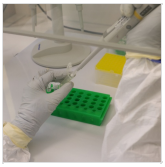

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Ancient DNA Extraction from Skeletal Material

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1 Works for me dx.doi.org/10.17504/protocols.io.baksicwe

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ABSTRACT

Silica-based total DNA extraction protocol optimised for the recovery of ultra-short DNA molecules from skeletal material powder (e.g. bone, teeth), modified from Dabney *et al.* (2013) *PNAS* (doi: 10.1073/pnas.1314445110).

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KEYWORDS

Dabney, aDNA, tooth powder, bone powder, extraction, skeleton, DNA extraction, nucleic acids, tooth, teeth, bone, ancient DNA, palaeogenetics, archaeogenetics, paleogenetics, archeogenetics

IMAGE ATTRIBUTION

Matthäus Rest

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31090

GUIDELINES

Working in an Ancient DNA Laboratory

- All steps of the protocol should take place in a clean room facility specifically designed for ancient DNA.
- The researcher performing lab work should wear correspondingly suitable lab-wear, such as:
  - full-body suit with hood (e.g., Tyvek)
  - hairnet
  - face mask
  - two pairs of clean gloves
  - clean shoes
  - protective glasses
- Sample processing should be carried out in separated work benches with integrated UV irradiation (e.g. Dead Air

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PCR work bench)
- Surfaces and equipment should be regularly decontaminated with e.g. bleach solution or Thermofisher's DNA AWAY (or similar) and irradiated with UV.

Please see the following for more detailed guidance:

Llamas, B. et al., 2017. From the field to the laboratory: Controlling DNA contamination in human ancient DNA research in the high-throughput sequencing era. *STAR: Science & Technology of Archaeological Research*, 3(1), pp.1–14. Available at: https://doi.org/10.1080/20548923.2016.1258824.

Definitions
Stock-aliquot refers to a personal 'stock' (e.g. in a 50ml Falcon Tube) of reagents you can use across multiple sessions of this protocol. An 'aliquot' refers to a sub-aliquot of the stock, that is used for a single session of this specific protocol.

Protocol Specific Guidelines
This protocol requires the use of two rooms - a dedicated PCR-free ultra-clean buffer preparation room and a DNA extraction room.

MATERIALS

NAME	CATALOG #	VENDOR
Parafilm	743311	Biozym
pH indicator strips MQuant® Supelco®	1.09535.0001	Merck Millipore
Safe-Lock Tubes 1.5 ml PCR clean DNA LoBind	0030108051	Eppendorf
Safe-Lock Tubes 2 ml PCR clean DNA LoBind	0030108078	Eppendorf
50 ml CELLSTAR® Polypropylene Tube 30/115 MM Conical Bottom Blue screw cap sterile skirt	210261	greiner bio-one
EDTA (0.5 M) pH 8.0-500 mL	AM9261	Thermo Fisher Scientific Australia
Ethanol Absolute	1009831011	Merck Millipore
Guanidine hydrochloride for molecular biology >=99%	G3272-500g	Sigma Aldrich
2-Propanol for Analysis	1070222511	Merck Millipore
Proteinase K from Tritirachium album lyophilized powder >=30 units/mg protein	P6556-100MG	Sigma Aldrich
Sodium Acetate buffer solution 3 M pH 52 for molecular biology	S7899-500ML	Sigma Aldrich
TE buffer (1X) pH 8.0 low EDTA for molecular biology 500ml	A8569,0500	Panreac AppliChem
TWEEN 20 for molecular biology viscous liquid	P9416-50ML	Sigma Aldrich
Water HPLC Plus	34877-2.5L-M	Sigma Aldrich
High Pure Viral Nucleic Acid Large Volume Kit	05114403001	Roche

MATERIALS TEXT

Lab Equipment
PCR work bench (e.g. AirClean Dead Air PCR Werkbank, 48´´)
UV irradiation box or cross linker (e.g. Vilber Lourmat Bio-Link BLX-254)
Incubator with natural convection (e.g. Thermo Scientific Heratherm General Protocol Inkubator IGS100)
Overhead tube rotator (e.g. Stuart SB2/SB3 Rotator)
Centrifuge 50 ml (e.g. Thermo Scientific Heraeus Megafuge 8)
Centrifuge Rotor 50 ml (e.g. Thermo Scientific TX-400)
Centrifuge 1.5/2.0 ml (e.g. Eppendorf 5424)
Centrifuge Rotor 1.5/2.0ml (e.g. Eppendorf F-45-24-11)
Balance (e.g. Ohaus Adventurer balance AX1502)
Vortex mixer (e.g. Scientific Industries Vortex-Genie® 2)

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Microwave (Optional)
Glass bottle (e.g., 500 ml)

Generic Reagents
Solution of household bleach (2-6% NaClO, then diluted to a working solution concentration of 0.2-0.5% NaClO)
Thermofisher DNA AWAY
Paper towels or tissues

SAFETY WARNINGS

Reagents
Household bleach solution (2-6%) diluted to a working concentration of 0.2-0.5 % NaClO in total
- H290 May be corrosive to metals.
- H314 Causes severe skin burns and eye damage.
- H411 Toxic to aquatic life with long lasting effects.
- EUH206 Warning! Do not use together with other products. May release dangerous gases (chlorine). Remove from surface after recommended incubation time with water-soaked tissue.

