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Chapter 17

Analysis of Ancient DNA in Microbial Ecology

Olivier Gorgé, E. Andrew Bennett, Diyendo Massilani, Julien Daligault, Melanie Pruvost, Eva-Maria Geigl, and Thierry Grange

Abstract

The development of next-generation sequencing has led to a breakthrough in the analysis of ancient genomes, and the subsequent genomic analyses of the skeletal remains of ancient humans have revolutionized the knowledge of the evolution of our species, including the discovery of a new hominin, and demonstrated admixtures with more distantly related archaic populations such as Neandertals and Denisovans. Moreover, it has also yielded novel insights into the evolution of ancient pathogens. The analysis of ancient microbial genomes allows the study of their recent evolution, presently over the last several millennia. These spectacular results have been attained despite the degradation of DNA after the death of the host, which results in very short DNA molecules that become increasingly damaged, only low quantities of which remain. The low quantity of ancient DNA molecules renders their analysis difficult and prone to contamination with modern DNA molecules, in particular via contamination from the reagents used in DNA purification and downstream analysis steps. Finally, the rare ancient molecules are diluted in environmental DNA originating from the soil microorganisms that colonize bones and teeth. Thus, ancient skeletal remains can share DNA profiles with environmental samples and identifying ancient microbial genomes among the more recent, presently poorly characterized, environmental microbiome is particularly challenging. Here, we describe the methods developed and/or in use in our laboratory to produce reliable and reproducible paleogenomic results from ancient skeletal remains that can be used to identify the presence of ancient microbiota.

Key words Ancient DNA, NGS, Double-stranded library, Single-stranded library, IonTorrent, Illumina, Contamination

1 Introduction

Ancient DNA (aDNA) preserved in skeletal remains from past organisms can be a rich source of information on the evolution of species, of both the organism itself and its pathogens (for a typical ancient skeleton *see* Fig. 1). aDNA, however, is often highly degraded and the techniques for its analysis need to be optimized in order to ensure the production of authentic results. Indeed,

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Fig. 1 Skeletal remains from a 7000-year-old Neolithic burial from Berry-au-Bac "le Vieux Tordoir" (Aisne, France). Excavation and photograph: CNRS, UMR 8215 "Trajectoires"

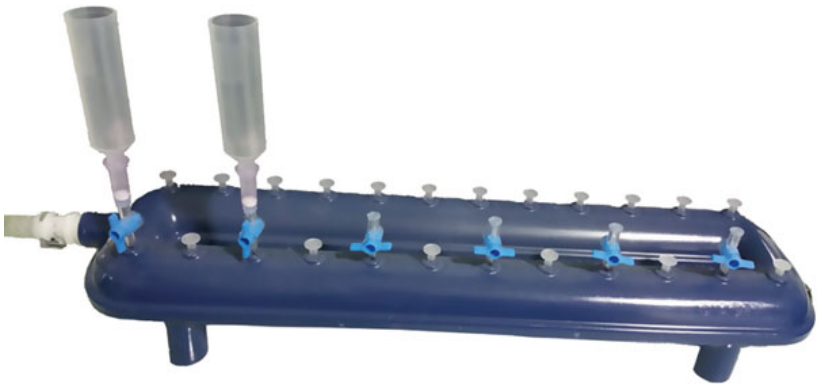

Fig. 2 Qiavac Manifold equipped with Qiaquick Spin columns and extenders to purify DNA from large-volume extracts

working with aDNA requires precautions that need to be applied even before samples enter the paleogenetic laboratory. Archaeologists should be taught the constraints of aDNA research so that they can adapt their working procedures to increase the likelihood of obtaining reliable aDNA results. For example, a common practice in archaeology is to wash bones after excavation. This washing leads to the dilution and degradation of the ancient DNA molecules as well as to contamination with environmental DNA [1, 2]. This is particularly problematic if only very low amounts of DNA are preserved in the skeletal remains. To increase the likelihood of DNA preservation in the sample, it is preferable to use freshly excavated remains for paleogenetic analyses [1]. This will be possible, however, only in a limited number of cases and does not concern previously excavated and curated remains. Moreover, DNA is heavily transformed after the death of an organism. Between several hours,

and possibly up to the first several years, after death, DNA is hydrolyzed enzymatically into small fragments leading to a median size of 50–70 bp (Guimaraes et al., unpublished) [3]. Over time, DNA bases become modified; in particular the cytosines become deaminated, which occurs preferentially close to the molecule ends [4]. The quantity of endogenous aDNA varies among samples, and even in different locations in the same skeletal remains. Although there may be other factors, temperature in particular has been characterized as playing a major role in aDNA preservation [5].

In order to study the genomes of pathogens that are associated with an animal (vertebrate) at the time of its death, one must consider all the events taking place during the diagenetic transformation of biomolecules, including DNA, following death. The body and its constituents will begin to decompose, mostly due to the action of microorganisms and insects. They not only metabolize biomolecules, but also deposit their own DNA, becoming the first contaminants of skeletal remains. Once the soft tissues and accessible organic parts of the bones have been consumed, the skeletal parts will enter a slow decay phase involving mostly chemical processes. When bones are buried, either intentionally at the time of death or simply due to natural burial of the skeleton over time, there will be a slow but regular exchange of biomolecules between bone and soil. Thus, at the time of excavation, the DNA that can be recovered may contain (1) DNA from the initial organism; (2) the DNA of the microbes, as well as parasites, that were associated with the organism during its lifetime (some of which may possibly have been the cause of its death); (3) the DNA of the organisms that have contributed to the decomposition of the body following its death; (4) and the DNA of the soil organisms that have penetrated into the bone. If at the time of excavation no special precautions are taken, and the bones are handled and washed as is routinely done, the bone can be further contaminated with fresh modern DNA, mostly of human and microbial origin, as well as from various other sources. The microbial composition of skeletal remains therefore reflects the microbial composition of the burial environment, showing that fossilizing skeletal remains resemble environmental samples. Indeed, when DNA retrieved from ancient bones is sequenced with a shotgun approach, typically only a few percent, or tenths of percent, of the sequenced DNA correspond to the initial organism, the rest being identified as “environmental DNA.” Most of this “environmental DNA” cannot be mapped to sequenced genomes, and remains as “unknown” [6]. Since older DNA is increasingly degraded until complete disappearance, one could expect that most of the environmental DNA recovered from the skeletal remains is of recent origin. The DNA decay rate depends on the environment; however, special “molecular niches” within the bone may offer more protected DNA-stabilizing microenvironments [7–9]. This can explain the exceptional preservation found in a limited number of

bones [10–13]. The more the microbiome is intimately associated with the bone and teeth matrix, the better the likelihood that it can also benefit from such "preserving molecular niches". Thus, the DNA of pathogens that can be spread to bones and teeth with the bloodstream, like *Yersinia pestis*, *Mycobacterium leprae*, and *Mycobacterium tuberculosis*, can be retrieved from well-preserved ancient skeletal remains (e.g., [14–16]). Similarly, the DNA of ancient buccal microbiomes can be retrieved from dental plaque, which appears to offer a suitable mineralized environment for long-term DNA preservation [17]. It remains to be determined if other organisms of the microbiome can also deposit their DNA into favorable "preserving molecular niches" allowing long-term DNA preservation. Such microorganisms are the first players to colonize the body through the blood vessels and it is yet unknown whether most of the "environmental DNA" that can be retrieved from ancient bones is of recent or ancient origin. In fact, it is likely that the unique taphonomic history (the history of *postmortem* decay) of each bone, in both macro- and microniches, shows sufficient bone-to-bone diversity to allow very different outcomes in terms of DNA preservation, and of the age of the DNA that is recovered.

