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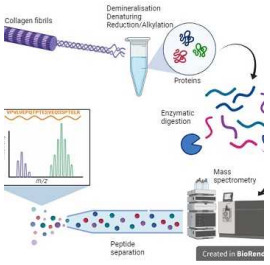
Shotgun proteomics for archaeological bone material - in solution protocol for bone samples from tropical sites



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We use this protocol and it's working

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Abstract

The analysis of bone ancient proteins (palaeoproteomics) is revolutionising the design of research strategies for zooarchaeological assemblages worldwide. By providing means to identify species presence in sites from samples that are not identifiable macroscopically through traditional methods, palaeoproteomics is offering a new exciting approach to understand people's past interactions with their environments.
Nevertheless, this technique is not depleted of challenges. Proteins preserved in ancient bones have undergone several modifications, most of which we do not know yet how they change the organic bone component. As a relatively new technique, much progress is needed in the optimization and development of laboratory protocols tailored to different taphonomic histories.
With a focus in European context, they are currently just few examples of the application of this technique in non-temperate contexts.
This protocol presents a modified version of a common pipeline to prepare bone samples documented in tropical archaeological or palaeontological sites for LC MS/MS analysis. The main differences with other laboratory protocols consist of reducing washings steps, avoiding reduction/alkylation processes, optimizing protein digestion and concentrating eluted samples.
This protocol has been successfully tested at the Australian National University, obtaining positive results for archaeological bone samples from Northwest Australia, Timor-Leste, PNG and Indonesia.

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Guidelines

1. Perform all steps in a standard wet chemistry laboratory, as a minimum biosafety level
2. Wear PPE while conducting all the steps (laboratory coat, gloves, hairnet and safety googles)
3. Be aware of any specific regulation in the handling, storage and disposal of reagents and chemical waste

Materials

- Standard glass bottles in different sizes (500 ml, 250 ml, 100 ml, 50 ml, 20 ml)
- Glass screw top microvials with insert for <2ml samples, e.g. SureSTART 0.3ml Thermo Scientific
- 9mm Screw caps with septum, e.g. SureSTART 9mm Screw Caps Thermo Scientific
- Pipette tips for different voluminal (0.5 µl - 5 ml) e.g. from Corning Deckworks
- Pipettors with different voluminal ranges (0.5- 10 µl, 2-20 µl, 20-200 µl, 100-1000 µl, 0.5-5 ml), e.g. Eppendorf Research® plus
- LoBind Microcentrifuge tubes e.g 1.5 ml and/or 2.0 ml, safe lock, Eppendorf
- Standard tube racks for microcentrifuge
- pH strips, e.g. VMR (ranges from 0-14)
- Scale for laboratory use, e.g. Metler Toledo precision scale
- Centrifuge with a rotor for 1.5 ml/2.0 microcentrifuge tubes, e.g. Eppendorf 5425 with rotor FA-24×2
- Reagent reservoirs, e.g. disposable reagent reservoirs, VWR
- Ultrapure water system, e.g. Elga Purelab Quest, Type 1 water system with UV lamp
- Vaccum centrifuge, e.g. Labconco Centrivap Acid Resistant Centrifugal Vacuum Concentrator
- Incubator, e.g. Eppendorf Thermomixer C
- Clean Spot PCR workstation 240v
- Agate mortar and pestle
- Ultrasonic bath, eg. Vevor 30L

Protocol materials

- Pierce Trypsin Protease **Thermo Fisher Scientific** **Catalog #**90057
- 10% Bleach
- Glacial acetic acid **Fisher Scientific** **Catalog #**A38-500
- Trifluoroacetic acid (TFA) **Bio Basic Inc.** **Catalog #**TC8960.SIZE.100mL
- Formic acid, LC-MS grade **Thermo Fisher Scientific** **Catalog #**28905
- Acetonitrile (ACN) **Fischer Scientific** **Catalog #**10660131
- 70% isopropyl alcohol
- 70% Ethanol
- Hydrochloric acid **Merck MilliporeSigma (Sigma-Aldrich)**
- Ammonium bicarbonate (NH4HCO3) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**AC393212500

Safety warnings

Be aware of your country and facility specific chemical safety guidelines.
This protocol uses several solvents, acids and other chemicals which need special precaution. Please be aware of the international GHS hazard statements (listed below) and follow your country and institute specific precautions/guidelines.

