Benchmark

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The influence of sample quantity and lysis parameters on the success of ancient DNA extraction from skeletal remains

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ABSTRACT

DNA extraction is of utmost importance in archaeobiology, as it determines the success of further DNA analyses. This study concentrates on the success of ancient DNA extraction using silica spin columns and PCR-based analysis from archaeological skeletal material and investigates the influence of sample quantity, lysis time and lysis temperature during sample preparation. The results show that lysis times ranging from 2 to 48 h are suitable, and that lysis should be carried out at a constant temperature of 56°C. Concerning sample quantity, 10 mg for mitochondrial DNA and 50 mg for chromosomal DNA are sufficient for high quality analyses. Thus invaluable sample material can be saved, and time of sample preparation can be reduced considerably.

METHOD SUMMARY

An optimized protocol for sample preparation for the extraction of ancient DNA from archaeological skeletal material is presented. The tested and optimized parameters were sample quantity, lysis duration and lysis temperature.

KEYWORDS:

ancient DNA extraction ● archaeological skeletal remains ● chromosomal DNA ● lysis duration ● lysis temperature ● mitochondrial DNA ● sample amount ● sample preparation parameters ● sequence analysis ● STR typing

Working with ancient DNA (aDNA) remains a great challenge because, in contrast to DNA from contemporary sample materials, aDNA is present in lower quantities, reveals stronger fragmentation and chemical changes [1] and includes higher levels of microbial DNA contamination. DNA extraction, including all the preparation steps, is therefore one of the most important and limiting steps within the analysis of aDNA [2,3]. We sought to test whether it is possible to reduce the sample amount and shorten the lysis duration without compromising the quantity and quality of extracted DNA. Especially in the case of valuable archaeological samples, it is useful and important to work with smaller sample quantities. Furthermore, shortening the lysis step, which is by far the most time-consuming step, would considerably accelerate the entire process of DNA extraction from skeletal samples.

Although samples have widely varying requirements for DNA extraction due to the diverse environmental conditions of the burials [4], silica-based methods predominate with skeletal remains due to their easy handling, less dangerous chemicals and suitable performance. In the field of anthropological aDNA research, Rohland and Hofreiter investigated aDNA extraction parameters in detail through a large-scale study [5]. Their efforts concentrated on a comprehensive comparison of published extraction methods and a systematic examination of the individual reagents' influences. They showed that the use of solely EDTA and proteinase K for sample lysis and the use of silica for DNA isolation produces the best results for paleontological samples. Similar results, with a focus on complete demineralization during the lysis process, were found in later studies using contemporary skeletal material [6,7]. Ultimately, optimized DNA extraction is not only relevant in the field of anthropology and archaeozoology, but is equally important for forensic research [e.g., 8,9].

In the present study, we investigated the influence of sample quantity, lysis duration and lysis temperature on the DNA amplifiability using a silica-based extraction procedure and PCR-based autosomal short tandem repeat (STR) and mitochondrial DNA typing [10]. The experiments were carried out on one femur from the skeletal series Goslar (GS97, 250 years old) and one from the burial site Lichtenstein cave (DO902.01, 3000 years old); both were known from earlier studies to show excellent DNA preservation (Supplementary Material 1). By restricting the study to samples with excellent DNA preservation, we expected a reliable correlation between the tested parameters and the results. In the case of samples with poor DNA preservation, the results would be suspected to occur stochastically due to too few intact targets.

In an initial experiment (pretest), we compared our current DNA extraction protocol [11], which involved an 18-h lysis at 37°C followed by a 2-h lysis performed at 56°C, with an 18-h lysis at a constant temperature of 56°C for all sample quantities. For the main experiment we used a 4 × 5 design to test sample quantities ranging from 10 to 250 mg and lysis durations ranging from 2 to 48 h (Supplementary

