**Supplementary information for:**

**A method for the temperature-controlled extraction of DNA from ancient bones**

**Supplementary protocol on protocol.io:**

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**Materials and methods:**

All pre-PCR sample preparation steps were performed in a dedicated ancient DNA clean room laboratory following stringent measures to reduce the introduction of contamination.

Proof-of-principle experiment

In the first experiment, 77 and 53 mg bone powder were obtained from a whale bone from the bottom of the North Sea (SP3554, [1]) and a bovid bone from Denisova Cave in southern Siberia, Russia (SP3391, [1]), respectively, by drilling with a sterile dentist drill. To facilitate resuspension of the bone powder during the subsequent incubation and wash steps, 3-4 ceramic beads (2.8 mm in diameter, VWR International) were added to the sample material. The sample powder was subjected to serial 15-minute incubations in 0.5 ml sodium phosphate buffer (0.5 M sodium phosphate buffer, pH 7.0, 0.1% Tween-20) at 21, 37, 60, 70, 80 and 90 °C, respectively. Two incubation steps were performed at each temperature, followed by one wash in 0.5 ml Tris-Tween buffer (10 mM Tris-HCl, pH 8.0, 0.1% Tween20) to reduce the carry-over of DNA between fractions. Finally, the sample powder was digested in Lysis buffer (0.45 M EDTA, pH 8.0, 0.05% Tween-20 and 0.25 mg/ml proteinase K) at 37 °C with rotation overnight (8 – 16 h). Prior to each incubation, wash or lysis step, the sample powder was resuspended by vigorous vortexing.

Decontamination methods comparison

To test whether the gradual DNA extraction method provides advantages over regular DNA extraction and previously described decontamination methods, we performed a second experiment in which we applied the different decontamination methods to a set of three samples. Additional bone powder (593 and 543 mg, respectively) was obtained from the whale and bovid bones used in the previous experiment by drilling with a sterile dentist drill, as well as from a 230,000-year-old straight-tusked elephant bone fragment from Weimar-Ehringsdorf [2] (527 mg). Bone powder from each sample was thoroughly mixed and divided into fifteen 30-45 mg sub-samples in 2.0-ml Eppendorf DNA LoBind tubes using a sterile, antistatic spatula. To facilitate resuspension of the bone powder during the subsequent incubation and wash steps, 3-4 ceramic beads were added to the sample material. Each experimental condition was performed in triplicates.

Gradual DNA extraction was performed as described above but limiting incubation temperatures to 37, 60 and 90 °C and performing only one wash with Tris-Tween buffer (after the second 90 °C incubation). Bleach treatment was performed following Korlevic et al. (2018) [1]by adding 1 mL 0.5% bleach solution to the sample powder, vortexing and incubating for 15 minutes at room temperature under rotation, followed by three washing steps with 1 mL water to remove excess bleach. Room temperature phosphate pre-treatment was performed as described earlier [1] using three consecutive wash steps in 1 mL sodium phosphate buffer (0.5 M sodium phosphate buffer, pH 7.0, 0.1% Tween-20) at room temperature for 15 minutes, followed by one wash in 1 ml Tris-Tween buffer (10 mM Tris-HCl, pH 8.0, 0.1% Tween20) to reduce the carry-over of phosphate into the final digestion. Pre-digestion was performed using the method of Damgaard et al. (2015) [3] by adding 0.25 ml digestion buffer (0.5 M EDTA, 18 mg/mL Proteinase K and 0.5% N-Laurylsarcosyl) to the sample powder, vortexing and incubating for 60 minutes at 37 °C under rotation, followed by a final digestion in 0.25 mL digestion buffer overnight at 37 °C under rotation.

DNA extraction, library preparation, sequencing and data processing

DNA extractions were performed following the method by Glocke and Meyer (2017) [4] as described in detail by Rohland et al. (2018) [5] (binding buffer option ‘G’). Supplementary Table 1 details the fractions of the lysates/phosphate washes that were purified. Final volume of all DNA extracts was 50 µl. Single-stranded DNA libraries were prepared using 20% of the DNA extract as input, following the protocol for library preparation, quantification and indexing by Gansauge et al. (2020) [6]. Indexed libraries were pooled with libraries from other projects and subjected to shallow shotgun sequencing on Illumina’s HiSeq2500 platform using a paired-end double-index configuration (2x 76 + 2x 7 cycles) [7].

Adapters were trimmed and overlapping paired-end reads merged into single-molecule sequences using leeHom [8]. We used the Burrows-Wheeler Aligner [9] (BWA, https://github.com/mpieva/network-aware-bwa) to align merged sequences to a suitable reference genome (turTru1.75, bosTauUMD3.1, loxAfr4) using ancient parameters (“-n 0.01 –o 2 –l 16500”) allowing more mismatches and indels [8]. Further analyses were restricted to sequences longer than 35 bp. Sequences with the same start- and end-coordinates were merged into single consensus sequences using bam-rmdup (<https://github.com/mpieva/biohazard-tools>). Summary statistics were generated using samtools [10] (see Supplementary Tables 1 and 2).

**References**

1. Korlević P, Gerber T, Gansauge M-T *et al*. Reducing microbial and human contamination in DNA extractions from ancient bones and teeth*.* *Biotechniques* 59(2), 87-93 (2015).

2. Meyer M, Palkopoulou E, Baleka S *et al*. Palaeogenomes of Eurasian straight-tusked elephants challenge the current view of elephant evolution*.* *eLife* 6 e25413 (2017).

3. Damgaard PB, Margaryan A, Schroeder H, Orlando L, Willerslev E, Allentoft ME. Improving access to endogenous DNA in ancient bones and teeth*.* *Sci. Rep.* 5 11184-11184 (2015).

4. Glocke I, Meyer M. Extending the spectrum of DNA sequences retrieved from ancient bones and teeth*.* *Genome Res.* 27(7), 1230-1237 (2017).

5. Rohland N, Glocke I, Aximu-Petri A, Meyer M. Extraction of highly degraded DNA from ancient bones, teeth and sediments for high-throughput sequencing*.* *Nat. Protoc.* 13(11), 2447-2461 (2018).

6. Gansauge M-T, Aximu-Petri A, Nagel S, Meyer M. Manual and automated preparation of single-stranded DNA libraries for the sequencing of DNA from ancient biological remains and other sources of highly degraded DNA*.* *Nat. Protoc.* 15(8), 2279-2300 (2020).

7. Kircher M, Sawyer S, Meyer M. Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform*.* *Nucleic Acids Res.* 40(1), e3-e3 (2011).

8. Renaud G, Stenzel U, Kelso J. leeHom: adaptor trimming and merging for Illumina sequencing reads*.* *Nucleic Acids Res.* 42(18), e141-e141 (2014).

9. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform*.* *Bioinformatics* 25(14), 1754-1760 (2009).

10. Li H, Handsaker B, Wysoker A *et al*. The sequence alignment/map format and SAMtools*.* *Bioinformatics* 25(16), 2078-2079 (2009).