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PROTOCOL UPDATE

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# Extraction of highly degraded DNA from ancient bones, teeth and sediments for high-throughput sequencing

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**DNA preserved in ancient bones, teeth and sediments is typically highly fragmented and present only in minute amounts. Here, we provide a highly versatile silica-based DNA extraction protocol that enables the retrieval of short (≥35 bp) or even ultrashort (≥25 bp) DNA fragments from such material with minimal carryover of substances that inhibit library preparation for high-throughput sequencing. DNA extraction can be performed with either silica spin columns, which offer the most convenient choice for manual DNA extraction, or silica-coated magnetic particles. The latter allow a substantial cost reduction as well as automation on liquid-handling systems. This protocol update replaces a now-outdated version that was published 11 years ago, before high-throughput sequencing technologies became widely available. It has been thoroughly optimized to provide the highest DNA yields from highly degraded samples, as well as fast and easy handling, requiring not more than ~15 min of hands-on time per sample.**

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## Introduction

### Background and applications

The finding that DNA can survive for up to hundreds of thousands of years in skeletal remains[1,2] and even sediments[3,4] has greatly accelerated the use of ancient DNA analysis in evolutionary studies. However, isolation of small quantities of highly degraded DNA from such material is not trivial, as short DNA fragments are difficult to separate from other organic molecules, such as humic acids, which inhibit enzymatic DNA manipulations that must be performed prior to sequencing. The most widely used DNA purification and concentration technique for ancient remains is based on DNA adsorption to silicon dioxide (silica) particles[5]. This is achieved by supplementing a lysis buffer, which is used to release DNA from sample powder, with a high-salt binding buffer. DNA binding can be performed either by adding a silica suspension or by centrifugation through silica spin columns. Adsorbed DNA is then desalted with an ethanol-containing wash buffer and eluted into a low-salt buffer. Silica-based DNA extraction minimizes the co-extraction of inhibitory substances when used with chaotropic binding buffers[6,7], and offers ease of handling, especially with commercially available silica spin columns.

The protocol presented here is an update of a previous protocol[6] that was in wide use in ancient DNA research for DNA extraction prior to the genomic era. This era was marked by a revolution in sequencing technologies and associated sample preparation techniques. A key change was the replacement of earlier methods of amplification of short genomic targets by PCR followed by Sanger sequencing[8] with preparation of DNA libraries and high-throughput sequencing. This made it possible to access very short DNA fragments preserved in ancient samples that cannot be targeted directly with PCR[9]. Because there is an inverse, exponential correlation between fragment length and abundance[10–13], systematic efforts have been made to minimize losses of short molecules during DNA extraction and library preparation. For DNA extraction, an important step in this direction was made in 2013 by Dabney et al., who developed a silica-based DNA extraction technique that allows efficient recovery of DNA fragments as short as ~35 bp[9]. This was achieved mainly by modifying the composition of the binding buffer of a widely used silica-based DNA extraction method[6]. Combining this method with a single-stranded method for DNA library preparation[14,15] enabled the retrieval of DNA

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sequences from the 430,000-year-old bear and hominin remains from Sima de los Huesos[1,9,16], by far the oldest non-permafrost material that could be genetically characterized to date. More recently, the same method was the basis for the successful recovery of Neanderthal and Denisovan DNA from Pleistocene cave sediments[4], opening new possibilities for the study of human evolutionary history. The effectiveness of the Dabney method has also been demonstrated in combination with simpler double-stranded library preparation methods, for example, in studies reporting genome-wide sequence data from hundreds of human remains[17–20].

As attempts to push the temporal and geographical limits of ancient DNA research continue, further improvements to DNA extraction have been made. Glocke and Meyer have recently demonstrated that even shorter molecules (≥25 bp) can be isolated from ancient biological remains if the binding buffer recipe is further adjusted during silica-based DNA extraction[21]. Thus, the protocol provided here includes a choice of binding buffers that allows users to adjust the recovered fragment lengths to their needs. Moreover, we also adapt the technique to make possible the parallel processing of large numbers of samples. To this end, we provide a suspension-based protocol (Step 5B) that relies on silica-coated magnetic beads that can be separated from buffers without centrifugation. This protocol option is suited for automation on liquid-handling platforms. Silica-coated beads have been used before as a replacement for silica spin columns in ancient DNA extraction[22], but without thorough optimization and evaluation of their performance, as reported here.

The previous[6] and current versions of the protocol provided here use nearly identical lysis buffers, which are composed of ethylenediaminetetraacetate (EDTA) for decalcification of the bone and tooth matrix and release of DNA from the mineral fraction of sediments, as well as proteinase K, which is used for the digestion of bone and tooth collagen. The essential difference lies in the DNA-binding step, which was previously optimized for the recovery of fragments that are long enough to be targeted by PCR. The current protocol adopts the binding buffer recipes of the Dabney[9] and Glocke[21] methods, as well as changes in the ratios of volumes in which the lysis and binding buffers are combined prior to the binding step, thereby allowing recovery of shorter molecules. It also utilizes preassembled large-volume silica spin columns, which were introduced shortly after the first publication of the Dabney method[23], or silica-coated magnetic particles instead of silica suspension[6], and includes the addition of detergent to the lysis and elution buffers to prevent loss of DNA on tube walls[23].

## Comparison to other methods

Besides the Dabney and Glocke methods, other methods have been proposed for the extraction of short DNA fragments from ancient bones and teeth. Gamba et al. compared three published silica-based methods for ancient DNA extraction[24]; the Rohland method[6], which is updated here, the Dabney method[9] and a method developed by Yang et al., which uses Qiagen's PB buffer for binding[7]. The exact formulation of the PB buffer is proprietary, but its main components, guanidine hydrochloride and isopropanol, and their concentration appear to be the same as those used in the Dabney binding buffer. Although the performance of the Yang method was found to be very similar to that of the Dabney method[24], it is more complex in that it involves an additional DNA concentration step, which is performed with ultrafiltration spin columns. Another extraction method for short DNA fragments was proposed by Allentoft et al.[25]. The Allentoft method, which was not directly compared to the Dabney or Yang methods, recommends the use of a silica suspension instead of spin columns[25]. Although the Yang and the Allentoft methods may produce results that are similar to those obtained with the protocol presented here when the Dabney binding buffer is used, they both involve more handling steps than our spin-column-based protocol Step 5A, which makes these protocols less convenient and increases the risk of introducing contamination. In addition, none of these methods offer the possibility of automation.