In order to optimize the recovery of DNA from the ancient microbiome, improved methods adapted to the preferential recovery of the most damaged molecules must be developed. Their use should prevent, as much as possible, the introduction and incorporation of modern DNA molecules into the sequencing libraries. After sequence production, bioinformatics methods adapted to analyze ancient molecules should be used. Since the analysis of ancient microbiomes is presently in its infancy, there are not yet reliable, established procedures available to ensure the recovery of authentic data. For the moment, one has to rely on the more established procedures developed to analyze ancient host DNA.

Here, we provide some guidelines designed for the analysis of ancient microbiomes. First, it is essential to use all possible means to minimize contamination with modern DNA because the minute quantities of DNA in the ancient bone and tooth extracts can readily be contaminated with traces of modern DNA from the same or other species. Indeed, the scarcer the endogenous DNA, the higher the ratio of contaminating DNA likely to be found in the extract. This requires a high-containment laboratory for extraction, purification, and library construction of aDNA. In addition, very strict protocols to avoid carryover contamination and to decontaminate reagents must be applied. Carryover contamination results from molecules produced during previous amplification or library construction steps being reintroduced into another sample. Another source of contamination is trace DNA molecules present in reagents such as DNA from domestic animals, the proteins of which are often used to stabilize enzymes,

human DNA from employees at biotech companies, or bacterial DNA from either the bacteria used for enzyme production or the bacteria introduced from the environment during the production process. In order to ensure the authenticity of the results, reagents must first be decontaminated to eliminate as much reagent-borne DNA as possible before use [18]. Here we describe the different protocols that we have developed to reduce the level of contaminating DNA found in reagents.

Second, because DNA is damaged and degraded, one. must use library construction methods that allow the best possible recovery of the most damaged molecules, and, if possible, discriminate against the recovery of modern ones. We present herein experimental procedures allowing optimal recovery of short double-stranded and of highly damaged molecules, which are best recovered as single-stranded DNA [19]. In the case of amplicon sequencing (i.e., 16S rRNA gene), short regions must be targeted because of the reduced length of ancient DNA. When analyzing soils or sediments, the vast majority of DNA, and consequently 16S rRNA genes, is from modern organisms. As a consequence it is best to select for short DNA fragments, when possible, prior to amplifying targets.

Third, data produced must be analyzed using bioinformatic workflows designed to characterize ancient DNA and ancient microbiomes. We use leeHom [20], to quality trim and merge paired-end reads produced from short ancient DNA templates and mapDamage 2.0 [21] to assess the authenticity of the mapped DNA. To analyze shotgun sequencing reads or 16S rRNA amplicons, we use both MG-RAST (presented in Chapter 4) and homemade dedicated pipelines with leeHom to pre-process reads, and BWA [22] to map them against an in-house reference sequence consisting of concatenated bacterial genomes, before taxonomic characterization.

2 Materials

Buffers are stored at room temperature while reaction mixes, primers, and most enzymes are stored at −20 °C.

2.1 DNA Extraction Reagents

Prepare all solutions from autoclaved deionized water. We use household bleach (2.6 % sodium hypochlorite) and RNase away (Life Technologies, Carlsbad, CA, USA) as agents for decontamination and DNA removal.

1. Commercial soil extraction and purification kits are used, but the reagents are only opened in the high-containment laboratory, under controlled conditions to avoid contamination. We currently use MoBio PowerMax Soil DNA Isolation kit (MO

BIO, Carlsbad, CA, USA, ref. 12988), Qiagen Gel Extraction kit (Qiagen, Hilden, Germany, ref. 28704), and Qiagen PCR purification kit (ref. 28104).

2. Bone matrix disintegration and digestion buffer: 0.5 M EDTA pH 8, 0.25 M PO_4^{3-}, 0.14 M β-mercaptoethanol. 0.5 M EDTA pH 8 is prepared from EDTA powder and autoclaved water and pH is adjusted with NaOH pellets [2].
3. Buffer QG (Qiagen, ref. 19063), solubilization and binding buffer.
4. Buffer PE (Qiagen, ref. 19065), wash buffer.
5. Buffer EB (Qiagen, ref. 19086), elution buffer.

2.2 qPCR Reagents

1. MixG (homemade qPCR mix) [23]: To prepare 100 µL of 10× mixG, mix 19.5 µL γ-irradiated water (*see* **Note 1**), 6.25 µL 10 mg/mL bovine or horse serum albumin (BSA or HSA), 3 µL 10 % Lubrol-17A17 (SERVA Electrophoresis GmbH, Heidelberg, Germany), 50 µL 50 % glycerol, 1.25 µL 5 M KCl, and 20 µL 2.5 M AMPD (2-amino-2-methyl-1,3-propanediol) pH 8.3 (*see* **Note 2**). For volumes higher than 200 µL, aliquot 200 µL each in UV-transparent tubes (Qubit Assay tubes, Life Technologies, ref. Q32856) and treat with UV (*see* **Note 3**), dilute 10,000× SYBR-Green I (Life Technologies, ref. S-7585) 1/40 in DMSO and add 1 µL diluted SYBR-Green I per 100 µL mix. Freeze overnight at −80 °C (*see* **Note 4**).
2. BIOTEC buffer: To prepare 10 mL BIOTEC buffer, mix 200 µL 1 M Tris–HCl pH 7.5, 800 µL 25 mM $MgCl_2$, 20 µL 5 M NaCl, 5 mL 50 % glycerol, 10 µL 10 % Triton x100, complete to 10 mL with γ-irradiated water. Aliquot 540 µL each in UV-transparent tubes, UV irradiate 300 s on each side on a UV cross-linker (*see* **Note 3**).
3. Thermolabile double-strand DNase (2 u/µL hl-dsDNase) from ArcticZymes (Tromsø, Norway, ref. #70800). For a final activity of 0.02 u/µL, add 1 µL 2 u/µL of hl-dsDNase to 99 µL of BIOTEC buffer.
4. Decontaminated Taq DNA polymerase: 108 µL of 5 u/µL Hot Start Taq polymerase in its storage buffer is supplemented with 6 µL premixed 200 mM $MgCl_2$, 20 mM $CaCl_2$, and 2.45 µL 50 mM DTT, then incubated with 6 µL 2 u/µL hl-dsDNase for 30 min at 25 °C followed by a 20-min inactivation step at 50 °C. Aliquot to desired volumes. Final activity of decontaminated Taq is 4.4 u/µL.
5. Decontaminated dNTPs: 20 µL of dATP, dCTP, dGTP, and 40 µL of dUTP (100 mM stock solutions each) are mixed with 100 µL of γ-irradiated water. 40 µL of this dNTP mix is mixed with 35 µL γ-irradiated water, 4 µL 50 mM DTT, 20 µL 250

mM Tris pH 8, 80 μL 50 mM $MgCl_2$, and 20 μL 10 mM $CaCl_2$ and then incubated with 1 μL 0.02 u/μL hl-dsDNase for 30 min at 25 °C followed by a 30-min inactivation at 55 °C. The final dNTP concentration is 2 mM (4 mM for dUTP).

