relative fluorescence units above 50 were accepted for all dyes.

**RESULTS**

The quantification of nuclear DNA with the Quantiplex kit resulted in the determination of up to 66 ng DNA/g of powder from the bones and teeth, and up to 94 ng DNA/g of powder using the Quantifiler kit. In almost two thirds of the samples the results of quantification were higher using the Quantifier than using the Quantiplex kit. In nine of ten tibias the quantification was up to 6 times higher, in 21 of 27 femurs it was up to 5 times higher and in 5 of 17 teeth it was up to 3 times higher. No inhibition was detected in any of bone or tooth analysed, with CT values for IPC between 30 and 32 for the Quantiplex kit and between 26.5 and 27.5 for the Quantifier kit.

The detection limit of the Quantifier DNA quantification assay is 0.023 ng/μl [11] and 0.020 ng/μl for the Quantiplex kit [12]. We detected less than 0.023 ng DNA/μl in 10 bone and tooth samples using Quantifier kit and less than 0.020 ng DNA/μl in 15 bone and tooth samples using Quantiplex kit. We did not detect any DNA with at least one quantification kit in two femurs, one tibia and two teeth. Among them typing of autosomal STRs failed only for femur S24 and tooth S46, for femur S31 and tibia S1 we succeeded to obtain full autosomal profile, and for tooth S43 we managed to obtain partial autosomal STR profile (Table 1).

We obtained complete STR profiles from almost all samples analysed. The autosomal STR typing with the ESSplex Plus kit was successful in 52 out of the 54 samples which represent a 96% success rate, and very few allelic drop-outs were observed. We obtained full autosomal STR profiles for 47 samples and all of them were in concordance to the genetic profiles previously obtained with other kits. Partial profiles with four loci missing were obtained for femur S37, with three loci missing for tooth S43, and with one locus missing for femur S23, tibia S3, and tibia S6. In femur S24 and tooth S46 autosomal STR typing failed (Table 1).

In the process of STR typing of 70 years old skeletal remains, we minimized the possibility of contamination during genetic investigations. No contamination was noted in the extraction and PCR-negative controls. The genetic profiles of the bones and teeth did not match any person from the elimination databases.

**DISCUSSION AND CONCLUSIONS**

Different environmental factors, combined with underground burial for almost 70 years, detrimentally affected the ability to recover intact and uncompromised DNA from WWII skeletal remains. Since methods used
for DNA extraction from old skeletal remains have a strong effect on the amplification success, it is important to use efficient extraction procedure. In the present study we used total demineralization process linked together with the extraction protocol previously validated for old skeletal remains in our laboratory and already successfully used for identification of WWII mass grave victims in Slovenia [13, 31].

As shown also from other studies [6, 8] full demineralization significantly increases the proportion of full profiles reflecting with better DNA quality. EZ1 Biorobot DNA purification system used was very efficient since no inhibition was detected in any of bone and tooth sample analysed. High purification efficacy of Biorobot EZ1 (Qiagen) was observed also from other researchers [32-34] analysing various casework samples. Advanced extraction and purification techniques were found to be essential tools for obtaining sufficient DNA from 70 years old bones and teeth.

According to different length of DNA fragments amplified using the Quantifier and the Quantiplex quantification kits the degree of degradation of bone and tooth samples was estimated. The quantities determined with amplification of 62 bp fragment (Quantifier kit) were up to 6 times higher in almost two thirds of the samples than the quantities determined with amplification of 146 bp fragment (Quantiplex kit), implicating degradation of nuclear DNA. Degradation was observed in 90% of tibias, 80% of femurs and only 30% of teeth, indicating DNA in bones is more degraded than DNA in teeth from Slovenian WWII victims analysed. These findings are in concordance with our previous study using different amplification kits where more full autosomal STR profiles were obtained from old teeth than from old bones [20].

Among different types of skeletal elements teeth together with femurs provided the best STR typing success rates also in identifying the victims of armed conflict in the former Yugoslavia [35].

We obtained STR ESSplex Plus profiles in 96% of samples. In bone and tooth samples where DNA typing failed no detectable DNA was observed with at least one quantification kit. On the other hand partial or full profiles were obtained also from samples where no detectable DNA was observed after quantification. We can conclude that the Investigator ESSplex Plus kit can be used for STR typing of old skeletal remains without any changes to the manufacturers’ PCR amplification protocols and with the DNA extraction method optimised in our laboratory. The Human Quantifier kit and Investigator Quantiplex kit together can be used for estimation of the degree of DNA degradation in compromised old bone samples.

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References