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Automated DNA extraction from recent and archaeological human skeletal remains using QIAGEN's EZ2 Connect Fx Instrument - an experience report

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Abstract

The aim of this study was to investigate the efficiency of the automated DNA extraction method using the EZ2 Connect Fx instrument (QIAGEN), showcasing its ability to achieve swift and reliable results from bone samples. Genomic DNA extraction from 16 archaeological and recent bone samples was performed with a commercial EZ1&2[®] DNA Investigator[®] Kit (QIAGEN). Prior to PCR amplification, DNA concentration was quantified using Qubit[™] Fluorometer (Thermo Fisher Scientific). Amplification was carried out with the Investigator 24plex Kit (QIAGEN). DNA profiles were generated using 3500 Genetic Analyzer (Applied Biosystems) and analysed with GeneMapper[™] ID-X 1.6 Software (Applied Biosystems). For nine archaeological dental samples, eight exhibited partial profiles, and one showed no amplified loci. Among the seven recent femoral bone samples, one exhibited a partial profile, while six presented complete DNA profiles. In this study, we investigated the benefits of automated DNA extraction, particularly its ability to generate timely and reliable results. Additionally, this method reduces pipette and tip usage, almost completely minimising the risk of human error, exogenous DNA contamination and cross-contamination. Such efficiency is of paramount importance, especially when working with skeletal remains, namely archaeological skeletal remains that pose a challenge for molecular genetic analyses.

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Introduction

From the forensic genetics standpoint, choosing the most suitable DNA extraction method is a crucial step, which is particularly important when working with archaeological samples (Saiz et al., 2022). Old skeletal remains often contain DNA that is damaged, degraded or present in low concentrations (Latham & Miller, 2019; Pajnič, 2020). The analysis of DNA extracted from these samples is not only affected by the period following the organism's death but more significantly the environmental conditions, encompassing both abiotic and biotic elements. Taking into consideration the extrinsic factors that affect the preservation of skeletal remains, the optimal conditions for well-preserved DNA are low temperature and humidity, high salt concentration, slightly basic to neutral pH of soil and absence of microorganisms, as well as the absence of UV radiation. Additionally, effective preservation of DNA is shaped by individual characteristics, including race, sex, age and the type of skeletal element itself (Higgins & Austin, 2013; Pajnič, 2020). Considering these optimal conditions, the deterioration of DNA in archaeological samples originates from geological and chemical properties of soil which skeletal remains were excavated from. Other contributing factors include presence of salt, low pH, oxygen levels and moisture, exposure to radiation, high temperature as well as microorganisms representing the biotic variable (Loreille et al., 2007; Campos et al., 2012; Latham & Miller, 2019; Haarkötter et al., 2022). In addition to numerous environmental factors, different PCR inhibitors (e.g. humic and fulvic acid) that directly affect the preservation of DNA contribute to the degradation of skeletal elements.

Likewise, various inhibitors can be added during sample processing or DNA extraction, such as powder from gloves, sterilising agents, different salts (e.g. potassium and sodium chloride), detergents and other organic molecules like ethylenediaminetetraacetic acid (EDTA), sarkosyl,

phenol chloroform, and isopropyl alcohol (Latham et al., 2021; Haarkötter et al., 2022). The anthropogenic effect cannot be disregarded, considering that improper post-excavation storage of skeletal remains, such as exposure to higher temperatures, generates poorer results during DNA analysis (Zupanič Pajnič, 2020).

Now having recognized the delicate nature of DNA in skeletal samples, chiefly archaeological ancient DNA (aDNA), the significance of selecting a suitable extraction method becomes evident. Although conventional organic phenol-chloroform method has repeatedly been shown as efficient in DNA extraction from different skeletal samples (Davoren et al., 2007; Džehverović et al., 2021; Jusić et al., 2022; Džehverović et al., 2023; Haarkötter et al., 2023), using this method has some disadvantages - namely the usage of toxic reagents, potential loss of DNA during sample transfer between tubes, ongoing contact among technicians and different samples leading to potential cross-contamination and contamination with exogenous DNA. Another disadvantage arises from the additional step of decalcification standardised in this conventional method (Loreille et al., 2007) leading to a longer extraction time. This approach is also susceptible to potential human error as its execution relies significantly on the individual carrying out the procedure. This dependence arises due to the possibility the reagents used might integrate into the final product, acting as PCR-inhibitors. Consequently, there is an increased risk of uneven sample analysis (Khan et al., 2022).