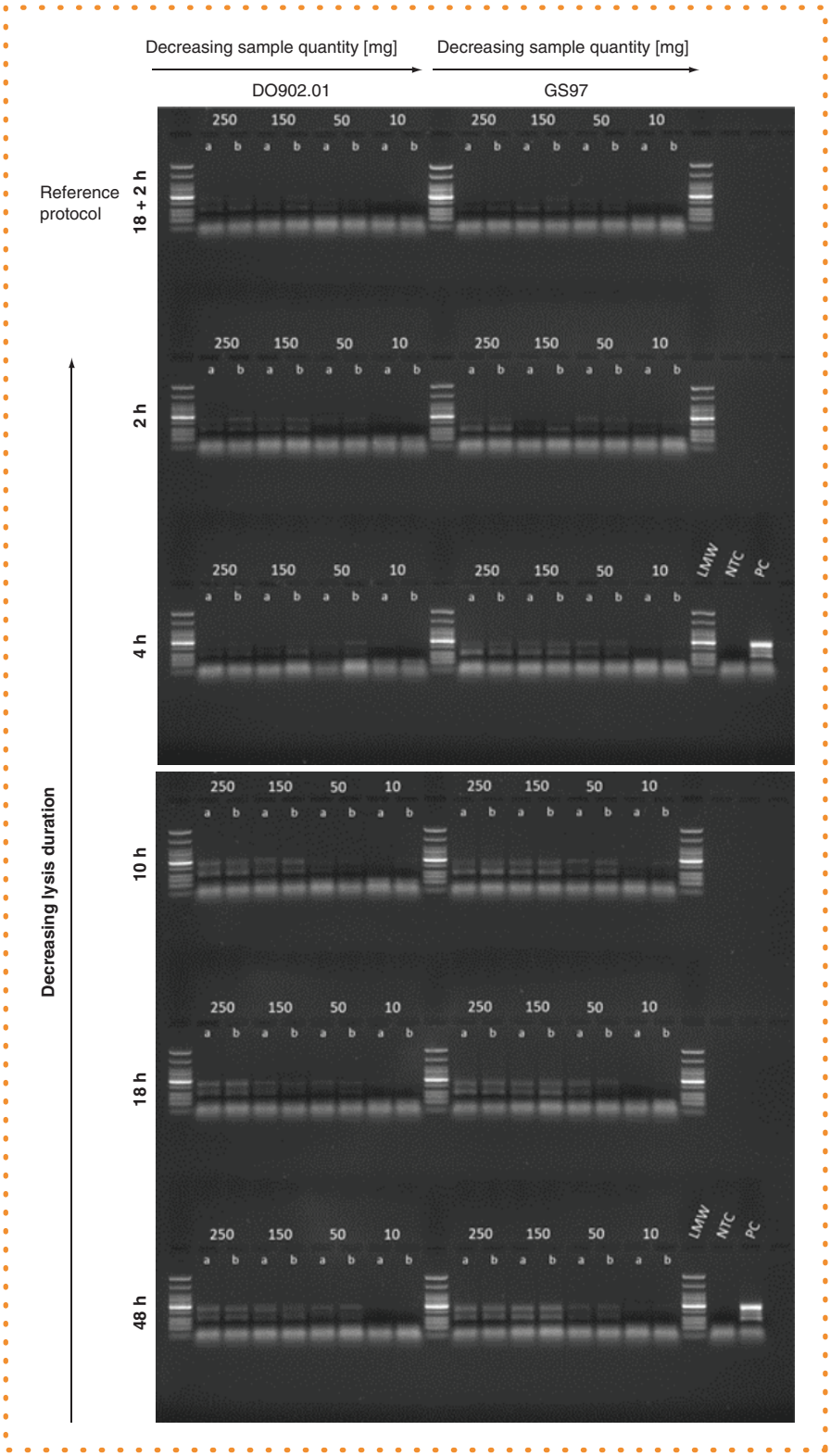

Figure 1. Agarose gel electrophoresis for nuclear DNA amplifications. Analysis shows a successful overall amplification for all analyzed parameters, although the samples revealed considerably weaker signals when 10 mg of bone powder was used. PCR was performed in duplicates with 5 µl of DNA extract.
LMW: Low-molecular-weight DNA ladder; NTC: Negative control; PC: Positive control.