## Limitations of the method

Similar to the preceding protocol[6], the current version of the protocol has been optimized for the most abundant sources of ancient DNA, namely bones and teeth, and now also sediments. Other types of material, such as hair, dried soft tissue, dental calculus, seeds or other ancient plant remains, may not yield optimal results with the EDTA/proteinase K lysis buffer described here. It is likely, however, that changes in the lysis buffer composition can make the protocol compatible with DNA extraction from these or even other sources of highly degraded DNA, such as formalin-fixed tissue. For instance, it has been recently demonstrated that highly degraded plant DNA can be efficiently

recovered from herbaria if lysis in *N*-phenacylthiazolium bromide buffer is combined with the purification steps of the Dabney method[26].

It should further be noted that no more than 50 mg of sample material can be used in 1 ml of lysis buffer for a single DNA extraction without risking suboptimal DNA recovery. However, it is generally not advisable to use much larger quantities of bone or tooth powder, even when faced with extremely poor DNA preservation. This is because endogenous ancient DNA content and contamination with microbial and human DNA can vary substantially within one sample, even on a microscale. Processing several subsamples and analyzing each individually therefore increases the chance of producing at least one DNA extract of sufficient quality for data generation. The situation is similar in sediments, in which the content of DNA from the species of interest can be highly variable, requiring screening of dozens of samples from different areas of a site to identify, for example, the presence of ancient hominin DNA[4].

## Experimental design

### Selection of binding buffer

The protocol options presented here (see Fig. 1 for an overview) provide a high degree of flexibility with regard to the fragment lengths retrieved. The most important decision to make is the choice of binding buffers. Binding buffer D (that of the Dabney method) yields fragment length distributions with a mode ~35 bp and a long tail of larger fragments ('Anticipated results', Fig. 2). The recovery of shorter DNA fragments is often not desirable if enough DNA fragments can be extracted with this buffer. This is because the vast excess of microbial DNA sequences present in most ancient samples can make it difficult to distinguish authentic sequences of the organism under study from spurious alignments if sequences are shorter than ~35 bp[16], although appropriate length cutoffs vary across samples[27]. Fragment length distributions obtained with binding buffer G (that of the Glocke method) exhibit a mode ~25 bp, with the majority of fragments (41–91%) being shorter than 35 bp ('Anticipated results', Figs. 2 and 3). Despite the dominance of short fragments, binding buffer G also doubles the number of DNA fragments >35 bp as compared with those obtained with binding buffer D[21]. However, this holds true only if DNA extracts are converted into libraries using a single-stranded method[21] and if library preparation is not impaired by inhibitory substances ('Anticipated results', Fig. 4). No substantial increase in informative sequence yield has been reported for double-stranded libraries prepared from such extracts[21].

The use of binding buffer G comes with further caveats: first, it can slightly increase the co-extraction of inhibitory substances[21] (Supplementary Table 1, conversion rate of oligonucleotide spike-in), which may require the conversion of smaller aliquots of DNA extract into libraries to mitigate the impact of inhibition. Second, binding buffer G must be used in larger excess than binding buffer D, which allows only half the amount of lysis buffer to be used for DNA extraction or requires two DNA extracts to be created from a single lysate. Binding buffer G is thus preferred only for samples from unfavorable climatic conditions, for which the DNA is expected to be particularly degraded, or if yields need to be maximized, for example, if only minuscule amounts of sample material are available. Molecules that are too short and non-informative can be depleted by hybridization capture of genomic targets[28] or gel excision of short library molecules[15] after library preparation. For the majority of ancient skeletal material or if double-stranded library preparation methods are used, we recommend using binding buffer D. Similarly, we advise using binding buffer D for sediment samples due to the more frequent presence of inhibitory substances in this sample type.

### Choice of purification matrix

Another choice to make is between the centrifugation-based protocol (Step 5A), which uses large-volume silica spin columns from a commercially available nucleic acid extraction kit (Roche)[23], and the suspension-based protocol (Step 5B), which uses silica-coated magnetic particles for DNA binding. Step 5B was developed with laboratory automation in mind. However, it can also be used in manual DNA extraction as a means of reducing costs (list price in Germany is €0.23 per sample for silica magnetic beads and €7.29 for a single large-volume spin column). Because bead-based DNA extraction cannot easily be performed in large volumes, especially on liquid-handling systems, this option allows only smaller volumes of lysate (150 µl when used with binding buffer D; 75 µl with binding buffer G, Fig. 1) to be purified per reaction. If sample powder is incubated in 1 ml of lysis buffer, as recommended, this corresponds to only 15% and 7.5% of the lysate, respectively. This contrasts with Step 5A, in which all (binding buffer D) or half of the lysate (binding buffer G) is