In contrast, automated DNA extraction yields results within a shorter time-frame, utilising silica-coated magnetic bead technology. Specifically, the QIAGEN's EZ2 Connect Fx Instrument is equipped with preinstalled protocols for DNA purification, appropriate for samples containing limited amount of DNA, which is often the case with archaeological bone samples. However, despite numerous advantages offered by automated DNA extraction protocol, including its

rapidness, minimised risk of contamination and avoidance of hazardous chemicals (Rubinstein, 2021), recent study conducted by Zupanič Pajnič et al. (2023a) indicates that an additional step of demineralisation in automated DNA extraction protocol ensures larger amounts of DNA to be obtained. Additionally, a number of studies emphasise the importance of removing calcium and mineral ions in the process of DNA extraction from skeletal samples (Loreille et al., 2007; Lee et al., 2010; Amory et al., 2011; Jakubowska et al., 2012; Booncharoen et al., 2021). Thus, the optimisation of DNA extraction protocols when working with skeletal remains may be necessary for enhanced subsequent analyses. The optimisation holds the most potential to improve DNA profile success rates for this type of samples (Zoranjic et al., 2021; Haarkötter et al., 2023).

In this study, we aim to showcase the efficiency of automated DNA extraction method by analysing nine archaeological skeletal samples excavated from various mediaeval Bosnian necropolises and seven recent skeletal samples.

Material and methods

Skeletal specimen preparation

In total 16 human skeletal samples were processed, including nine archaeological dental samples (Figure 1) originating from various necropolises from the period of mediaeval Bosnia (Sample A2, Sample A7 and Sample A8 – Travnik (Regional Museum of Travnik); Samples A4-A6 – Tuzla, Sample A3 – Čelebići, Sample A1 – Bobovac (Crkvice), Sample A9 – locality near Trebinje), along with seven recent femoral bone samples (Figure 2). To eradicate any potential contaminants, the bone surfaces underwent a cleaning step utilising a Dremel rotary (Dremel, Wisconsin, USA) tool with a grinding stone attached. Subsequently, all samples underwent a thorough washing procedure according to standardised protocol (5% Na-hypochlorite 3x, distilled water 3x and absolute ethanol 3x), followed by a drying period of five to seven days.

Next followed the grinding step where teeth samples were turned into a fine dental powder using a sterilised IKA tube mill (IKA®-Werke GmbH&Co.KG, Germany). Femur samples were ground using a Dremel rotary. To prevent any risk of contamination, grinding was carried out using sterilised grinding equipment within a negative pressure lab hood customised for work with skeletal samples.



Figure 1. Archaeological dental samples used in this study



Figure 2. One of the recent femoral bone samples used in this study

DNA extraction and quantification

Approximately 400 mg of dental or bone powder per sample underwent a four-hour pre-treatment process involving disruption and lysis on 56°C, according to the "Bone Extra Large Volume Protocol" (QIAGEN, 2022). Lysis reagent mix was prepared with G2 Buffer, Proteinase K, both supplied in the EZ1&2® DNA Investigator® Kit,

and 0.5M EDTA. Then, DNA extraction and purification proceeded as per the same protocol on the EZ2 Connect Fx Instrument, with a 20µL elution volume. The quantification of DNA extracts was performed in accordance with the manufacturer's recommendations, employing the Qubit® dsDNA HS Assay Kit and the Qubit® Fluorometer (Thermo Fisher Scientific). Also, negative extraction controls were included in the process in order to detect potential contamination during the extraction procedure.

PCR amplification and post-amplification

The amplification of the isolated DNA was carried out on the QIAamplifier 96 (QIAGEN, Hilden, Germany) using the Investigator® 24plex QS Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations (QIAGEN, 2021). The detection of PCR products using capillary electrophoresis was carried out using the 3500 Genetic Analyzer (Applied Biosystems™, Massachusetts, USA). Subsequently, DNA profiles were generated using the GeneMapper™ ID-X 1.6 Software (Applied Biosystems™, Massachusetts, USA), with RFU threshold of 50.

To detect potential contamination, PCR negative controls were included into the process. Furthermore, all PCR and post-amplification procedures were conducted in different laboratory sections, each equipped with sterilised laboratory tools dedicated to PCR and post-amplification work, respectively.