6. 1u/μL codUNG (ArcticZymes, ref. #70500): codUNG is a uracil-DNA glycosylase from Atlantic cod that is completely and irreversibly inactivated by moderate heat treatment [18].

2.3 Library Preparation for PCR Products

1. Oligonucleotides
   (a) Oligonucleotides are those proposed by Life Technologies for genomic DNA Fragment Library preparation[1] (*see* **Note 5**).
   (b) Annealing buffer 10×
   - 25 μL 5 M NaCl
   - 100 μL 250 mM Tris
   - 50 μL 250 mM $MgCl_2$
   - 75 μL γ-irradiated water (*see* **Note 1**)
   - 40 μM annealed adapters (*see* **Note 6**)
2. End-repair enzymes (3 u/μL T4 DNA polymerase and 10 u/μL T4 polynucleotide kinase), such as NEBNext end repair module (ref. E6050, New England Biolabs (NEB), Ipswich, MA, USA)
3. Commercial purification kit, based on silica columns (Qiagen or Macherey-Nagel, Düren, Germany) or SPRI magnetic beads (Ampure XP, Agencourt Technologies, Beverly, MA, USA, ref. 16388 or NucleoMag NGS clean-up and size selection, Macherey Nagel ref. 744970)
   (a) DNA purification kit for 96 samples (96 silica column plate or SPRI magnetic beads)
   (b) Individual DNA purification kit (silica column or magnetic beads)
4. Quick ligase, such as NEBNext Quick ligation module (NEB, ref. E6056, 2000 u/μL T4 DNA ligase)
5. Size selection reagents for E-gel or Caliper XT devices
6. OneTaq Hot Start 2× Master Mix with Standard Buffer (NEB, ref. M0484)

2.4 Double-Stranded DNA Library Preparation

Use γ-irradiated water for all solutions and buffer preparations as well as for any dilution or elution steps (unless otherwise indicated) (*see* **Note 1**).

[1] Appendix E, p. 56–57, Publication Part Number MAN0009847; Revision C.0 Date 29 April 2014.

1. Oligonucleotides (*see* **Note 7**)
   (a) 10× Annealing buffer (*see* Subheading 2.3, **item 1**) UV-irradiated (*see* **Note 3**)
   (b) Annealed adapters 40 µM (*see* **Note 6**)
2. Deaminated cytosine repair
   1 u/µL USER enzyme (NEB, ref. M5505)
3. End repair
   NEBNext End Repair Module (NEB, ref. E6050)
4. Purification of repaired ancient DNA extract
   (a) MinElute Column (Qiagen ref. 28604) (*see* **Note 8**)
   (b) Qiaquick Gel Extraction Kit (Qiagen ref. 28704)
   (c) QG Buffer (Qiagen ref. 19063)
   (d) Isopropanol
5. Blunt-end double-stranded adapter ligation
   (a) 40 µM Double-stranded adapters
   (b) NEBNext Quick Ligation Module (NEB ref. E6056)
6. Elongation and pre-amplification
   (a) OneTaq Hot Start 2× Master Mix with Standard Buffer (NEB, ref. M0484)
   (b) 10 µM Illumina amplification forward and reverse modified primers P5s/P7s (*see* **Note 9**)
7. Purification and size selection
   (a) NucleoMag NGS clean-up and size selection kit (Macherey Nagel ref. 744970)
   (b) Freshly prepared 80 % ethanol
   (c) DNase/RNase-free water
8. qPCR determination of the optimal number of cycles for library amplification
   FastStart DNA Master^{PLUS} SYBR Green I mix (Roche Applied Science, ref. 035158)
9. Final library amplification
   (a) FastStart Taq DNA Polymerase, dNTPack (Roche Applied Science, ref. 04738)
   (b) DNase/RNase-free water
10. Library characterization and purification
    Agilent Bioanalyzer high sensitivity DNA kit (Agilent ref. 5067-4626)

2.5 Single-Stranded DNA Library Preparation

Use γ-irradiated water for all solutions and buffer preparations as well as for any dilution or elution steps (unless indicated otherwise) (*see* **Note 1**).

1. Oligos (*see* **Note 10**)
   (a) 10× Annealing buffer UV-irradiated (*see* **Note 3**)
   (b) 40 μM Annealed CL53/CL73 adapters (*see* **Note 6**)
2. DNA preparation
   (a) 100 u/μL Circligase II ssDNA ligase with 10× Circligase buffer and 50 mM $MnCl_2$ solution (Epicentre, Chicago, IL, USA, ref. CL902)
   (b) 10 u/μL Endonuclease VIII (NEB, ref. M0299)
   (c) 1 u/μL codUNG from ArticZymes (optional)
   (d) 1 u/μL FastAP (Thermo Scientific, Waltham, MA, USA, ref. EF065), a thermosensitive alkaline phosphatase
3. First adapter ligation
   (a) 50 % PEG 4000 (Sigma-Aldrich, St. Louis, MO, USA, ref. 95904)
   (b) Dynabeads MyOne Streptavidin C1 (Life Technologies, ref. 6500)
   (c) Bead binding buffer: 1 M NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 0.05 % Tween-20, 0.5 % SDS. Prepare buffer just before use and discard immediately. Buffer has no shelf life after adding SDS.
   (d) Wash buffer A: 100 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 0.05 % Tween-20, 0.5 % SDS. Can be stored at room temperature for a month.
   (e) Wash buffer B: 100 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 0.05 % Tween-20. Can be stored at room temperature for a year.
   (f) Stringency wash buffer: 0.1× SSC, 0.1 % SDS. Can be stored at room temperature for a month.
   (g) 10× ThermoPol Buffer (NEB, ref. B9004)
   (h) Bst 2.0 DNA Polymerase (NEB ref. M0537)
   (i) 10× Tango buffer (Thermo Scientific, Waltham, MA, USA, ref. BY5)
   (j) 1 % Tween-20 (Sigma-Aldrich, St. Louis, MO, USA, ref. P2287)
   (k) T4 DNA polymerase (Thermo Scientific, ref. EP006)
   (l) Stop solution: 0.5 M EDTA pH 8.0, 2 % Tween-20

4. Second adapter ligation
   (a) T4 DNA ligase (Thermo Scientific, ref. EL001)
   (b) EBT: 10 mM Tris–HCl pH 8.0, 0.05 % Tween-20
5. Library amplification
   (a) qPCR master mix such as LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science)
   (b) Either MinElute PCR purification kit (Qiagen), AMPure XP (Agencourt Technologies), or NucleoMag NGS Clean-up and Size Select kit (Macherey-Nagel)