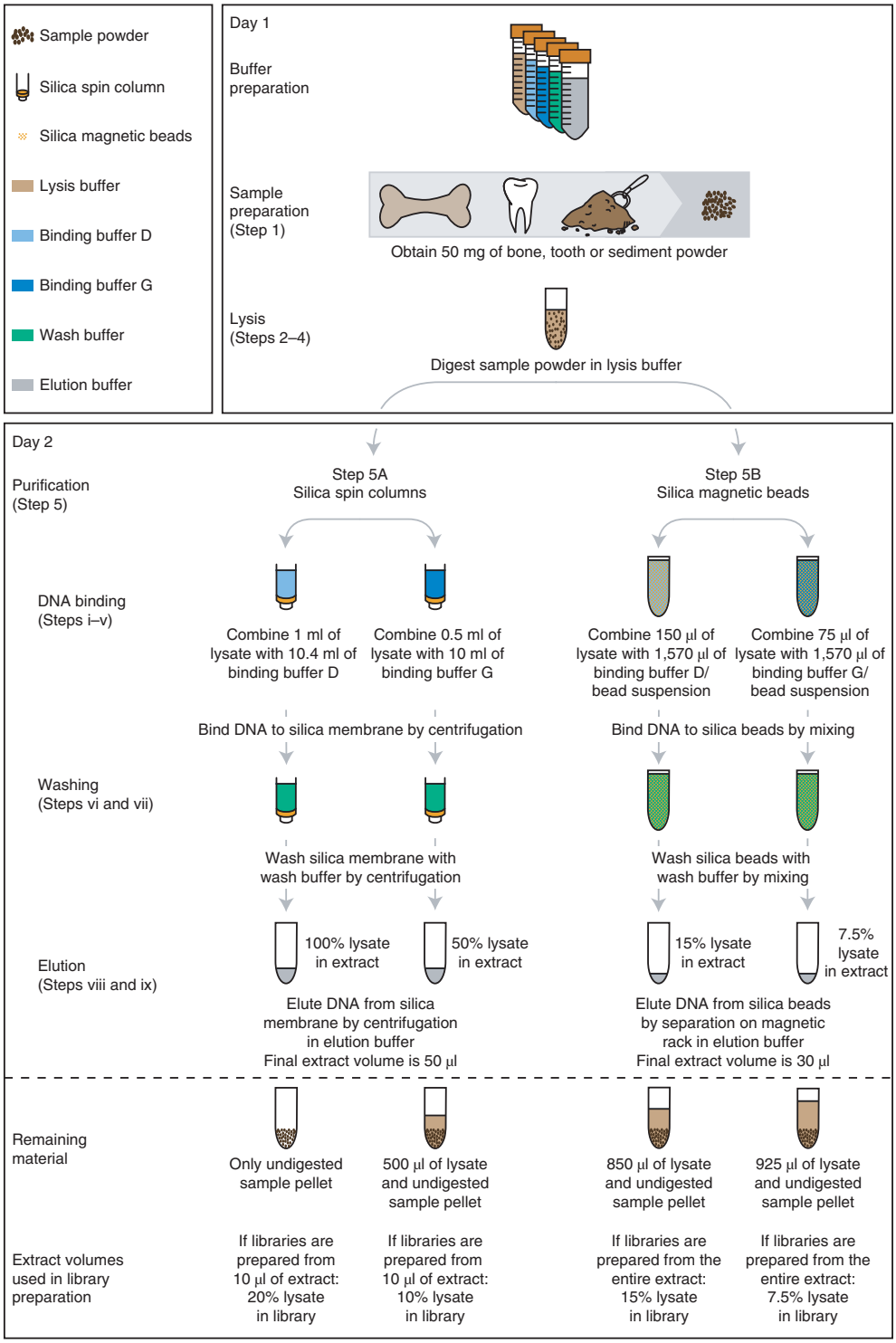


**Fig. 1 | DNA extraction workflow.**

subjected to purification. However, DNA libraries are typically not prepared from the entire extract volume (50 µl) obtained with Step 5A, but in multiple batches using 10-µl aliquots or smaller aliquots. Each 10-µl aliquot contains quantities of DNA that correspond to 20% (binding buffer D) or 10% (binding buffer G) of the lysate, depending on the binding buffer chosen. Thus, the reduction in DNA yield during DNA extraction with Step 5B can be compensated for during library preparation by using the entire DNA extract volume as input instead of an aliquot. With this approach for Step 5B,

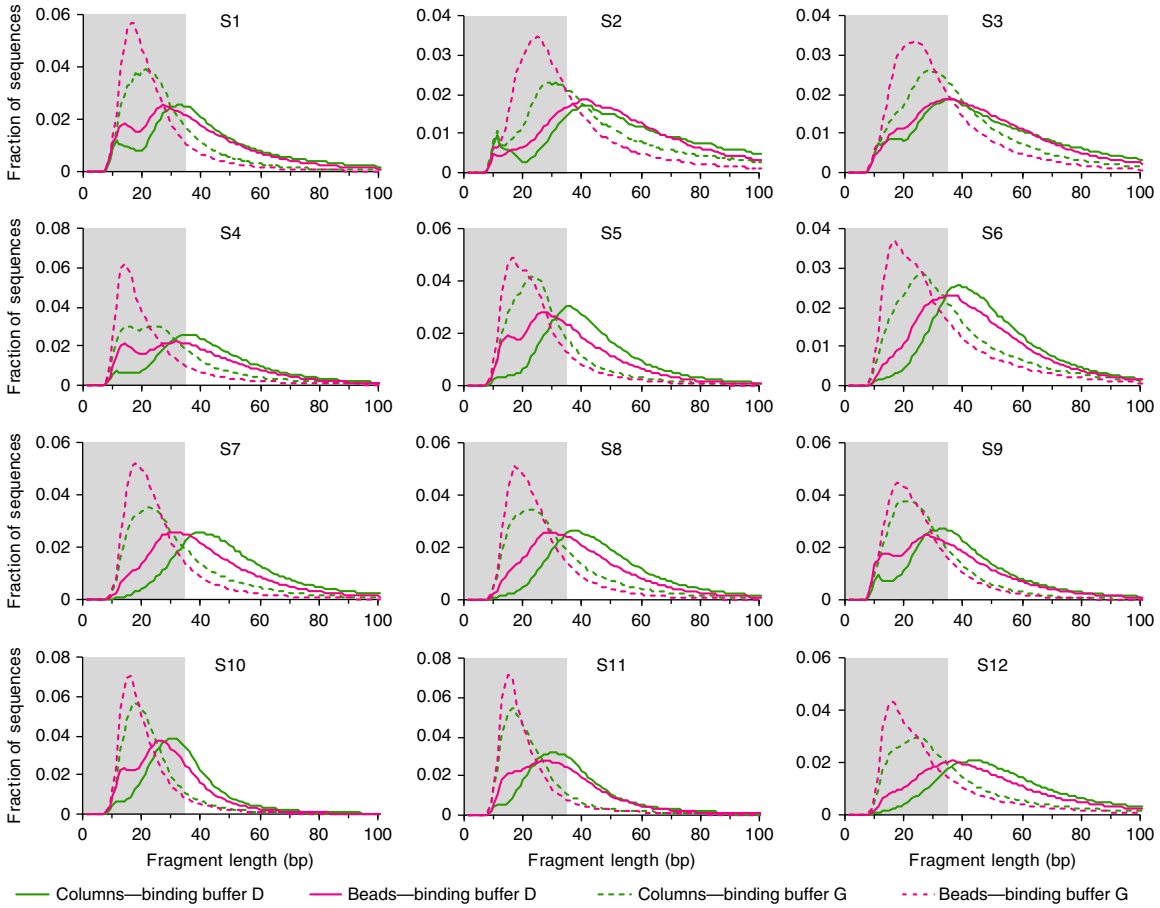


**Fig. 2 | DNA fragment length distribution in libraries prepared from extracts generated with the protocol options described here.** Binding buffer G recovers much shorter fragments than does buffer D. Magnetic silica beads also increase the recovery of short DNA fragments as compared to that for silica spin columns, but the difference is rather subtle. Peaks ~10 bp are artifacts of library preparation. Gray boxes highlight fragments <35 bp, which are often excluded from data analysis. Note that the distributions are plotted as fractions of sequences; they do not represent total yields. Columns = Step 5A; beads = Step 5B. S1–S6: bone and tooth samples; S7–S12: sediment samples.

residual DNA is stored at the lysis step and not in the form of a DNA extract, providing the option to generate additional DNA extracts later and possibly to reconsider the choice of the binding buffer, taking the outcome of the initial experiment into account. Note that the length cut-off of silica-based DNA extraction is not determined solely by the buffer composition used in the DNA-binding step but also by the type and geometry of the silica in the spin columns or silica beads used. As a result of this, the bead-based protocol (Step 5B) on average recovers slightly shorter DNA fragments than the column-based protocol (Step 5A) ('Anticipated results', Fig. 2, Supplementary Table 1).