Results and Discussion

The selection of an optimal DNA extraction method is pivotal in the analysis of skeletal remains, particularly in the case of archaeological samples where natural DNA degradation poses an occurring obstacle. In addressing this difficulty, automated DNA extraction methods have been documented in existing literature as a highly efficient approach for obtaining genomic DNA from both archaeological and recent samples,

offering faster results with higher-quality DNA. For instance, Karija Vlahović and Kubat (2012) compared the efficacy of manual silica-based DNA extraction with automated DNA extraction from skeletal remains with different stages of decomposition. Based on the removal of inhibitors and the quality of resulting STR profiles, they concluded that the automated extraction protocol can definitely be used instead of manual DNA extraction procedure. A study conducted by Zupanič Pajnič et al. (2016a) confirmed that the automated extraction method can provide robust and reliable results, and thus can be used for efficient genomic DNA extraction from old and contemporary skeletal remains of various ages, especially with demineralisation stage included. The study examined 24 recent bone samples, applying the standardised automatic DNA extraction protocol to one series of samples and introducing an additional demineralisation step to another group. The results showed an 8.4% increase in the number of obtained full DNA profiles in samples that underwent the demineralisation process, as opposed to those treated according to the standardised procedure. Barbaro et al. (2021) compared two QIAGEN automated protocols out of which one was modified, subsequently leading to a conclusion that the modified automated extraction protocol with EDTA demineralisation of powdered skeletal remains within 5 to 7 days allows high recovery of DNA that is free of inhibitors. Recently, Zupanič Pajnič et al. (2023a) used an extraction instrument EZ1 Advanced XL (QIAGEN) and EZ1 Investigator Kit (QIAGEN), similar to the one used in this study, to extract DNA from different Second World War skeletal remains. This study confirmed the effectiveness of this particular method when processing small amounts of bone or dental powder, once again emphasising the importance of full-decalcification/demineralisation step. Likewise, Zupanič Pajnič et al. (2023b) successfully obtained STR profiles from mediaeval skeletal remains and predicted eye and hair colour based on the DNA extracted using the

previously mentioned automated DNA extraction procedure. Haarkötter et al. (2023) conducted a comparative study of five different methods for DNA extraction from aged human skeletal remains including, inter alia, manual phenol-chloroform DNA extraction procedure and automated DNA extraction protocol using the PrepFiler™ BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific) in AutoMate™ Express (Thermo Fisher Scientific), where automated protocol showcased as a good quality procedure for routine high-throughput DNA extraction from degraded human remains. Other studies also reported obtaining reliable results from skeletal remains after automated DNA extraction (Cafiero et al., 2019; Duijs and Sijen, 2020; Rubinstein, 2021). Aforementioned findings regarding the benefits of demineralisation with EDTA are consistent with the conclusions of other studies (Loreille et al., 2007; Lee et al., 2010; Amory et al., 2011; Jakubowska et al., 2012; Booncharoen et al., 2021), although Loreille et al. (2007) highlighted the loss of DNA after each step of washing in EDTA.

To evaluate the efficiency of automated DNA extraction, we performed STR profiling on nine archaeological teeth samples, out of which eight were partial (two samples showing less than 50% amplified loci and six displaying over 50% amplified loci), while Sample A2 showed no amplification (Table 1). Among the seven recent femoral bone samples, one exhibited a partial profile, while the remaining six profiles were complete with all autosomal loci amplified, as shown in Table 2. This observation suggests that the prevalence of partial archaeological profiles may be primarily attributed to incomplete DNA preservation. However, to confirm this, it is essential to repeat the process with a prior demineralisation stop. In contrast, recent STR profiles show a higher completeness rate, amounting to 86% for full DNA profiles. This underscores the importance of factors such as age, suboptimal DNA preservation, and environmental conditions in the excavation site soil as contributing factors to the increased occurrence of partial profiles in archaeological samples (Zupanič Pajnič, 2020).

Table 1. DNA profiles of nine archaeological samples

STR locus	Sample A1	Sample A2	Sample A3	Sample A4	Sample A5	Sample A6	Sample A7	Sample A8	Sample A9
TH01	6 -	...	9	6 9.3	8 9.3	6 6	6 6	6 6	6 8 9
D3S1358	...	...	17	15 18	16	15 16	14 16	16	...
vWA	...	...	...	18	16	...	17 19	...	...
D21S11	...	...	...	27 28	...	...	29	...	...
TPOX	...	...	8	8 11	8	8 11	9 11	8 11	8 9 11
DYS391	...	...	...	11	11	11	9	9	...
D1S1656	19.3	...	12	13 15	15 17.3	11 17.3	11 19.3	12 16	...
D12S391	...	...	17 18	18	...	20 21	18 23	...	...
SE33	...	...	...	18 29.2	...	18	20	...	...
D10S1248	...	...	...	13 13	16	13 13	14 15	12 16	12 13
D22S1045	...	...	...	15 15	16	14 16	11 16	14 15	...
D19S433	...	...	...	11 13	16	16	14 16	12 13 14	...
D8S1179	...	...	13	13	...	...	13 13	...	...
D2S1338	...	...	...	...	...	...	...	...	...
D2S441	...	...	11 11.3 12	11 14	11	11 14	10 10	11	10 14
D18S51	16	...	15	16 17	16	13 17	12 13	18 21	16
FGA	...	...	19 22	21	...	20 23	20 26	23	...
D16S539	...	...	11	9 12	12	10 12	11 11	11 12	...
CSF1PO	10	...	12	10	8 10	10 11	10 11	11 12	...
D13S317	...	...	12	8 12	...	11 11	8 12	11 12	11
D5S818	...	...	...	...	11	13	11 12	...	...
D7S820	...	...	...	8	...	10	10 10	...	...
Amelogenin	...	...	X Y	X Y	X Y	X Y	X Y	X Y	X Y