2.6 Equipment (other than common devices for molecular biology laboratories)

1. qPCR-capable thermocycler such as LightCycler 2.0 (Roche Applied Sciences)
2. UV-crosslinker such as Spectrolinker XL 1500 UV-crosslinker (Spectronics Corp., Westbury, NY, USA)
3. Multi-purpose rotating tool such as Dremel 9100 Fortiflex Heavy Duty Flex Shaft Tool (Robert Bosch GmbH, Stuttgart, Germany) with diamond cutting wheel (ref. 545) and high-speed cutting or drilling bits (e.g., ref. 194)
4. Freezer-mill such as Spex Certiprep 6770 Freezer/Mill® (SPEX, Metuchen, NJ, USA)
5. Electrophoresis system for DNA sizing and purification, such as E-Gel SizeSelect (Life Technologies), Caliper Labchip XT (Perkin-Elmer, Waltham, MA, USA) or Pippin Prep (Sage Science, Beverly, MA, USA)
6. Lab-on-chip electrophoresis system, such as Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA)
7. Fluorimeter for DNA quantification, such as Qubit 2.0 (Life Technologies)
8. Optional: Robotic platform, such as a TECAN EVO 100 (Tecan, Maennedorf, Switzerland), to facilitate high-throughput sample treatment, but all steps can be performed manually
9. Heating/cooling block with mixing capability, such as an Eppendorf Thermomixer Comfort (Eppendorf, Hamburg, Germany) (*see* **Note 11**)
10. Ice-water bath
11. Magnetic rack for 1.5 mL tubes

3 Methods (*See* Note 12)

3.1 DNA Extraction and Purification

1. DNA extraction from soil
   To extract and purify DNA from soil (*see* **Note 13**), we found PowerMax Soil DNA Isolation kit from MoBio useful for a wide range of samples (*see* **Note 14**).

Typically, 10 g of soil are processed following the guidelines recommended by the manufacturer.

After extraction and purification, DNA is dissolved in 5 mL resuspension buffer. It is subsequently concentrated and repurified with a modified Qiagen Gel Extraction protocol as follows:

(a) Set an incubator (e.g., heating block) or water bath to 50 °C.

(b) Heat an aliquot of EB to 50 °C (150 μL per sample to be extracted).

(c) Add 30 mL of QG and 20 mL of isopropanol to the DNA solution.

(d) Pass through columns mounted on a manifold device (QIAvac 24 Plus, Qiagen, ref. 19413) equipped with extension tubes (Qiagen ref. 19587) (*see* Fig. 2).

(e) Once all the samples have passed through the columns (*see* **Note 15**), remove extenders to wash them with autoclaved water and install them in place again.

(f) Wash columns with 2 mL PE.

(g) Break vacuum and stop vacuum pump.

(h) Remove columns from the manifold and transfer them in clean 2 mL collection tubes.

(i) Centrifuge at 9300 × *g* for 2 min to dry the columns.

(j) Transfer columns to new clean 1.5 mL microcentrifuge tubes.

(k) Carefully dispense 100 μL of preheated (50 °C) elution buffer (EB or molecular grade water) and incubate for 1 min at room temperature.

(l) Centrifuge at 9300 × *g* for 1 min, discard columns, and store eluate (*see* **Note 16**).

2. DNA extraction from bone

Ancient bones and teeth can be considered as environmental samples (*see* **Note 17**), since they are a matrix in which bacteria, fungi, and other organisms reside. The dedicated procedures developed to access the endogenous DNA also extract the degraded bacterial (as well as fungal and eukaryotic) DNA present in the ancient bone (*see* **Note 18**).

The cleaning and powdering steps of the skeletal remains are performed in a high-containment laboratory [19] (*see* **Note 19**). The surface of the remains is removed in a UV-irradiated protective hood. Bone is then drilled or ground to fine powder in a freezer mill (Spex Certiprep 6750). Further processing of the bone powder is performed as described [19]. Blank extractions are carried out for each extraction series.

(a) Decontaminate the hood and all tools with pure household bleach (use RNAse away for decontaminating metal tools).

(b) Cover the base of the working station with a sheet of aluminum foil.

(c) Remove the outer layer of the sample with a scalpel. Use a new scalpel for each sample, and discard used scalpels.

(d) Set up and turn on a vacuum cleaner during cutting to prevent the dispersal of bone powder in the working station.

(e) Sample preparation in a freezer mill:

- Use a Dremel multitool equipped with a diamond cutting wheel to cut pieces (up to 200 mg), adjusting the speed to the density/mineralization of the bone. Decontaminate the cutting wheel using the medium flame of a Bunsen burner at around 500 °C, but not above, to avoid damaging the diamond wheel.
- Weigh the bone fragment(s) and pulverize it in a freezer mill in liquid nitrogen for 1 min (10 impacts per second).
- After transferring the powder to a clean tube, clean freezer mill tubes. Metal parts: remove remaining bone powder in a bath of RNase away, rinse in a water bath, and dry under UV light. Plastic tubes: brush with water to remove remaining bone powder, rinse in a bleach bath, then with water and let them dry overnight on clean aluminum foil. Do not expose to UV light.

(f) Sample preparation using a drill

- Depending on the density of the bone, its shape, and the location of the sampling area, various drill bits can be chosen according to the user's needs. Assemble and decontaminate the drill bit using the medium flame of a Bunsen burner (ca. 500 °C). Drill at the lowest speed possible giving efficient bone powder production.
- Transfer the bone powder in a pre-weighed tube and weigh it.

(g) Cleaning

- Between preparations of each sample, change the aluminum foil, clean the working station with bleach, and change gloves. Flame the drill bit or the cutting wheel using the medium flame of a Bunsen burner (ca. 500 °C).
- After completion of the preparation series, decontaminate the Dremel tool with 70 % ethanol and RNase away (not bleach), and flame the drill bit or the cutting wheel using the medium flame of a Bunsen burner (ca. 500 °C). Clean and decontaminate with bleach the working station and the vacuum cleaner, place a UV

lightsource inside the working station so that it irradiates the surface of the bench at close proximity for at least 3 h. Clean the area around and wash floor with 10 % household bleach.

(h) Once powdered, the bone is mixed with digestion buffer (1 mL per 100 mg of bone powder) and incubated at 37 °C in an orbital shaker (10 RPM) for 24/48 h or upon complete dissolution of the bone powder.

(i) After disintegration of the bone matrix, DNA is purified on silica columns.

- Centrifuge the suspension for 10 min at 13,000 × *g*.
- Prepare manifold with QiaAmp spin columns and extenders as in Subheading 3.1, **step 1**.
- Transfer supernatant to 15 mL Falcon tubes and store pellets (−20 °C) for possible future re-extraction.
- Follow the modified Qiagen Gel Extraction protocol described in Subheading 3.1, **step 1**.

3.2 DNA Amplification

1. To test whether the DNA extracts from the ancient bone samples inhibit the PCR (*see* **Note 20**), serial dilutions of the extracts spiked with a known quantity of positive internal control are amplified [24]. Subsequently, the Ct (crossing point at threshold) is analyzed for each serial dilution of the same sample. We dilute the extract two- and fourfold at the highest, considering that further dilution of ancient DNA extracts potentially containing only few molecules could cause the loss of targets.
2. For DNA amplification (*see* **Note 21**), we systematically use a home-made decontaminated qPCR mix (mixG). Endogenous DNA detection relies on the amplification of DNA fragments that are informative enough to discriminate between animals or, for bacteria, between phyla, classes, orders, genera, or even species depending on the targeted genes. To assess bacterial diversity, the 16S rRNA gene is a powerful tool, thanks to its ubiquity and structure, which make it an optimal marker for the characterization of environmental samples (*see* **Note 22**).