### Controls

When designing an experiment, it is important to include appropriate controls. At least one extraction negative control, that is an aliquot of lysis buffer with no sample powder added, must be included in each experiment to assess the level of DNA contamination introduced during the extraction process. Adding more negative controls, especially with larger extraction sets (we recommend at least 1 control per 11 samples), increases the chance of detecting instances of sporadic contamination. DNA extracts generated from these controls must then be taken through all subsequent steps, that is, library preparation, hybridization capture, if applicable, and sequencing. We also recommend the addition of positive controls (see Box 1 for options), especially during the implementation phase of the protocol.

### Library preparation methods

Because only small amounts of DNA can be isolated from most ancient skeletal remains or sediment samples, DNA concentrations in the extracts are often too low to be determined

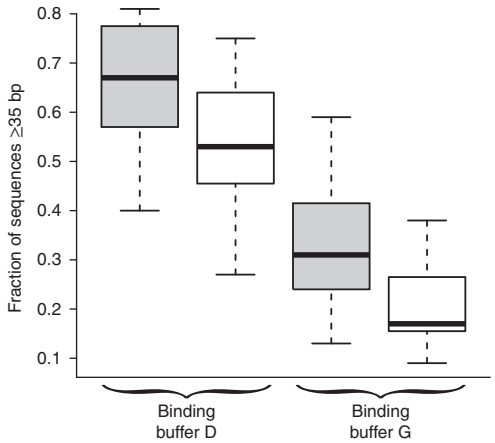


**Fig. 3 | Boxplots showing the fraction of fragments ≥35 bp in libraries prepared from extracts using the protocol options described here ($n = 12$).** Sequences <35 bp often cannot be reliably mapped to a reference genome and may have to be removed by gel excision after library preparation to make the best use of sequencing capacity. See Supplementary Table 1 for detailed results by sample. Gray boxplots = columns (Step 5A); white boxplots = beads (Step 5B). Thick black lines represent medians; boxes depict the lower and upper quartiles; and whiskers extend to values within 1.5 times the interquartile.

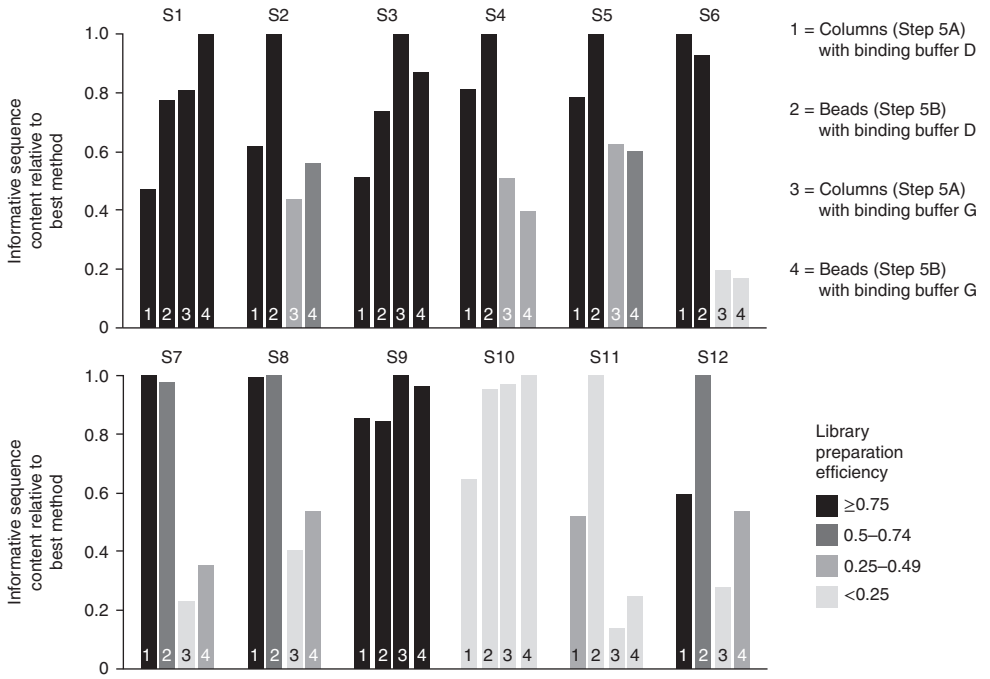


**Fig. 4 | Influence of the extraction method on the informative sequence content of the libraries.** The informative sequence content is shown, that is, the sum of nucleotides present in DNA fragments ≥35 bp whose sequences can be aligned to a reference genome, in each library relative to the library with the highest informative sequence content per sample (normalized to the same volume of lysate). The highest yields are obtained with binding buffer G if library preparation efficiency is ≥0.75. The informative sequence content obtained from sediment samples was approximated by mapping to a single mammalian genome (here, we mapped to the dolphin genome in order to target regions that are conserved across mammals). Library preparation efficiency was determined using qPCR measurements of a library control oligonucleotide that was spiked into each library preparation reaction. S1–S6: bone and tooth samples; S7–S12: sediment samples.

directly. DNA extracts are therefore best characterized by converting an aliquot into a DNA library and quantifying the number of library molecules by quantitative PCR (qPCR)[14] or digital PCR[23]. Numerous library preparation methods are available for this purpose. For the most highly degraded material, we recommend single-stranded library preparation as detailed in other protocols[14,29], as it

**Box 1 | DNA extraction positive controls ● Timing 10 min–25 h, depending on the approach**

As there is usually no a priori knowledge about the DNA content of an ancient sample, successful conversion of DNA extracts into libraries alone does not preclude the occurrence of inefficiencies during DNA extraction. Each of the following approaches can serve as an extraction positive control:

1 *DNA size marker*. When implementing the protocol for the first time, include one positive control reaction containing 2 µg of a DNA size marker (e.g., 4 µl of GeneRuler Ultra Low Range DNA Ladder, supplied at 500 ng/µl) at the lysis step (step 2 of the main Procedure) and follow the protocol as described. Visualize the recovery of the size marker by loading a volume of extract that corresponds to 500 ng of input DNA on an agarose gel (for the Ultra Low Range DNA Ladder, we recommend using a 4% (wt/vol) agarose gel). Load 500 ng of the stock solution of the DNA size marker on the same gel as reference. Band intensities give an estimate of the recovery of DNA of various lengths. In addition, the recovery of the size marker can be quantified by spectrophotometry, again using the stock solution or a dilution thereof as reference. Timing 10 min–2 h: 10 min if DNA size marker is quantified by spectrophotometry, 2 h if DNA size marker is visualized on a gel.