DNA AWAY
- H314 Causes severe skin burns and eye damage.

Note: Both bleach solutions and DNA AWAY are used for decontamintation. DNA AWAY is less corrosive than bleach and should be preferred for decontamination of sensitive equipments such as surfaces of electric devices.

GuHCl
- H302 Harmful if swallowed.
- H332 Harmful if inhaled.
- H315 Causes skin irritation.
- H319 Causes serious eye irritation.

Ethanol
- H225 Highly flammable liquid and vapour.
- H319 Causes serious eye irritation.

Isopropanol
- H225 Highly flammable liquid and vapour.
- H319 Causes serious eye irritation.
- H336 May cause drowsiness or dizziness.

EDTA
- H373 May cause damage to organs through prolonged or repeated exposure.

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Proteinase K
- H315 Causes skin irritation.
- H319 Causes serious eye irritation.
- H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled.
- H335 May cause respiratory irritation.

Sodium Acetate
- H139: Causes serious eye irritation

Kits
Check manufacturer's safety information for the High Pure Viral Nucleic Acid Large Volume Kit used in this protocol.

Equipment
UV radiation
- UV radiation can damage eyes and can be carcinogenic in contact with skin. Do not look directly at unshielded UV radiation. Do not expose unprotected skin to UV radiation.
- UV emitters generate ozone during operation. Use only in ventilated rooms.

BEFORE STARTING

Planning
This protocol takes 2 days.

Only the extraction buffer can be prepared within the DNA extraction room. All other home-made buffers must be prepared in a separate dedicated PCR-free ultra-clean room, and we typically UV-irradiate these for 30 min. Purchased kits should be DNA-free.

Check waste disposal guidance for all reagents in this protocol against your corresponding laboratory regulations.

Equipment
Make sure all necessary equipment is available (see Materials).

Abbreviations
EDTA = Ethylenediaminetetraacetic acid
GuHCl = Guanidinium chloride or Guanidine hydrochloride
HPLC = High Performance Liquid Chromatography (-Grade Water)
NaClO = Sodium hypochlorite
TE = Tris-EDTA
TET = Tris-EDTA-Tween (-buffer)
UV = Ultraviolet (radiation)

Samples

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Ensure sample aliquots of 30-50mg of bone powders (in 2ml tubes) are prepared in a dedicated sampling room, prior the day of this protocol.

Controls
Take along a positive control (sample of known performance) and a negative control (tube with HLPC water instead of DNA) in order to assess the performance of the protocol and the level of background contamination. Consider these two extra samples in your calculations for buffer preparations.

Day 1: Binding buffer preparation (Buffer Prep Room)

1 Prepare cleaned workspace with all necessary reagents and equipment.

If lab-wide large-batch pre-prepared reagent stores are used, ensure to have made personal stock-aliquot of reagents such as UV-Water, EDTA, sodium acetate, and proteinase K in amounts sufficient for this extraction.

2 Prepare binding buffer calculating **10 mL / reaction** .

Reagent [Stock Concentration]	Final Concentration	Volume/reaction
GuHCl (1 mol=95.53 g)	5 M	4.77 g
UV HPLC-water up to		6 ml
Isopropanol (100%)	40%	4 ml
Total		**10 ml**

2.1 Weigh GuHCl and transfer into a glass bottle.

If you want to clean the area where GuHCl was used, first use water and then bleach solution. **Do not** use bleach directly as it reacts with GuHCl to produce toxic gases!

2.2 Fill up with UV-irradiated HPLC water to final volume.

This reaction is endothermic and the tube will become very cold. Be aware of the unusual 'slushy' way of dissolving.

2.3 Gently shake horizontally in order to get the salt dissolved. If necessary, apply short (**00:00:10**) bursts in microwave (~400W) keeping the tube slightly unscrewed. Wait until bottle cools down between microwave bursts.

2.4 Pipette isopropanol to reach the final reaction volume (**10 mL**).

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3 Prepare wash buffer by pipetting **40 mL** ethanol to the wash buffer from the High Pure Viral Nucleic Acid kit following manufacturer's instuctions and make an aliquot calculating **900 µl / reaction** .

4 Prepare TET elution buffer by making an aliquot of TE-buffer calculating **100 µl / reaction** and pipette Tween-20 accordingly to reach **0.05 % (v/v)** concentration to make 'TET'.

Because Tween-20 is highly viscous, we dilute it 1:10 in UV-HPLC water, and use this 10% dilution to add Tween-20 to the TE-buffer

5 Irradiate all buffers with UV for **00:30:00** without the lids.

UV irradiation can be done together with solutions from steps 1 (binding buffer), 4 (wash buffer), and 5 (TET buffer).

6 Store binding buffer in a fridge at **4 °C** overnight for day 2.

Label the bottle accordingly with the name, date and for the calculated amount of reactions. Buffer can be stored in a fridge for up to four weeks. Seal bottle with parafilm to avoid evaporation.