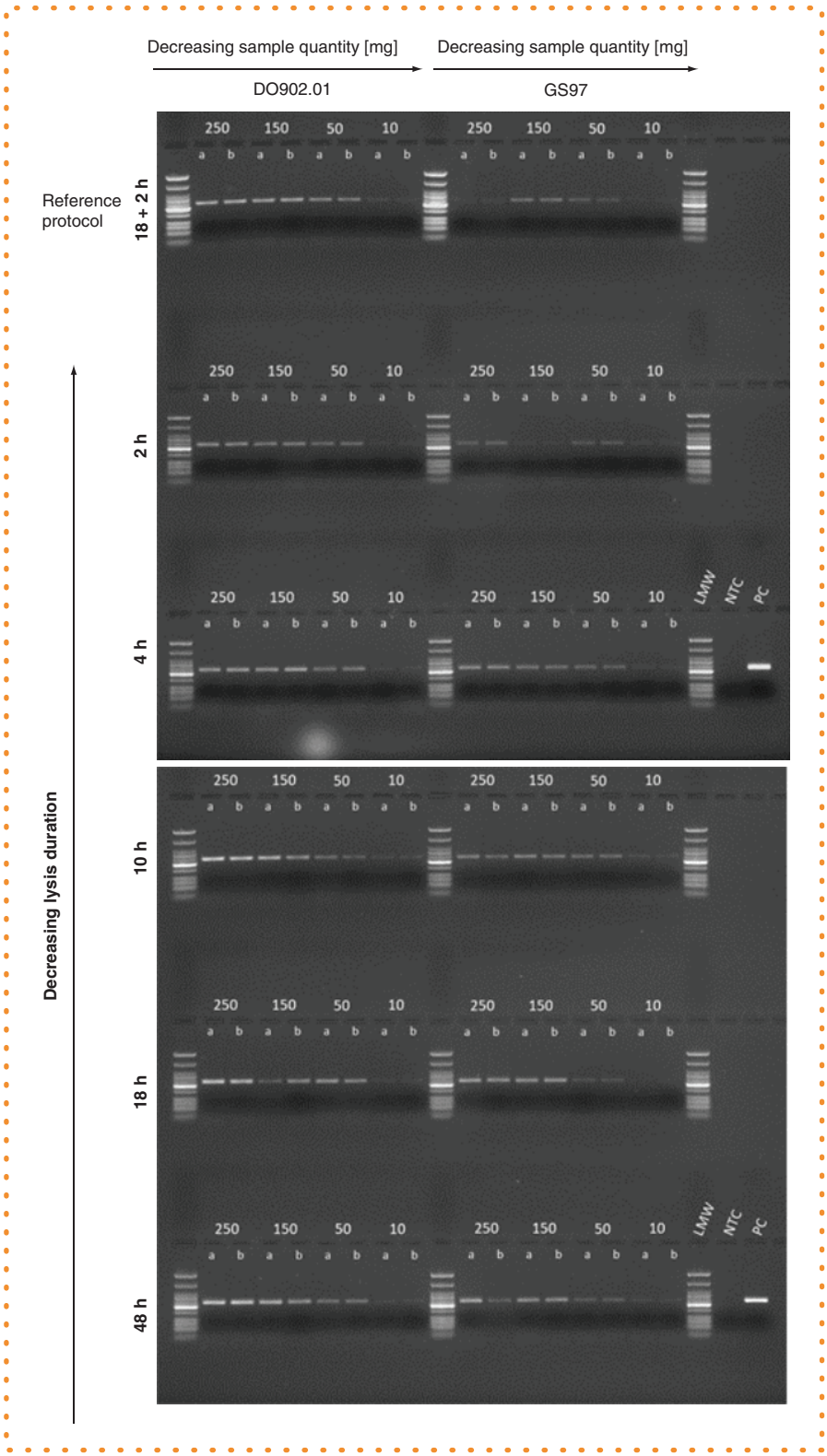

Figure 2. Agarose gel electrophoresis for mitochondrial DNA amplifications. Analysis shows a successful overall amplification for all analyzed parameters, although the samples revealed weak signals when 10 mg of bone powder was used. PCR was performed in duplicates with 5 µl of DNA extract.
LMW: Low-molecular-weight DNA ladder; NTC: Negative control; PC: Positive control.