2 *Control DNA fragment*. Create a double-stranded control DNA fragment in a 50-µl hybridization reaction containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM NaCl and 20 µM each of oligonucleotides CL200 and CL204 (ref. [21]) (see the sequences below). Incubate the reaction in a thermal cycler at 95 °C for 10 s and decrease the temperature to 14 °C at a rate of 0.1 °C/s. Dilute the fragment 200-fold to 0.1 µM with elution buffer. Add 1 µl (0.1 pmol) to a separate tube containing lysis buffer (step 2) and carry it through the DNA extraction protocol. Quantify the recovery of the control fragment in the extract by qPCR using primers CL201 and CL202 (ref. [21]) (see the sequences below) and 1 µl of DNA extract as template. Include in the measurement a separate dilution of the control fragment that corresponds to the concentration expected in the extract, assuming full recovery. Use a ten-fold dilution series of the control fragment in elution buffer as qPCR standard (ranging from $10^9$ to $10^3$ molecules per microliter). Set the annealing temperature to 60 °C and the number of PCR cycles to 45. Use the program of your qPCR system to calculate the number of molecules from the standard curve and Ct values. To calculate the recovery rate, divide the number of output molecules by the number of molecules in the reference dilution. A recovery rate >75% indicates that DNA extraction was successful. Timing 3 h: 30 min for setting up the qPCR, 2.5 h run time.
Dissolve all oligonucleotides in water.

| Name | Description | Sequence (5′-3′) | Purification |
|---|---|---|---|
| CL200 | Extraction control, strand 1 | [Phosphate]TATCCGCTCACAATTCCACACAAC ATACGAGCCGGAAGCATAAAGTGTAAAGCCTGG GGTGCCTA[phosphate] | HPLC |
| CL204 | Extraction control, strand 2 | [Phosphate]TAGGCACCCCAGGCTTTACACTTTAT GCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGC GGATA[phosphate] | HPLC |
| CL201 | qPCR primer | TATCCGCTCACAATTCCACA | Desalted |
| CL202 | qPCR primer | TAGGCACCCCAGGCTTTAC | Desalted |

3 *Control lysate*. Generate a large volume of lysate from a sample that previously tested positive for DNA preservation (e.g., lyse 5 g of sample powder in 100 ml of lysis buffer). Separate the lysate from the residual sample powder, prepare 1-ml aliquots and store them at −20 °C. The lysate aliquots can be stored for at least 1 year. Include one aliquot as positive control in each extraction set, starting from Step 5 of the main Procedure. If DNA extraction was successful, library yields should be similar to those of previous experiments. Timing 16–25 h: 1 h for sample preparation, 15–24 h for sample lysis and aliquoting (30 min hands-on time); preparation only needs to be done once and lasts until all lysate aliquots are used up.

4 *Control powder*. Generate a large amount (e.g., 5 g) of very fine sample powder from an ancient bone. Store the sample powder at the temperature at which the sample is stored (e.g., room temperature). Include a 50-mg subsample in every extraction set, starting with Step 2 of the main Procedure. Once the protocol has been successfully implemented, this control is preferred over the options above, as it monitors the efficiency not only of DNA purification but also of the lysis step. Timing 1 h: preparation only needs to be done once and lasts until all sample powder is used up.

increases the yield of library molecules by approximately one order of magnitude as compared to double-stranded methods[29–31]. However, the latter methods are less expensive, easier to implement and often sufficient for the processing of samples with moderate or good DNA preservation. Among the double-stranded methods, we recommend blunt-end ligation-based approaches, for example, the ones described in references[32–34], as they are less prone to artifact formation and loss of short molecules[14]. Following library preparation, library quantification and sequencing allow extrapolations of the content of the DNA of interest in each sample extract[29] and, importantly, also in the negative controls.

## Materials

### Biological materials

- Samples (ancient bones, teeth or sediment)

### Reagents

- Water, HPLC grade (Sigma-Aldrich, cat. no. 270733)
- EDTA solution, pH 8.0 (0.5 M; AppliChem, cat. no. A4892)
- Tris-HCl solution, pH 8.0 (0.5 M; AppliChem, cat. no. A4577)
- Proteinase K (10 mg/ml; Sigma-Aldrich, cat. no. P6556, or from Roche High Pure Viral Nucleic Acid Large Volume Kit, cat. no. 5114403001)
- Guanidine hydrochloride (Sigma-Aldrich, cat. no. G3272) **! CAUTION** Guanidine hydrochloride is harmful; wear protective gloves. Do not mix with bleach (sodium hypochlorite) because buffers containing guanidine hydrochloride can liberate toxic compounds upon contact with bleach.
- 2-Propanol (Merck, cat. no. 109634)
- Sodium acetate buffer solution, pH 5.2 (3 M; Sigma-Aldrich, cat. no. S7899)
- Buffer PE (Qiagen, cat. no. 19065)
- Ethanol, absolute, for analysis (Merck, cat. no. 100983)
- Tween 20 (Sigma-Aldrich, cat. no. T2700)
- Sodium chloride solution (5 M; Thermo Fisher Scientific, cat. no. AM9759)
- (Optional) Silica magnetic beads (G-Biosciences; VWR International, cat. no. 786-915) **▲ CRITICAL** Use of other silica magnetic beads may substantially alter the length spectrum of recovered DNA fragments and may even impair yields. Note that either silica spin columns (Step 5A) or silica magnetic beads (Step 5B) are needed.
- (Optional, Box 1) Ultra Low Range DNA Ladder (Thermo Fisher Scientific, cat. no. 10597012)
- (Optional, Box 1) Agarose (UltraPure Agarose; Thermo Fisher Scientific, cat. no. 16500100)
- (Optional, Box 1) Gel stain (SYBR Safe DNA Gel Stain; Thermo Fisher Scientific, cat. no. S33102)
- (Optional, Box 1) Oligonucleotides for determining the success of DNA extraction (Box 1, section 2)
- (Optional) Agilent DNA 1000 Kit (Agilent, cat. no. 5067-1504) or Agilent High Sensitivity DNA Kit (Agilent, cat. no. 5067-4626)