7 Dilute proteinase K powder (**100 mg**) to a concentration of **10 mg** / **1 mL** .

Remaining proteinase K solution should be stored in **-20 °C**

Day 1: Extraction Buffer Preparation (DNA Extraction Room)

8 Prepare extraction buffer calculating to a total of **1 mL / reaction** .

Reagent [Stock Concentration]	Final Concentration	Volume/reaction
EDTA [0.5 M]	0.45 M	0.9 ml
UV HPLC water		0.075 ml
Proteinase K [10 mg/ml]	0.25 mg/ml	0.025 ml
Total		**1 ml**

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8.1 Make an aliquot of EDTA ([M] **0.5 Molarity (M)** , pH **8.0**) calculating **0.9 mL / reaction** and irradiate with UV for **00:30:00** .

UV irradiation can be done together with solutions from steps 1 (binding buffer), 4 (wash buffer), and 5 (TET buffer).

8.2 Prepare extraction buffer by following the table (**go to step #8**).

Day 1: Decalcification and Protein Denaturation (DNA Extraction Room)

9 Pipette **1 mL** of from the extraction buffer aliquot to a **30 mg** - **50 mg** aliquot of bone powder.

Preparation of the bone or tooth powder ideally should be done prior to beginning of the extraction protocol in a dedicated sampling room, and stored in a labelled **2 mL** Safe-Lock tube.

10

Seal tubes with Parafilm, rotate **Overnight (12-18h)** with low overhead rotation speed (e.g., 12-16 rpm) at **37 °C** in the incubator.

Post-incubation lysate and pellet can safely be stored in a freezer (**-20 °C**) before isolation and clean-up.

After starting the incubation, we recommend beginning preparations for day 2, such as pre-labelling the falcon tubes that will be used in step 12.

Day 2: DNA isolation and clean-up (DNA Extraction Room)

11 Prepare cleaned workspace with all necessary reagents and equipment.

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12 For each reaction prepare one **50 mL** Falcon tube, one High Pure Extender Assembly (i.e. Falcon tube from kit containing funnel and purification column), two collection tubes from the kit, and one **1.5 mL** LoBind tube for final elution step.

13 In every **50 mL** Falcon tube pipette **10 mL** binding buffer and **400 µl** sodium acetate (UV-irradiated). Mix by inversion and measure pH (should be 5-6).

Add more sodium acetate if the pH is too high. If the pH is too low you can add sodium hydroxide.

14 

Remove parafilm from extraction tubes, then spin the tubes for **00:02:00** at **18500 x g** to pellet bone powder.

If pellet is not solid, repeat centrifugation.

15 Pipette supernatant to matching **50 mL** Falcon tube, mix contents by inversion. If pellet is too fragile, repeat centrifugation before transferring supernatant. Store the bone pellet at **-20 °C** .

16 Pipette binding buffer/extract mix to High Pure Extender Assembly.

17 

Spin at a maximum of **1500 rpm** for **00:08:00** .

This RPM is specific to a 50 ml Thermo Scientific TX-400 Swinging Bucket Rotor. As this is a swing rotor, the rpm value maybe inconsistent for other models. Therefore this value must be adjusted on a per-machine basis. Convert the rpm to rcf (g) and determine the appropriate rpm for your instrument.

You can also turn the tube 180^{o} after **00:04:00** to ensure the liquid does not get stuck on the inner rim of the funnel

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During this centrifugation step, we recommend preparing downstream steps, such as labelling of final elution tubes.

18 Pipette any liquids remaining in the funnel onto the column. Remove funnel from column and insert the column into a fresh collection tube and take off the funnel.

19

Dry spin the column in the collection tube for **00:02:00** at **18500 x g** .

20

Pipette **450 µl** wash buffer from the High Pure Viral Nucleic Acid kit and spin at **8000 x g** for **00:01:00** .

21 Remove column from the collection tube, discard the flow-through and the old collection tube, and put the column into a fresh collection tube.

22

Repeat washing step once (**go to step #19**) reusing the collection tube, and discard flow-through.

Discard flow-through in one of two following ways:

- Remove all liquid in the collection tube with a pipette, or
- Pour off the liquid into a fresh waste tube, and pat the rim of the collection tube dry on a paper tissue or towl. Use just one spot on the paper tissue per sample. Be careful not to touch the rim of the tube on the waste container. Be sure to clean the surface with DNA Away or bleach after discarding the paper.

23

Dry spin at **18500 x g** for **00:01:00**

To ensure the liquid does not get stuck on the inner rim of the funnel, you can optionally spin for **00:00:30** , turn the tube 180^{o}, and dry spin for another **00:00:30**

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Day 2: Elution (DNA Extraction Room)

24 

Transfer column into **1.5 mL** LoBind Tube, pipette **50 µl** of TET to the center of column to elute DNA, incubate for **00:03:00** and spin **00:01:00** at **18500 x g** .

Eluted DNA will be stored in this tube. Label tube on top and side accordingly.

25 Repeat elution step for a total elution volume of **100 µl** TET.

26 Store the DNA extracts at **-20 °C** until use.

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