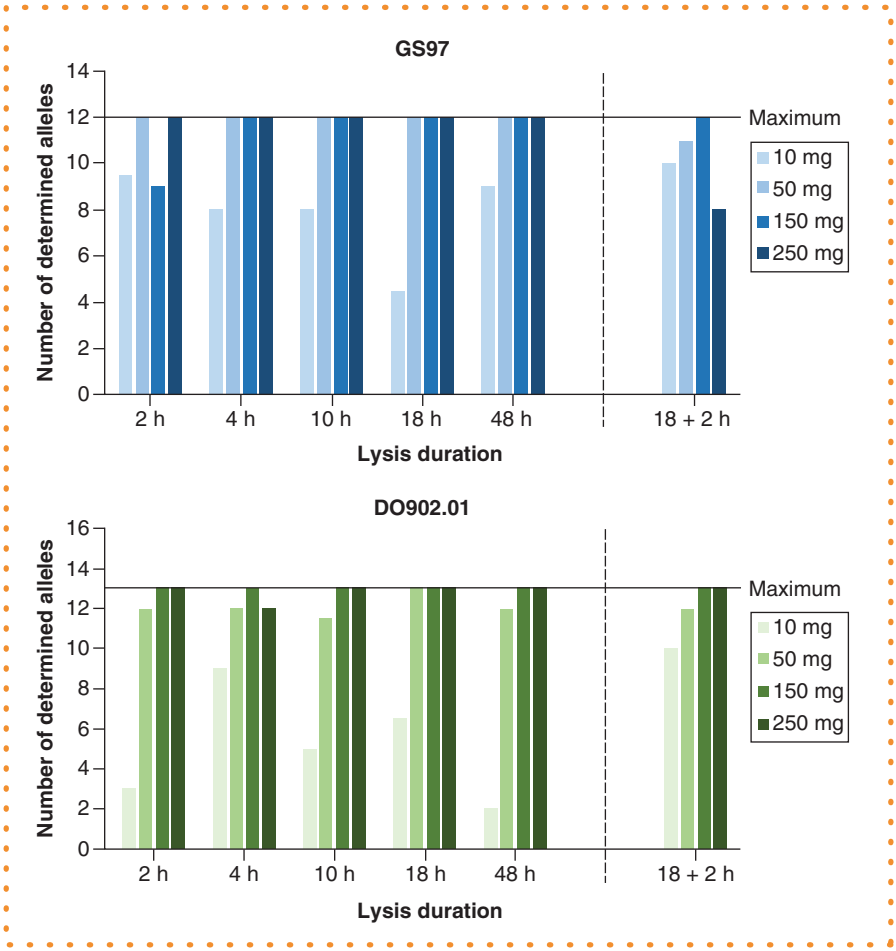

Figure 3. Number of determined alleles as a function of lysis duration and sample quantity for nuclear DNA.

Material 1). After DNA extraction and amplification, we used agarose gel electrophoresis to verify the overall amplification success. To assess the quality of the results, we performed capillary electrophoresis including software-based allele determination for the autosomal STR typing. The number of amplified STR alleles and their peak heights served as a measure of DNA extraction success. This approach is preferable to a quantification of the total amount of DNA because it correlates only to the numbers of intact target sequences in the extracted DNA – and these are influenced by the extraction procedure [12]. For mitochondrial DNA base calling, we performed Taq-cycle sequencing prior to capillary electrophoresis. Here we focused on whether the bases could be unambiguously determined in all cases. For this, the homogeneity of sequences and the signal-to-noise ratios were used as qualitative indicators. To ensure comparability of the results, all other parameters in the experiment were standardized during the analyses (Supplementary Material 1).

Recently, Hasap *et al.* published an improved method for forensic casework samples which proposes a lysis time of only 2 h [13]. We therefore considered this significant reduction in lysis time even for historical samples. Haffner *et al.* (manuscript in progress) obtained excellent results when analyzing mitochondrial DNA extracted from prehistoric bat bones while using as little as 6–8 mg bone powder. This is in accordance with other ancient DNA applications that were also successful using smaller sample quantities [9,14]. These results encouraged us to significantly reduce the amount of bone powder from human skeletal material to a minimum of 10 mg, thus deviating considerably from the 250–500 mg commonly used [2,7,8,15]. The aim was to achieve a total lysis as soon as possible. Therefore we decided not to reduce the buffer volume accordingly, as Dukes *et al.* [6] showed that a large buffer volume increases DNA yield and favors total lysis, which is known to maximize DNA recovery [7]. Many protocols in aDNA research carry out lysis at 37°C, mostly complemented by a short interval at a higher temperature (56°C) to avoid further DNA damage [11,14]. However, in forensic science, the entire lysis process is often carried out at 56°C [6,8]. Given that 56°C is the optimum temperature for proteinase K activity [16], from a theoretical point of view lysis temperature is a clear candidate for further assessment. Therefore, and in order not to extend the main experiment unreasonably, we decided to study this by a pretest as described above.