### Equipment

- LoBind tubes, 2.0 ml (Eppendorf, cat. no. 0030108078)
- LoBind tubes, 1.5 ml (Eppendorf, cat. no. 0030108051)
- Conical tubes, 50 ml, with screw cap (Greiner Bio-One, cat. no. 210261)
- (Optional) Drill (Komplett-Set EV410-230 Emax EVOlution; Mafra, cat. no. 8225) with exchangeable bits (New Technology Instruments, cat. no. H1S-010-RA, or other sizes) or cutting disks (Superflex; New Technology Instruments, cat. no. 806.104.355.524.190, or other sizes)
- (Optional) Homogenizer (Minilys; Peqlab, cat. no. 432-0274) with grinding tubes (Peqlab, cat. no. 432-3752, or other)
- (Optional) Mortar (VWR, cat. no. 410-0113 or other) and pestle (VWR, cat. no. 410-0121 or other)
- Balances to weigh reagents and sample powder (Adventurer AX124; Ohaus, cat. no. 30122610)
- Parafilm M, 10 cm wide (neoLab, cat. no. 3-1012)
- Incubator (Memmert, model no. Incubator IN55)
- Tube rotator for 2.0-ml tubes (VWR, cat. no. 444-0500)
- UV cross-linker (Vilber, model no. Bio-Link BLX 254)
- Vortex mixer (Vortex Genie 2; Scientific Industries, cat. no. SI-0236)
- Microcentrifuge (MiniStar silverline; VWR, cat. no. 521-2844)
- Microwave (Samsung, model no. GE89MST-1/XEG)
- Coverall (BFL Handelsgesellschaft, cat. no. 200-5905160-B)
- Gloves (textured single-use nitrile gloves; Ansell Health Care, cat. no. 588783; and Rotiprotect-latex gloves, type 2, powder-free; Roth, cat. no. L950.1)
- Face mask (activated carbon mask; Roth, cat. no. 8401.2)
- Hair net (High Five Spunbond Bouffant Caps; Thermo Fisher Scientific, cat. no. 19-156-211)
- Face shield (F800 face shield; VWR, cat. no. SCTS2014617)
- (Optional) Preassembled silica spin columns and collection tubes (High Pure Viral Nucleic Acid Large Volume Kit; Roche, cat. no. 5114403001; reagents from the kit are not used, except for proteinase K)

▲ CRITICAL Use of other silica spin columns may substantially alter the length spectrum of recovered DNA fragments and may even impair yields. Note that either silica spin columns (Step 5A) or silica magnetic beads (Step 5B) are needed.

- (Optional) Centrifuge for 50-ml conical tubes (Heraeus Megafuge 40 R; Thermo Fisher Scientific, cat. no. 75004518)
- (Optional) Table-top centrifuge (Centrifuge 5424; Eppendorf, cat. no. 5424000010)
- (Optional) Magnet for 2.0-ml tubes (DynaMag-2 Magnet; Thermo Fisher Scientific, cat. no. 12321D)
- (Optional) Centrifuge/Vortex Multispin, model no. MSC-6000 (Biosan, cat. no. BS-010211-AAL); this facilitates and speeds up handling during purification with silica magnetic beads because it combines mixing and centrifugation.
- (Optional) 2100 Bioanalyzer Instrument (Agilent, cat. no. G2939BA)
- (Optional) Qubit 4 Quantitation Starter Kit (Thermo Fisher Scientific, cat. no. Q33227)
- (Optional, Box 1) Electrophoresis system (Sub-Cell GT Cell system; Bio-Rad, cat. no. 1704401; PowerPac Basic Power Supply; Bio-Rad, cat. no. 1645050)
- (Optional, Box 1) Gel imaging system (Typhoon FLA 7000; GE Healthcare Life Sciences, cat. no. 28955209)
- (Optional, Box 1) Spectrophotometry device (NanoDrop 3300; Thermo Fisher Scientific, cat. no. ND-3300)
- (Optional, Box 1) Thermal cycler (Veriti Thermal Cycler; Thermo Fisher Scientific, cat. no. 4375786)
- (Optional, Box 1) qPCR system (CFX96 Touch Real-Time PCR Detection System with Starter Package; Bio-Rad, cat. no. 1855196)

## Reagent setup

▲ CRITICAL Decontaminate LoBind tubes (with closed lids) and collection tubes before use by UV-C irradiation (254-nm wavelength) with an energy density of 7 kJ/cm$^2$ in a UV cross-linker. Unless otherwise indicated, all buffers are irradiated the same way. Silica spin columns and beads are not irradiated. ▲ CRITICAL Prepare excess buffer for extraction negative controls. We recommend including at least one extraction negative control per 11 samples. ▲ CRITICAL When adding Tween 20 to buffers, pipette slowly to ensure the correct volume is taken up, as Tween 20 is highly viscous.

### Lysis buffer

To prepare 25 ml for 24 reactions, combine 22.5 ml of 0.5 M EDTA (pH 8.0), 1.863 ml of water, 12.5 µl of Tween 20 and 625 µl of 10 mg/ml proteinase K. ▲ CRITICAL UV- irradiate the solution before adding proteinase K. This buffer can be stored at room temperature (20–25 °C) for at least 1 year before adding proteinase K; this buffer cannot be stored after the addition of proteinase K.

### Binding buffer D

Binding buffer D is 5 M guanidine hydrochloride, 40% (vol/vol) 2-propanol, 0.12 M sodium acetate and 0.05% (vol/vol) Tween 20 (260 ml for 24 reactions (Step 5A) or for ~160 reactions (Step 5B)). In a glass or plastic bottle, weigh 124.2 g of guanidine hydrochloride and fill up with water to 150 ml. Heat briefly in a microwave until the buffer is warm to the touch and shake until the salt is fully dissolved. Add 104 ml of 2-propanol, 10.4 ml of 3 M sodium acetate buffer solution (pH 5.2) and 130 µl of Tween 20. This buffer can be stored at room temperature for up to 4 weeks. Seal the bottle with Parafilm to avoid evaporation.

### Binding buffer G

Binding buffer G is 2 M guanidine hydrochloride, 70% (vol/vol) 2-propanol and 0.05% (vol/vol) Tween 20 (250 ml for 24 reactions (Step 5A) or for ~150 reactions (Step 5B)). In a glass or plastic bottle, weigh 47.75 g of guanidine hydrochloride and fill up with water to 75 ml. Mix by shaking until the salt is dissolved; heating is not necessary. Add 175 ml of 2-propanol and 125 µl of Tween 20. This buffer can be stored at room temperature for up to 4 weeks. Seal the bottle with Parafilm to avoid evaporation. ▲ CRITICAL Only one binding buffer is needed: choose between binding buffer D and binding buffer G. Binding buffer D is optimized to recover DNA fragments ≥35 bp. Binding buffer G recovers fragments ≥25 bp.

### Wash buffer

Make 500 ml of wash buffer for >300 reactions (Step 5A) or for >600 reactions (Step 5B). To 100 ml of buffer PE concentrate, add 400 ml of ethanol. This buffer can be stored at room temperature for at least 1 year.

**Elution buffer**

Make 50 ml of elution buffer for 1,000 reactions. Combine 49.4 ml of water, 500 µl of 1 M Tris-HCl (pH 8.0), 100 µl of 0.5 M EDTA (pH 8.0) and 25 µl of Tween 20. This buffer can be stored at room temperature for at least 1 year.

## Procedure

### Sample preparation ● Timing 10–30 min per bone or tooth sample; 5–10 min per sediment sample

▲ CRITICAL Carry out all steps of the experiment in a dedicated ancient DNA clean room[35] spatially separated from post-PCR areas. Wear disposable protective gear (gloves, face mask, hair net, coveralls and eye protection such as a face shield) to prevent contamination of samples, reagents and equipment.

▲ CRITICAL Carry out all centrifugation steps at room temperature.