Table 2. DNA profiles of seven recent bone samples

STR locus	Sample R1	Sample R2	Sample R3	Sample R4	Sample R5	Sample R6	Sample R7
TH01	6 6	7 9.3	6 8	7 -	9 9.3	6 9.3	9 9.3
D3S1358	15 15	16 16	15 16	15 17	16 16	15 17	16 18
vWA	15 16	17 18 19	15 19	19 -	14 17	15 18	15 20
D21S11	29 31	29 30	29 29	28 31.2	29 31.2	28 31	29 30
TPOX	11 12	11 11	8 8	11 11	8 8	8 10	8 8
DYS391	...	11	10	...	11	11	...
D1S1656	11 13	15 16	12 12	11 17	14 18	12 16	16 16
D12S391	16 21	18 22	17 18	17.3 23	21 22	18 22	16 22
SE33	25.2 28.2	19 27.2	19 24.2	15 27.2	19 29.2	20 27.2	17 18
D10S1248	14 15	13 16	15 15	13 14	13 14	12 16	12 15
D22S1045	15 18	15 17	11 15	11 15	16 19	11 15	15 17
D19S433	15	14 14	14 16	13 16	14 14	12 12	14 14
D8S1179	14 16	13 13	13 13	...	13 13	13 13	10 12
D2S1338	13 23	17 20	20 23	18 -	16 17	24 -	17 25
D2S441	11 14	14 15	11 13	14 14	14 14	10 10	10 11
D18S51	15 18	13 18	14 15	12 14	13 18	14 14	15 17
FGA	22 25	20 22	19 21	25 26	24 25	24 -	22 23
D16S539	11 14	9 9	11 12	9 14	11 12	12 13	19 12
CSF1PO	11	10 10	11 13	10 14	10 12	11 11	10 11
D13S317	8 12	11 12	9 12	11 14	11 12	8 9	8 12
D5S818	9 11	11 12	12 15	11 12	11 12	12 13	12 12
D7S820	10 12	11 11	11 12	10 11	10 11	11 11	8 11
Amelogenin	X X	X Y	X Y	X X	X Y	X Y	X X

We posit that the introduction of an extra decalcification step, designed to enhance DNA recovery by softening tissues and facilitating more effective action of lysis solutions (Barbaro et al., 2021), is likely to enhance the probability of obtaining complete DNA profiles from archaeological bone samples. This observation aligns with findings from various mentioned studies that have already demonstrated the same effect in the context of recent bone samples. The STR profiles obtained were cross-referenced with elimination database profiles, revealing no matches, which implies the absence of contamination. The STR profiles obtained were cross-referenced with elimination database profiles, revealing no matches, which implies the absence of contamination. Additionally, no profiles were obtained from the extraction and PCR negative control samples. Concerning archaeological skeletal remains, the lowest measured DNA concentration was ≤ 0.010 ng/ μ L (Sample A1), whereas the highest measured DNA

concentration was 28.80 ng/ μ L (Sample A7), and DNA concentration in Sample A6 was too low to detect. Among the recent samples, Sample R3 displayed the highest DNA concentration at 80.20 ng/ μ L, while Sample R2 showed the lowest detected DNA concentration at 0.010 ng/ μ L. DNA concentration of each sample is depicted in Table 3. With DNA profiles and concentrations now established for both archaeological and recent samples through automated DNA extraction, attention will be directed toward a comparative analysis of this contemporary method and the conventional phenol-chloroform extraction. The objective is to clarify distinct attributes of each method and their relevance to forensic genetic analysis. The first distinction lies in the fundamental principles of the two methods, where automated DNA extraction is based on combination of different reagents and silica-coated magnetic particles (Nagy et al., 2005; QIAGEN, 2022), whereas phenol-chloroform method uses often hazardous organic solvents (McKiernan &

Danielson, 2017; Shen, 2023). Following is the difference in rapidness, which is one of the main qualities of automated DNA extraction, simultaneously generating results from multiple samples within a few short hours.