   Amplification is performed with a LightCycler 2.0 (Roche Applied Sciences). Prepare a PCR mix using 1 μL of DNA extract, 1 μL of mixG, 1 mM $MgCl_2$, 1 μM each of primer, 0.01 u of codUNG, 200 μM dNTPs (with dUTP in place of dTTP), 0.1 u of Taq, and complete to 10 μL with γ-irradiated water.

   Hybridization times and temperatures are primer dependent and elongation times are defined according to user's needs.
3. With qPCR, amplification is reported by fluorescence emission of SYBR Green I intercalated in double-strand DNA. Non-specific products, which are sometimes synthetized during qPCR and are mainly primer-dimers, also lead to fluorescence

emission. A first control is the use of several non-template controls (NTCs) to monitor the cycle number required to amplify such dimers and to determine the Tms of the various possible dimers. If similar Cts and Tms are observed for both samples and NTCs, the amplification of dimers can be suspected. Primer-dimers, however, may sometimes have a Tm close to that of the desired product. Gel visualization is then needed to discriminate between primer-dimers, other non-specific products, and the desired product. Direct sequencing of products is a mandatory step to authenticate ancient DNA PCR results. When amplifying a target gene present only in the species of interest, such direct sequencing can be done by Sanger sequencing (i.e., Eurofins Genomics, Ebersberg, Germany). When amplifying genes like the 16S rRNA, the PCR product is a mix of thousands of different sequences best analyzed through high-throughput sequencing (HTS). The IonTorrent platform is well suited for this.

3.3 Library Preparation for PCR Products

1. For each sample, mix 20 μL of PCR product (diluted if necessary—*see* **Note 23**), 5 μL of 10× enzyme buffer, 0.1 μL of end repair enzyme, and 24.9 μL of γ-irradiated water. Gently pipet the total volume up and down 1–2 times to mix and incubate for 30 min at 25 °C (*see* **Note 24**).
2. Purify end-repaired products using a 96-well plate system (for high-throughput) or in tubes by magnetic beads or silica columns according to the manufacturer's instructions (*see* **Note 25**). The final elution volume is usually 50 μL. *Samples may be frozen at –20 °C at this point.*
3. In a 96-well plate, add 1 μL of a different barcoded adapter mix (A + P1) combination to each well to be used. Distribute a premix composed of 6 μL of 5× ligation buffer, 1 μL of Quick ligase, and 2 μL of γ-irradiated water to each well and add 20 μL of each purified end-repaired sample (*see* **Note 26**). Gently pipet up and down 1–2 times to mix and incubate for 30 min at 16 °C.
4. Immediately after the ligation, add binding buffer to each well according to the manufacturer's recommendation (60 μL of NT buffer, Macherey-Nagel), mix well, and pool all samples before loading on a silica column. The binding, washing, and drying steps are performed as recommended. Elute in a volume between 30 and 50 μL. *Samples may be frozen at –20 °C at this point.*
5. Use an electrophoresis system for DNA sizing and purification to size-select your library and eliminate adapter dimers or multimers of amplicons. We use the E-Gel SizeSelect or the Caliper Labchip XT depending on the size range of amplicon size

(*see* **Note 27**). To determine the size range to select, add the length of the two adapters (85–87 bp depending on the barcode length, *see* **Note 5**) to your minimal and maximal amplicon size.

6. Nick repair and amplification are made sequentially with the same reaction mix (*see* **Note 28**). Add 8 μL of size-selected sample, 1 μL of 10 μM Primer A, 1 μL of 10 μM Primer P1, and 10 μL of 2× OneTaq Hot Start Master Mix with buffer. Gently pipet up and down 1–2 times to mix, incubate for 20 min at 68 °C (nick repair step), and then amplify the library using the following program: initial denaturation at 94 °C for 5 min (94 °C for 15 s, 60 °C for 15 s, 68 °C for 40 s) for six cycles, final elongation at 68 °C for 5 min (*see* **Note 29**).
7. Purify the amplified libraries with a silica column or SPRI magnetic beads. Final elution volume is usually 30 μL. *Samples may be frozen at –20 °C at this point.*
8. Qualitative analysis on a Bioanalyzer is recommended to check the final library product (*see* **Note 30**). Products saved at intermediate steps (sizing and amplification) can also be run on the same chip and compared. Libraries are quantified using a Qubit 2.0 to determine the concentration and adapt it to the emulsion PCR for IonTorrent PGM sequencing. A qPCR is also recommended to compare the new library to a known reference to ensure that the Qubit measurement corresponds to samples ligated with the two adapters.
9. Follow the manufacturer's recommendations to prepare the chip for sequencing. Depending on the heterogeneity of the PCR product(s) and the number of samples analyzed, either 314, 316, or 318 chips (V2) can be used.

3.4 Double-Stranded DNA Library Preparation

1. Mix in a 1.5 mL tube 1–500 ng of DNA extract, 3 μL of 10× NEBNext end repair buffer, 1.5 μL of 1 u/μL USER enzyme, and complete to 28.5 μL with γ-irradiated water. Mix by pipetting and incubate for 1 h at 37 °C in a heating block.
2. After the cytosine deamination repair step, add 1.5 μL of NEBNext End Repair Enzyme Mix directly to the tube. Mix well by pipetting gently times and incubate for 30 min at 20 °C.
3. The extract is purified with Qiagen MinElute kit, following the manufacturer's protocol, except that elution is done twice with 17 μL of γ-irradiated water (preheated at 50 °C).
4. Add 10 μL of 5× Quick ligation reaction buffer, 1 μL of the 40 μM annealed P50X adapter, 1 μL of the 40 μM annealed P7XX adapter (*see* **Note 31**), 2 μL of Quick DNA ligase, and 6 μL of γ-irradiated water to the sample. Gently pipet 1–2 times to mix and incubate for 30 min at 20 °C.