1 Remove the surface of the bone or tooth sample with a disposable drill bit or another abrasive disposable device or tool. Drill into the cleaned area using a fresh disposable drill bit and collect up to 50 mg of sample powder into a 2.0-ml LoBind tube. If using a homogenizer or mortar and pestle, grind the cleaned sample to fine powder and collect up to 50 mg into a 2.0-ml LoBind tube. When extracting DNA from a sediment sample, weigh out up to 50 mg of material into a 2.0-ml LoBind tube.

▲ CRITICAL STEP The surfaces of ancient skeletal remains are often heavily contaminated with human DNA, consolidants and dirt. Exchange the drill bit after removal of the surface to avoid contaminating the sample with surface material.

■ PAUSE POINT The protocol can be interrupted here, and the sample powder can be stored infinitely at room temperature, 4 °C or −20 °C, depending on how the specimen itself is stored.

### Sample lysis ● Timing handling time is 30 min for 24 samples; overnight incubation takes 15–24 h

2 Add 1 ml of lysis buffer to each 50-mg sample. Include extraction negative controls by adding 1 ml of lysis buffer to empty tubes; include at least one negative control per 11 samples. Optionally, replace one sample with a positive control (Box 1).

3 Suspend the sample powder by vortexing for 10 s. Seal the tube with Parafilm and incubate overnight (15–24 h) at 37 °C in an incubator under constant rotation on a tube rotator at 18 r.p.m.

4 Centrifuge the tubes for 2 min at 16,400 *g* in a table-top centrifuge to separate the lysate from undigested sample material, and transfer the lysate to a fresh tube or directly to the binding buffer (Step 5).

▲ CRITICAL STEP The undigested sample pellet can be stored at −20 °C for later experiments for at least 1 year.

■ PAUSE POINT The lysate (or excess of lysate when using <1 ml) can be stored at −20 °C for at least 1 year. Thaw the tube at room temperature, mix by vortexing and repeat the centrifugation step before proceeding with the protocol.

### DNA purification

5 DNA can be isolated from the lysates using silica spin columns (option A) or silica magnetic beads (option B).

(A) **Silica spin column purification ● Timing 2 h for 24 samples**

(i) When using binding buffer D: for each sample and control, transfer 10.4 ml of binding buffer D to a 50-ml tube. When using binding buffer G: for each sample and control, transfer 10 ml of binding buffer G to a 50-ml tube.

(ii) When using binding buffer D: transfer the entire lysate (~1 ml) to the binding buffer. Mix by inverting. When using binding buffer G, transfer 500 µl of lysate to the binding buffer. Mix by inverting.

▲ CRITICAL STEP To avoid carryover of undigested sample material, transfer <1 ml of lysate if the pellet is unstable.

(iii) Pour the entire lysate/binding buffer mixture into the extender of the silica spin column assembly and close the tube with a screw cap. Spin for 4 min at 500*g* in a centrifuge with a swing bucket rotor.

? TROUBLESHOOTING

(iv) Remove the screw cap and place the silica spin column assembly into a collection tube. Carefully remove the extension reservoir and close the cap of the silica spin column. Label the spin column. Close the 50-ml tube containing the flow-through with a screw cap and store it at −20 °C until the experiment is successfully finished.

(v) Dry-spin the silica column for 1 min at 3,400*g* in a table-top centrifuge and place the column into a fresh collection tube.

(vi) Add 750 µl of wash buffer to the spin column, spin at 3,400*g* for 30 s, and place the spin column into a fresh collection tube. Repeat this step for a total of two washes.

(vii) Dry-spin the column for 1 min at 16,400*g*. Transfer the spin column to a fresh collection tube.

▲ **CRITICAL STEP** Handle the spin column carefully when placing it into a fresh collection tube, in order to avoid carryover of wash buffer.

(viii) Add 50 µl of elution buffer on top of the silica membrane and incubate it for 5 min. Spin for 1 min at 16,400*g* in a table-top centrifuge. Repeat this step by transferring the eluate back onto the silica membrane. The final elution volume remains 50 µl.

▲ **CRITICAL STEP** The elution buffer should be pipetted to the center of the silica membrane without touching the membrane to maximize elution efficiency.

(ix) Transfer the eluate (the DNA extract) to a fresh 1.5-ml LoBind tube and store it at −20 °C until used for library preparation. DNA extracts can be stored for at least 1 year.

(B) **Silica magnetic bead purification** ● **Timing 2 h for 24 samples**

(i) Fully resuspend the stock suspension of silica beads by vortexing. For each reaction, transfer 10 µl of silica bead suspension to a 2.0-ml LoBind tube. Include an excess of 5% (e.g., use 252 µl of silica bead suspension for 24 reactions). Place the tube on a magnet to collect the beads, then pipette off and discard the supernatant. Remove the tube from the magnet, add 500 µl of elution buffer and resuspend the beads by vortexing for 8 s. Spin the tubes briefly (2,000*g*, 2 s) in a microcentrifuge to collect the suspension at the bottom, and place the tubes on the magnet. Pipette off and discard the supernatant. Repeat this step for a total of two washes. Resuspend the beads in a volume of elution buffer equivalent to the initial volume (e.g., 252 µl of elution buffer for 24 reactions).

(ii) For each reaction, transfer 1.56 ml of the respective binding buffer to a 2.0-ml LoBind tube and add 10 µl of bead suspension.

(iii) When using binding buffer D, pipette 150 µl of lysate into the binding buffer D/bead mixture. When using binding buffer G, pipette 75 µl of lysate into the binding buffer G/bead mixture.

(iv) Vortex the tubes for 5 s. Rotate the tubes for 15 min at room temperature to bind the DNA to the silica beads.

(v) Spin the tubes briefly (2,000*g*, 2 s) in a microcentrifuge to collect the suspension at the bottom and place them on the magnet. Pipette off the supernatant and store it at −20 °C until the experiment has been successfully finished.

(vi) Remove the tubes from the magnet, add 250 µl of wash buffer and vortex for 8 s. Spin the tubes briefly (2,000*g*, 2 s) in a microcentrifuge to collect the suspension at the bottom and place the tubes back on the magnet. Pipette off and discard the supernatant. Repeat this step twice for a total of three wash steps. Vortexing and centrifugation in the wash step can be carried out using a centrifuge/vortex multispin device to speed up the process.

(vii) To ensure that all wash buffer is removed, aspirate any remaining drops of liquid using a small-volume pipette, for example, a 20- or 100-µl pipette. Dry the beads for 20 min at room temperature by leaving them on the magnet with open lids.

(viii) Remove the tubes from the magnet, add 15 µl of elution buffer, vortex the tubes until all beads have been resuspended and spin briefly to collect the beads on the bottom. Incubate for 5 min at room temperature. Place the tubes back on the magnet and transfer the supernatant (the first eluate) to a fresh 1.5-ml LoBind tube.