The results of the pretest show that comparable (DO902.01) or superior (GS97) results (as indicated by the completeness of STR typing) were obtained for the 18-h lysis at a constant temperature of 56°C as compared with an 18 + 2-h lysis at predominantly 37°C (Figures 1–3). We therefore decided to carry out the main experiments with the constantly elevated lysis temperature of 56°C.

The results of the main experiment testing sample quantity and lysis duration are presented in Figure 3. The agarose gel electrophoresis, which served as a verification for the overall amplification success, shows that DNA could be successfully extracted and amplified from all samples and gives a first impression of the tested parameters' general suitability (Figures 1 & 2).

Capillary electrophoresis was used for precise fragment length analysis of the STR alleles (STR typing) and sequence analysis (mitochondrial DNA base calling). The number of amplified STR alleles and their peak heights served as a measure for nuclear DNA extraction success (Supplementary Material 3). In STR typing, complete sets of alleles were obtained for all lysis durations and a sample quantity of at least 50 mg. In contrast, the DNA samples derived from quantities of only 10 mg of bone powder showed numerous allelic dropouts (Figure 3 & Supplementary Material 2). In mitochondrial DNA base calling, however, it was possible to determine the sequence from all tested lysis durations and sample quantities, including the 10-mg aliquots, and the bases could be determined unambiguously. To evaluate whether the tested parameters nevertheless influence the quality of the sequencing results, we compared the quality of the electropherograms in terms of signal-to-noise ratio and homogeneity of the sequences. There seemed to be no obvious correlation between the tested parameters and our chosen indicators for sequence quality (Supplementary Materials 4 & 5). The difference observed in the analysis of mitochondrial and nuclear DNA from the 10-mg samples can be explained by the much higher content of mitochondrial DNA in a defined volume of sample material.

In summary, despite the small sample size, our results suggest that suitable allele determination and sequencing data can be achieved with a lysis time ranging from 2 to 48 h at 56°C for aDNA from skeletal remains using a silica-based extraction and PCR-based analysis. Further, our experiments reveal that sample quantity can be considerably reduced. 10 mg of well-preserved material for mitochondrial DNA analysis, or 50 mg for chromosomal DNA analysis, may be sufficient. Although our study would benefit from follow-up experiments involving more degraded samples and the testing of the suitability for next-generation sequencing applications, these findings already allow flexibility in lab work with respect to lysis duration, which should be oriented toward achieving total lysis and help to preserve invaluable sample material.

Future perspective

The analysis of ancient and degraded DNA has found its way into numerous scientific fields. This implies the need for ongoing optimization and adaptation to the specific source materials. In general, it is expected that minimally destructive methods will be in demand in many new fields of application, such as provenance research. The same applies to time-saving methodological approaches, which will be increasingly in demand in forensic applications.

Executive summary

- We tested an optimized protocol for ancient DNA extraction from archaeological skeletal material (Early Modern to Bronze Age).
- Extracted DNA samples from bone underwent mitochondrial and chromosomal DNA analysis (hypervariable region sequencing and short tandem repeat typing).
- We demonstrated the feasibility of reducing the starting sample quantity to 10 mg for mitochondrial DNA and 50 mg for chromosomal DNA analysis.
- We demonstrated that lysis times ranging from 2 to 48 h are suitable.
- We showed that a permanent increase of the lysis temperature to 56°C achieves satisfactory results.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2020-0169

Author contributions

A Euskirchen: acquisition, analysis and interpretation of data; writing of the manuscript. L Hartmann: acquisition, analysis and interpretation of data; writing of the manuscript. J Mazanec: project design, interpretation of data, revision of the manuscript. P Wittmeier: acquisition of data, revision of the manuscript. S Hummel: project design, interpretation of data, writing of the manuscript. All authors critically reviewed the manuscript and approved the final version.

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