5. Add to the sample 3 μL of each of the 10 μM Illumina amplification forward and reverse primers P5s and P7s and 50 μL of OneTaq 2x Master Mix. Transfer the 100 μL reaction solution into a 0.2 mL PCR tube, and run in a thermocycler with the following program: 15 min OneTaq elongation step of the ligated products at 68 °C, followed by six cycles involving denaturation for 20 s at 95 °C, annealing for 35 s at 60 °C, and primer extension for 70 s at 72 °C.
6. The purification of the six-cycle-amplified library and the removal of potential artifacts (primer-dimers) are done using the NucleoMag NGS clean-up and Size Select kit:
   (a) Add 130 μL (1.3×) Macherey-Nagel (MN) beads to the 100 μL of amplified library.
   (b) Vortex and let sit for 5 min at RT.
   (c) Quick spin and place on a magnetic rack; let sit for 2 min or until supernatant is clear.
   (d) Discard liquid.
   (e) Add 500 μL 80 % ethanol (freshly made).
   (f) Twist tubes two or three times, until beads no longer stick to the tube's surface, and let sit for 2 min or until supernatant is clear.
   (g) Discard ethanol with a pipet, being careful to remove as much as possible.
   (h) Let dry for 2 min at RT on the magnetic rack.
   (i) Remove from the magnetic rack, add 52 μL γ-irradiated water, and pipet ten times to mix well.
   (j) Let stand for 2 min at RT away from the magnetic rack.
   (k) Quick spin and place on the magnetic rack; let sit for 2 min or until supernatant is clear.
   (l) Remove 50 μL to new tubes.
   (m) Add 65 μL (1.3×) MN beads.
   (n) Repeat **steps b–h**.
   (o) Remove from the magnetic rack, add 25 μL γ-irradiated water or EBT, and pipet ten times to mix well.
   (p) Let stand for 2 min at RT away from the magnetic rack.
   (q) Quick spin and place on the magnetic rack; let sit for 2 min.
   (r) Remove 22 μL of purified sample and place in a new tube.
7. To obtain a sufficient quantity of DNA while avoiding over-amplification (*see* **Note 30**), qPCR quantification of the pre-amplified library is performed using three serial dilutions (1:10, 1:100, 1:1000) and the modified Illumina P5 and P7 primers. For each sample, the amplification curves are analyzed and the

cycle number at the point between exponential phase and saturation is determined. This value is used to calculate how many cycles are needed to amplify the library. For a typical reaction, the correct amplification of the library will be 7–8 cycles less than the value determined with the 1/100 diluted sample (*see* **Note 32**).

8. To further amplify the library, mix 20 μL of the library, 61 μL of γ-irradiated water, 2 μL of 5 mM dNTPs (A,T,C,G), 3 μL of each 10 μM Illumina amplification primers P5s and P7s, 10 μL of the 10× PCR reaction buffer containing 20 mM $MgCl_2$, and 1 μL of 5 u/μL FastStart Taq DNA Polymerase. Gently pipet 1–2 times to mix and amplify the library using the following program: (95 °C for 20 s, 60 °C for 35 s, 72 °C for 70 s) for the appropriate number of cycles.
9. Purify the PCR using the Qiagen PCR purification kit.
10. Quantify the amplified libraries using a fluorescence-based quantification method; observe the size distribution of the amplified library and possible presence of artifacts on an Agilent Bioanalyzer 2100 (*see* **Note 33**).

3.5 Single-Stranded DNA Library Preparation

Prepare all enzymatic mixes prior to each step to avoid letting the beads dry between steps.

1. Dilute the purified DNA extract (between 1 fmol and 1 pmol of DNA) with γ-irradiated water to a final volume of 29 μL (*see* **Note 34**).
2. To optimize damaged DNA recovery prior to library preparation, add 29 μL of diluted DNA extract, 8 μL of 10× Circligase buffer, 4 μL of 50 mM $MnCl_2$, and 0.5 μL 10 u/μL Endonuclease VIII to a 1.5 mL tube and incubate for 1 h at 37 °C (*see* **Note 35**).
3. Add 1 u of FastAP to the above reaction and mix. Spin briefly and incubate for 10 min at 37 °C. Incubate the reaction for 2 min at 95 °C to heat denature the DNA, then transfer tube to an ice-water bath, and leave for 1 min. Spin briefly.
4. To ligate the first adapter
   (a) Add 32 μL 50 % PEG-4000, 1 μL 10 μM adapter oligo CL78, and 1 μL 100 u/μL Circligase II. Incubate tube for 1.5–3 h at 60 °C. *Samples may be frozen safely at –20 °C at this point.*
   (b) For each sample, transfer 20 μL of MyOne C1 dynabeads into a 1.5 mL tube. Use additional tubes for more than five samples. Wash beads by placing tube on a magnetic rack for 2 min. Discard supernatant and wash beads twice with 500 μL bead binding buffer (*see* **Note 36**).

(c) Resuspend beads in 250 µL bead binding buffer per sample (*see* **Note 37**), vortex, and transfer 250 µL of beads to each ligated sample.

(d) Rotate tubes slowly on a rotating wheel, making sure that the beads stay in suspension, for 20 min at room temperature.

(e) Spin tubes briefly, place on a magnetic rack for 2 min, and discard supernatant.

(f) Add 200 µL wash buffer A.

(g) Place tubes on the magnetic rack, wash by twisting tubes (*see* **Note 36**), and then leave tubes on the rack for 2 min. Discard supernatant.

(h) Add 200 µL wash buffer B.

(i) Place tubes on the magnetic rack, wash by twisting tubes, and then leave tubes on the rack for 2 min. Discard supernatant.

5. To create the second strand

(a) Mix together 38.5 µL γ-irradiated water, 5 µL 10× Thermopol buffer, 2.5 µL dNTP mixture (5 mM of each), and 1 µL 100 µM extension primer CL9.

(b) Add mixture (47 µL) to beads in each tube. Vortex tubes and spin down briefly.

(c) Incubate tubes for 2 min at 65 °C, and then cool for 1 min in an ice bath.

(d) Place tubes in an Eppendorf thermomixer set at 15 °C, and then add 3 µL Bst 2.0.

(e) Incubate at 15 °C for 30 min, mixing 1000 RPM (*see* **Note 11**).

(f) Place tubes on the magnetic rack for 2 min, and discard supernatant.

(g) Add 200 µL wash buffer A.

(h) Place tubes on the magnetic rack, wash by twisting tubes, and then leave tubes on the rack for 2 min. Discard supernatant.

(i) Add 100 µL stringency wash buffer, and incubate for 3 min at 45 °C in a Thermomixer, mixing at 1000 RPM.

(j) Spin tubes briefly, then place on the magnetic rack for 2 min, and discard supernatant.

(k) Add 200 µL wash buffer B.

(l) Place tubes on the magnetic rack, wash by twisting tubes, and then leave tubes on the rack for 2 min. Discard supernatant.

6. Remove 3′ overhangs remaining from the extension step
   (a) Mix 84.5 μL γ-irradiated water, 10 μL 10× Tango buffer, 2 μL dNTP mix (5 mM each), 2.5 μL 1 % Tween-20, and 1 μL T4 DNA polymerase.
   (b) Add mixture (100 μL) to beads in each tube, vortex, and spin down briefly.
   (c) Incubate for 15 min at 25 °C in a Thermomixer, mixing at 1000 RPM.
   (d) Add 10 μL stop solution to each tube.
   (e) Place tubes on the magnetic rack for 2 min, and discard supernatant.
   (f) Add 200 μL wash buffer A.
   (g) Place tubes on the magnetic rack, wash by twisting tubes, and then leave tubes on rack for 2 min. Discard supernatant.
   (h) Add 100 μL stringency wash buffer, and incubate for 3 min at 45 °C in a Thermomixer, mixing at 1000 RPM.
   (i) Spin tubes briefly, place on the magnetic rack for 2 min, and discard supernatant.
   (j) Add 200 μL wash buffer B.
   (k) Place tubes on magnetic rack, wash by twisting tubes, and then leave tubes on rack for 2 min. Discard supernatant.
7. To ligate the second adapter
   (a) Mix 71 μL γ-irradiated water, 10 μL 10× T4 DNA ligase buffer, 10 μL 50 % PEG-4000, and 2.5 μL 1 % Tween-20.
   (b) Add mix (93.5 μL) to each tube, vortex, and spin down briefly.
   (c) Add 5 μL 40 μM annealed adapters CL53/CL73 and 2 μL T4 DNA ligase to each tube, vortex, and spin down briefly.
   (d) Incubate for 1 h at 25 °C in a Thermomixer, mixing at 1000 RPM.
   (e) Place tubes on the magnetic rack for 2 min. Discard supernatant.
   (f) Add 200 μL wash buffer A.
   (g) Place tubes on the magnetic rack, wash by twisting tubes, and then leave tubes on rack for 2 min. Discard supernatant.
   (h) Add 200 μL wash buffer B.
   (i) Place tubes on the magnetic rack, wash by twisting tubes, and then leave tubes on rack for 2 min. Discard supernatant.