▲ **CRITICAL STEP** If the beads cannot be fully resuspended by vortexing, spin the tube briefly (2,000*g*, 10 s) in a microcentrifuge and resuspend the beads manually by pipetting.

(ix) Repeat the previous step, using an additional 15 µl of elution buffer, and add the second eluate to the first eluate (from Step 5B(viii)) to obtain 30 µl of extract.

■ **PAUSE POINT** The DNA extract can be stored at −20 °C for at least 1 year until it is used for library preparation.

**Assessment of DNA extraction success ● Timing single-stranded library preparation: 9 h; double-stranded library preparation: 5 h**

6 DNA isolated from ancient material is often too low in quantity to allow direct concentration measurements. Such measurements may be attempted using systems based on intercalating dyes (e.g., the Qubit 4 Fluorometer) or capillary gel electrophoresis (e.g., the 2100 Bioanalyzer) but are likely to fail even for well-preserved samples. We therefore recommend proceeding directly to library preparation and sequencing to determine the quantity and composition of the DNA that was isolated from each sample and the negative controls ('Experimental design'). We recommend using the single-stranded library preparation[14,29] for binding buffer G. DNA purified with binding buffer D (Step 5A or B) can be converted into libraries using either single- or double-stranded[32–34] methods. In addition, positive controls provide information about the efficiency of the DNA extraction procedure (Box 1).

**? TROUBLESHOOTING**

## Troubleshooting

Troubleshooting advice can be found in Table 1.

**Table 1 | Troubleshooting table**

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 5A(iii) | Lysate/binding buffer mixture does not flow through | Silica membrane is clogged with undigested sample powder | Centrifuge at 500*g* until all liquid has passed the membrane |
| | | | In subsequent extractions, transfer a smaller volume of lysate to the binding buffer to prevent carryover of undigested sample powder |
| 6 | Library preparation fails or has low yields | Inhibitors in the extract | Decrease the volume of extract used in library preparation |
| | | | Use binding buffer D |
| | | No or little DNA in the extract | Repeat extraction with fresh reagents and include a positive control (Box 1) |
| | Extraction negative control is contaminated | Contaminated reagents or cross-contamination from other samples | Repeat extraction with freshly prepared reagents |

## Timing

Step 1, sample preparation: 4–12 h (for 24 bone or tooth samples); 2–4 h (for 24 sediment samples)
Steps 2–4, sample lysis: 15–24 h (30 min hands-on time for 24 samples plus overnight incubation)
Step 5A, lysate purification using silica spin columns: 2 h
Step 5B, lysate purification using silica magnetic beads: 2 h
Step 6, single-stranded library preparation: 9 h; or double-stranded library preparation: 5 h
Box 1, DNA extraction positive controls: approach 1: 10 min–2 h (10 min if DNA is quantified by spectrophotometry, 2 h if DNA size marker is visualized on a gel); approach 2: 3 h (30 min for setting up qPCR and 2.5 h run time); approach 3: 16–25 h (1 h for sample preparation and 15–24 h for sample lysis and aliquoting with 30 min hands-on time; the preparation for approach 3 needs to be done only once and lasts as a positive control until all aliquots of lysate are used up); approach 4: 1 h (the preparation for approach 4 needs to be done only once and lasts as a positive control until all sample powder is used up)

## Anticipated results

To illustrate how the choice of the binding buffer and the silica type influences the yield of library molecules and their length distribution, we extracted DNA from five bone, one tooth and six sediment samples (Supplementary Table 1), using both protocol options and both binding buffers described here, as well as manual processing. Aliquots of these extracts (Supplementary Table 1) were converted into single-stranded libraries[29] using automated liquid handling[4], and

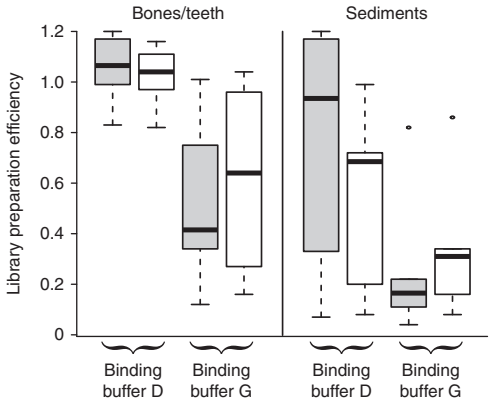


**Fig. 5 | Boxplots showing the influence of the extraction method on library preparation efficiency, as inferred from the conversion rate of the control oligonucleotide that was spiked into each library preparation reaction ($n = 6$).** Library preparation efficiency is often reduced with binding buffer G, especially when applied to sediments. Gray boxplots = columns (Step 5A), white boxplots = beads (Step 5B). Thick black lines represent medians; boxes depict the lower and upper quartiles; whiskers extend to values within 1.5 times the interquartile range; and circles represent outliers.

the yield of library molecules was determined by qPCR[14]. We also estimated library preparation efficiency by including small amounts of a control oligonucleotide in each library preparation reaction (spike-in)[21]. Libraries were indexed with two sample-specific barcodes each[36], then pooled and sequenced using paired-end sequencing (2 × 76 cycles) on two lanes of a HiSeq 2500 Rapid flow cell (Illumina).

Figure 2 shows that the fragment length distribution in the libraries (reconstructed from full-length molecule sequences obtained by overlap-merging of paired-end reads) depends primarily on the binding buffer chosen during DNA extraction. If binding buffer G is used, a substantial fraction of sequences are <35 bp (Figs. 2 and 3, Supplementary Table 1). The choice of the silica type also influences the fragment length distribution, with silica-coated magnetic beads retaining more short fragments than silica spin columns (Figs. 2 and 3).

Based on the number of unique molecules in each library and the fraction and length of sequences that could be mapped to an appropriate reference genome, we estimated the informative sequence content of each library, that is, the total number of nucleotides present in inserts ≥35 bp that produce alignments to the reference genome[29]. This analysis reveals that DNA extracted using binding buffer G contains more DNA fragments from the respective source organism than that extracted with binding buffer D, but only if no inhibitory substances are co-extracted (library preparation efficiency >0.75, Fig. 4). In addition, yields are slightly higher when using beads instead of silica spin columns with binding buffer D, presumably due to the increased recovery of shorter molecules. Inhibition occurs more frequently with binding buffer G, especially if DNA is extracted from sediments (Fig. 5), making this buffer a nonideal choice for this sample type.

## Reporting Summary

Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

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## Author contributions

N.R., I.G., A.A.-P. and M.M. designed experiments. N.R., I.G. and A.A.-P. performed experiments. N.R., I.G. and M.M. analyzed the data and wrote the paper.

## Competing interests

The authors declare no competing interests.

## Additional information

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**Key references using this protocol**

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