In contrast, phenol-chloroform extraction involves a more time-consuming preparation step, including the process of decalcification, cell lysis, protein degradation, and a 48-hour incubation, totalling an estimated 5 to 7 days (Edson & McMahon, 2016). However, opting for this methodology greatly

Table 3. DNA quantification results for archaeological and recent bone samples

Sample type	Sample code	DNA concentration (ng/μl)
Archaeological bone samples	Sample A1	≤ 0.010
	Sample A2	5.70
	Sample A3	9.92
	Sample A4	7.78
	Sample A5	5.10
	Sample A6	...*
	Sample A7	28.80
	Sample A8	14.7
	Sample A9	24
Recent bone samples	Sample R1	11.30
	Sample R2	0.010
	Sample R3	80.20
	Sample R4	5.96
	Sample R5	5.18
	Sample R6	9.38
	Sample R7	12.60

*non-detectable DNA concentration

reduces the overall cost of the experiment. Some of the benefits of the automated DNA extraction offers are avoiding pipette and tip usage (with the exception of setting up sample incubation pre-isolation), thus effectively minimising the potential for exogenous DNA contamination and cross-contamination.

On the other hand, the elevated costs associated with equipment and necessary chemicals are greater than in conventional methods, which is depicted in Figure 3 alongside other attributes of each method.

Conclusion

The objective of this study was to examine the efficiency of automated DNA extraction and its suitability for the analysis of skeletal remains, both of recent and archaeological origin, particularly in terms of speed and contamination risk reduction, we established the differences between this approach and the conventional phenol-chloroform DNA extraction method, and reached the following conclusions.

Automated DNA extraction has demonstrated high efficiency in analysing recent skeletal remains, yielding a full DNA profile in 86% of cases. However, none of the analysed archaeological samples generated a full DNA profile, indicating a 0% success rate for obtaining complete profiles. Thus we predict adding a pre-extraction decalcification step to the existing automated DNA extraction protocol may enhance results when working with samples containing low DNA concentration. Furthermore, the study clearly displays the advantage of automated DNA extraction over organic DNA extraction with its ability to yield results faster, reduce the potential for cross-contamination and exogenous DNA contamination, all while avoiding toxic chemical usage standardised in organic DNA extraction protocols.

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Conflict of interest

The authors declare no conflict of interest.

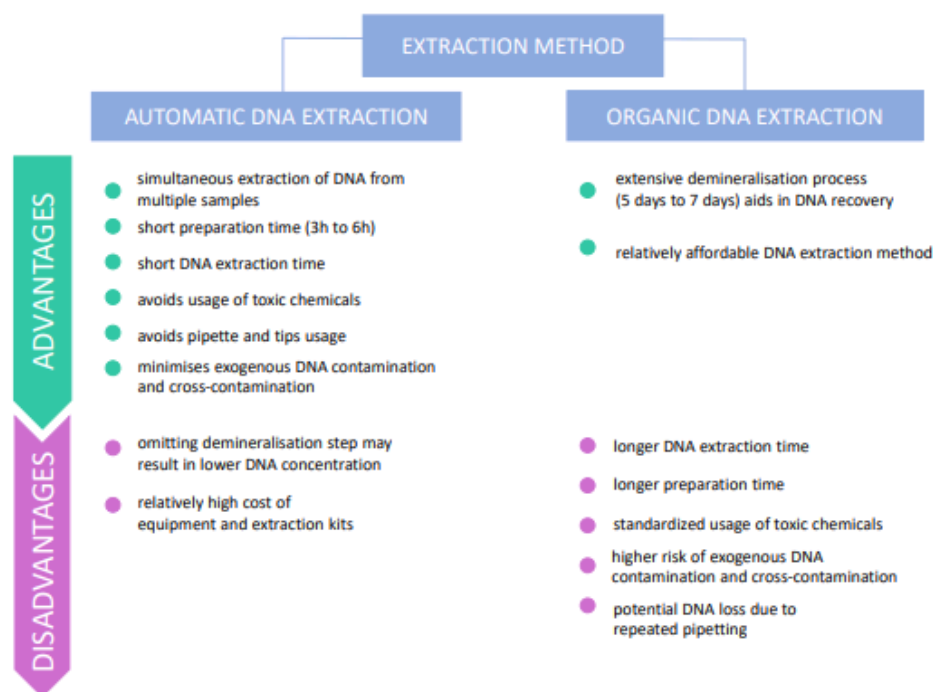


Figure 3. Advantages and disadvantages of automated and organic DNA extraction methods

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