(j) To elute the single-stranded library, add 50 μL EBT buffer to each tube and resuspend the beads by pipetting gently up and down.

(k) Incubate each tube for 1 min at 95 °C in a Thermomixer, mixing at 1000 RPM, and immediately move tube to the magnetic rack.

8. Transfer the supernatant, which contains the single-stranded library molecules, to a new tube. This non-indexed, single-stranded "proto"-library must be amplified with P7 and modified P5 indexing primers (preferably barcoded) before being ready for sequencing. Libraries can be stored at −20 °C for several months.
9. Follow the same protocol as for double-stranded library preparation (Subheading 3.4, **steps 5–10**) but using indexing primers P7 and modified P5.
10. When setting up the Illumina sequencing run, be sure to replace the Read 1 sequencing primer with the custom primer CL72 using the manufacturer's instructions for custom primers.

4 Notes

1. **Water was decontaminated by γ**-Irradiation (at least 2 kGy) using a ^{37}Cs source that had been calibrated by Fricke dosimetry [18].
2. MixG (home-made qPCR mastermix) is prepared with a high amount of BSA or HSA to allow its use with the LightCycler Carousel systems, since those systems use glass capillaries and BSA/HSA are needed to minimize DNA binding to the glass. We use the Carousel system for its sensitivity and its rapidity and to minimize carry-over contamination, each sample being in its own tube and capped after filling. The source of the albumin depends on the analyzed species. HSA is used for ancient bovine remains, and BSA in all other cases.
3. UV irradiation decontamination of reagents is performed in thin-wall, UV clear tubes for 300 s on each side in a Spectrolinker XL 1500 UV cross-linker device equipped with 254 nm UV light bulbs (Spectronics Corp., Westbury, NY, USA) corresponding to a total energy of 4.8 J/cm^2 [18]. The UV bulb must be at a close distance (we use 5 cm) from the tubes since the efficiency of the destruction of DNA molecules with UV light is a function of the square of the distance [18].
4. SYBR-Green I can be replaced by EvaGreen 25 mM diluted 1/80 in DMSO [18]. The −80 °C freezing step is essential.

5. Primer sequences for IonTorrent library preparation
   For sequencing on an Illumina sequencer, the adapters described in **Note 7** should be used instead.
   (a) A barcoded adapter:
      - Long strand: 5′ CCATCTCATCCCTGCGTGTCTCC GACTCAGXXXXXXXXXXCGAT 3′
      - Short strand: 5′ ATCGXXXXXXXXXX 3′
   (b) P1 adapter:
      - Long strand: 5′ CCACTACGCCTCCGCTTTCCTC TCTATGGGCAGTCGGTGAT 3′
      - Short strand: 5′ ATCACCGACTGCCC 3′
   (c) Primer PCR A: 5′ CCATCTCATCCCTGCGTGTCTC 3′
   (d) Primer PCR P1: 5′ CCACTACGCCTCCGCTTTCCT CTCT 3′
6. Hybridize both strands to make double-stranded adapters by mixing 20 μL of each adapter (100 μM), 5 μL of annealing buffer (10×), and 5 μL of γ-irradiated water. Incubate for 30 s at 95 °C in a heating block, then turn off the heat block, and allow the tubes to come to room temperature. Annealed oligos may be stored at −20 °C. Final concentration of adapter is 40 μM. For IonTorrent adapters, since only the A adapter is barcoded, it is more convenient to prepare a premix of equal volume of A and P1 adapters for each barcode.
7. Primers for the double-strand library preparation are modified from the Illumina sequencing primers, Nextera or TruSeq barcodes, and amplification primers, to change the Y-shape adapter design back to the initial Solexa design with two different adapters, as this design minimizes dimer background when working with very low DNA amounts [19]. For sequencing on an IonTorrent sequencer, the adapters described in **Note 5** should be used instead. In the example below, amplification primers are in italics, barcodes are bold, and sequencing primers are in roman fonts.
   (a) D7XX construct:
   CAAGCAGAAGACGGCATACGAGAT **XXXXXXXX**GT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
   (b) D50X construct:
   AATGATACGGCGACCACCGAGATCTACAC **XXXXXXXX** ACACTCTTTCCCTACACG ACGCTCTTCCGATCT
   (c) SLP5P7: 5′-AGATCGGAAGAG-3′
8. MinElute is used to allow small elution volume (here 17 μL). Columns are purchased independently of the purification kit.

9. P5s sequence CAAGCAGAAGACGGCATACGAGAT
   P7s sequence: AATGATACGGCGACCACCGAGAT
10. Oligonucleotides [10, 25]
    (a) CL78: single-stranded adapter: 5′[Phosphate]-AGATCGGAAGXX-[TEG-biotin] (X = C18 spacer). Alternatively, ten C3 spacers can be used in the place of two C18 spacers.
    (b) CL9: 5′-tailed extension primer: 5′GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT.
    (c) CL53: double-stranded adapter strand 1: 5′CGACGCTCTTC-[ddC] (ddC = dideoxy cytidine).
    (d) CL73: double-stranded adapter strand 2: 5′[Phosphate]-GGAAGAGCGTCGTGTAGGGAAAGAGTGTA.
    Some protocols specify four phosphorothioate bonds at the 3′ end of CL73 [25]. We obtain good results without this modification.
    (e) Modified P5 indexing primer: 5′ AATGATACGGCGACCACCGAGATCTACAC[barcode]ACACTCTTTCCCTACACGACGCTCTTCC
    Insert standard Illumina barcode of choice in the place of [barcode]. The modified P5 indexing primer with the P7 indexing primer is used to create, via PCR, full-length barcoded Illumina adapters flanking the initial single-stranded library products.
    IMPORTANT! Modifications in the P5 barcoded primer/adapter necessitate a corresponding custom sequencing primer, CL72, to be used in place of the Illumina Read 1 sequencing primer during sequencing (CL72: 5' ACACTCTTTCCCTACACGACGCTCTTCC [25]).
    (f) P7 indexing primer: Use a standard, non-annealed, barcoded Illumina P7 adapter oligo as the P7 indexing primer.
11. Alternatively, a standard heating block or thermocycler with a heated lid can be used instead of Eppendorf Thermomixer Comfort, but tubes containing beads must be manually mixed periodically to prevent the beads from settling.
12. All pre-PCR work is carried out in a physically isolated high-containment laboratory in a part of the building where no DNA amplification is performed. Rooms are under positive air pressure with a gradient from low to high positive pressure from the airlock, through the extraction and purification rooms, to the pre-PCR room with the highest positive pressure. Incoming air is filtered. This installation minimizes contamination with airborne environmental DNA.

