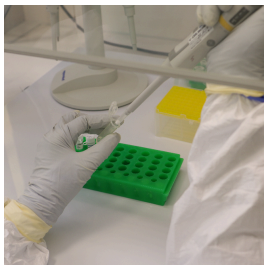
Dec 11, 2020

Ancient DNA Extraction from Skeletal Material



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DOI
dx.doi.org/10.17504/protocols.io.baksicwe

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.baksicwe

External link: https://doi.org/10.1371/journal.pone.0241883

Protocol Citation: Irina Velsko, Eirini Skourtanioti, Guido Brandt 2020. Ancient DNA Extraction from Skeletal Material. **protocols.io** https://dx.doi.org/10.17504/protocols.io.baksicwe

Protocol status: Working
We use this protocol and it's working

Created: December 17, 2019

Last Modified: December 11, 2020

Protocol Integer ID: 31090

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

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**).

Image Attribution

Matthäus Rest

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 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

MATERIALS

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

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)
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.

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 start

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
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.

Note

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.

Safety information

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.

Note

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).

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 [M] 0.05 % (v/v) concentration to make 'TET'.

Note

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.

Note

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.

Note

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 .

Note

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**

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 .

Note

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.

Safety information

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.

Note

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

Note

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.

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).

Note

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.

Note

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 .

Safety information

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.

Note

You can also turn the tube 180° after 00:04:00 to ensure the liquid does not get stuck on the inner rim of the funnel

Note

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.

Note

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

Note

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°, and dry spin for another 00:00:30

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 .

Note

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.