13. Many techniques exist to extract DNA from soil. Authors propose different buffers, but the general purpose is to homogenize and lyse the soil in a liquid buffer mechanically (Precellys, MoBio homogenizer, etc.) or using chemicals (SDS, NLS, etc.) while limiting the interactions between DNA and soil particles using a buffer of high ionic strength (such as NaCl 1.5 M). Different DNA extraction techniques have been found to modify the representation of bacterial populations [29, 30].
14. We use MoBio PowerSoil to obtain the least variable results and to be able to perform comparisons from one extract to another, since the various DNA purification protocols extract different bacterial populations [26].
15. Buffers usually pass through the column in a few minutes. With a vacuum pump capable of producing a vacuum of −800 to −900 mbar, buffers pass through the column in 5–10 min. Clogging of the silica membrane by particles remaining in the sample can sometimes occur during the DNA binding step. When this occurs, the remaining sample can be passed through one or more fresh columns and combined after elution. If clogging continues to occur, columns can be centrifuged multiple times (10,000 × *g* usually for 1 min) using 700 μL of buffer per centrifugation.
16. For maximal recovery, elution can be performed using 2× 75 μL preheated (50 °C) EB as follows:
    (a) Load spin columns with 75 μL preheated EB and incubate for 2 min.
    (b) Centrifuge for 1 min at 12,500 × *g*.
    (c) Load spin columns with 75 μL preheated EB and incubate for 1 min in the thermo-block at 50 °C.
    (d) Centrifuge for 2 min at 12,500 × *g*.
    (e) Pool the eluates and store at −20 °C.
17. The situation is more complicated since the skeletal remains are chemically not homogeneous but rather consist of multiple chemical microenvironments, each with its specific chemistry, in which DNA preservation can be variable.
18. Since there is no specific lysis procedure to open up microbial cells, it is likely that the DNA recovery from live microbial cells is low.
19. A complete overview of the process can be seen in a movie, available at http://www.univ-paris-diderot.fr/Mediatheque/spip.php?article246&var_mode=calcul, especially after the seventh minute.
20. In environmental samples, many soil compounds can interfere with molecular techniques used downstream, such as humic and fulvic substances.

21. The 16S rRNA gene includes both conserved regions, which can be used for designing amplification primers across taxa, and nine hypervariable regions (V1–V9), which can be effectively used to discriminate between taxa [27]. Nearly every hypervariable region or combination thereof has been studied. Owing to the short size of DNA fragments in degraded ancient samples, we selected V5 (28 bp long in *Escherichia coli*). Among published primers, we selected the pair providing the best coverage according to SILVA TestPrime and SILVA SSU refNR r114 [28]. We selected a forward primer (E786F) from Baker et al. [29] and a reverse primer (926r) from Watanabe et al. [30], the pair producing a 141 bp long fragment [29].
22. To prevent carry-over contamination, we systematically use dUTP instead of dTTP in qPCR mixes [31]. Incorporation of dUTP during PCR allows for elimination of amplicons from previous PCR steps when incubation with uracil-N-glycosylase (UNG) precedes each PCR. We selected codUNG from ArcticZymes (Tromsø, Norway) for its enhanced thermolability and high efficiency [18].
23. 20 pmol of adapters are present in subsequent preparation steps and must be in excess with respect to PCR products. A maximum amount of 5 pmol of amplicon products is recommended per sample.
24. This step will create blunt-ended 5′ phosphorylated DNA using two enzymes: T4 DNA polymerase and T4 polynucleotide kinase. T4 DNA polymerase fills in 5′-protruding ends and removes 3′-protruding ends, thus producing blunt ends. T4 polynucleotide kinase phosphorylates the 5′-ends of DNA.
25. We use a TECAN EVO 100 and NucleoSpin 96 PCR clean-up (Macherey-Nagel ref. 740658) to achieve automated 96-well purification.
26. This step ensures ligation of DNA fragments with adapters. We use Quick Ligase to increase the ligation reaction and reduce incubation time.
27. E-Gel is preferred when amplicons have similar sizes (within about 50 bp) whereas the Caliper XT is better suited when the amplicon sizes are more heterogeneous.
28. This step allows the removal of the small fragment of the adapters and the fill-in of the 3′-protruding end of the ligated adapter. We use OneTaq from NEB, a blend of Taq and Deep VentR™ DNA Polymerases.
29. Save 1 μL for bioanalyzer analysis if desired.
30. If libraries are amplified beyond the point at which PCR starts saturating, multimers of PCR products may form due to the

cross-hybridization of library molecules via their adapter sequences, both preventing the proper determination of fragment size distribution and DNA concentration using an electrophoresis-based system and causing abnormal chimeric sequences.

31. By using 1 μL of the 40 μM adapters, considering an average size of the ancient DNA fragment in the extract of 50 bp, the adapter excess is 2.5× if the starting input is 500 ng and 1250× if the starting input is 1 ng. The adapter concentration can be modified according to the quantity of the input material.
32. For example, considering a qPCR amplification plot obtained from quantifying the library with the 1:100 dilution: (1) qPCR was performed in a 10 μL reaction volume, whereas library amplification is performed in 100 μL. Thus, 3.5 cycles should be added to allow for ten times more end product. (2) One microliter of a 1:100 library dilution was used for measurement, whereas 20 μL of the library is used for the library amplification (2000 times more). This corresponds to roughly 11 cycles that should be deducted. Thus, 7.5 cycles should be deducted from the number of cycles just prior to the beginning of the saturation phase (around roughly 75 % of the plateau height) of the 1:100 dilution amplification curve. In this way we estimate the optimal cycle numbers for PCR.
33. If excessive adapter dimers or small inserts are present in your library, additional size selection may be desired following the library purification. If AMPure XP SPRI or NucleoMag beads are used, two rounds of purification using a bead volume of 1.3× sample volume are recommended to best remove adapter dimers while preserving inserts of 30 bp and above, as described in Subheading 3.4, **step 6**.
34. For optimal results, the purified DNA extract should first be quantified with a fluorescence-based quantification method, and length distribution can be observed in most cases using an Agilent 2100 BioAnalyzer. Positive and negative control libraries should be included with each procedure. A positive control oligonucleotide should be 5′-phosphorylated and have internal primers to allow quantification with qPCR. Use 29 μL of water for a negative control.
35. This step will remove DNA with abasic sites to maximize incorporation of damaged DNA molecules into the library. To additionally remove uracils, the result of DNA cytosine deamination, 0.5 μL codUNG (1 U) may also be added at this step. If uracils are not removed, some cytosines may be improperly rendered as thymines in the final sequence. 1 μL USER enzyme (NEB) may be used in place of Endonuclease VIII and UDG.

36. To wash beads, twist tube three turns while seated in the magnetic rack.
37. For example, if preparing three samples, resuspend beads in 750 μL.

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