The GHS hazard (H-) and precautionary (P-) statements for the chemicals used in this protocol, are:

Ethanol (for cleaning):

- H225 Highly Flammable liquid and vapor
- H319 Causes serious eye irritation
- P210 Keep away from heat, hot surface, sparks, open flames and other ignition sources. - No smoking)
- P264 Wash ... thoroughly after handling
- P280 Wear protective gloves/protective clothing/eye protection/face protection
- P303+P361+P353 IF ON SKIN (or hair): Take off Immediately all contaminated clothing. Rinse SKIN with water or shower
- P337+P313 IF eye irritation persists: Get medical advice/attention

Isopropyl alcohol (for cleaning):

- H225 Highly Flammable liquid and vapour
- H319 Causes serious eye irritation
- H336 May cause drowsiness or dizziness
- P210 Keep away from heat/sparks/open flames/hot surfaces. — No smoking.
- P261 Avoid breathing dust/fume/gas/mist/vapours/spray.
- P280 Wear protective gloves/protective clothing/eye protection/face protection.
- P303+P361+P353 IF ON SKIN (or hair): Remove/Take off Immediately all contaminated clothing. Rinse SKIN with water/shower.
- P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
- P337+P313 IF eye irritation persists: Get medical advice/attention.
- P370+P378 In case of fire: Use ... for extinction.
- P403+P235 Store in a well-ventilated place. Keep cool.
- P405 Store locked up.

Bleach (for bone decontamination)

- H314 Causes severe skin burns and eye damage.
- H400 Very toxic to aquatic life
- P260 Do not breathe vapour/ spray.
- P264 Wash contaminated skin thoroughly after handling.
- P273 Avoid release to the environment.

- P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.
- P301+P330+P331 IF SWALLOWED: Rinse mouth. Do NOT induce vomiting.
- P303+P361+P353 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/ shower.
- P304+P340 IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.
- P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
- P310 Immediately call a POISON CENTER or doctor/ physician.
- P321 Specific treatment
- P363 Wash contaminated clothing before reuse.
- P391 Collect spillage.
- P405 Store locked up.
- P501 Dispose of contents/ container in accordance with national regulations.

Hydrochloric acid:

- H290 May be corrosive to metals
- H314 Causes severe skin burns and eye damage
- H335 May cause respiratory irritation
- P280 Wear protective gloves/protective clothing/eye protection/face protection
- P301+P330+P331 IF SWALLOWED: Rinse mouth. Do NOT induce vomiting
- P305+ P351+ P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing
- P308+P310 IF exposed or concerned: Immediately call a POISON CENTER or doctor/physician

Ammonium bicarbonate (AmBic):

- H302 Harmful if swallowed
- P264 Wash thoroughly after handling
- P270 Do not eat, drink or smoke when using this product
- P301+P312 IF SWALLOWED: call a POISON CENTER/doctor/... IF you feel unwell
- P330 Rinse mouth

Acetonitrile:

- H225 Highly Flammable liquid and vapor
- H302+H312+H332 Harmful if swallowed, in contact with skin or if inhaled
- H319 Causes serious eye irritation
- P210 Keep away from heat, hot surface, sparks, open flames and other ignition sources. - No smoking
- P280 Wear protective gloves/protective clothing/eye protection/face protection
- P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing
- P403+P235 Store in a well-ventilated place. Keep cool.

Trifluoracetic acid:

- H318 Causes serious eye damage
- H314 Causes severe skin burns and eye damage
- H412 Harmful to aquatic life with long lasting effects
- H332 Harmful if inhaled
- H290 May be corrosive to metals
- P273 Avoid release to the environment
- P280 Wear protective gloves/protective clothing/eye protection/face protection
- P301+P330+P331 IF SWALLOWED: Rinse mouth. Do NOT induce vomiting
- P304+P340 IF INHALED: Remove person to fresh air and keep comfortable for breathing
- P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing
- P310 Immediately call a POISON CENTER or doctor/physician

Formic acid:

- H331 Toxic if inhaled
- H319 Causes serious eye irritation
- H318 Causes serious eye damage
- H315 Causes skin irritation
- H314 Causes severe skin burns and eye damage
- H302 Harmful if swallowed
- H226 Flammable liquid and vapour
- P405 Store locked up.
- P403+P233 Store in a well-ventilated place. Keep container tightly closed.
- P370+P378 In case of fire: Use ... for extinction.
- P337+P313 IF eye irritation persists: Get medical advice/attention.
- P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continuerinsing.
- P303+P361+P353 IF ON SKIN (or hair): Remove/Take off Immediately all contaminated clothing. Rinse SKIN with water/shower.
- P280 Wear protective gloves/protective clothing/eye protection/face protection.
- P260 Do not breathe dust/fume/gas/mist/vapours/spray.
- P210 Keep away from heat/sparks/open flames/hot surfaces. — No smoking.

Trypsin

- 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
- P261 Avoid breathing dust/fume/gas/mist/vapors/spray
- P264 Wash thoroughly after handling
- P271 Use only outdoors or in a well-ventilated area
- P272 Contaminated work clothing should not be allowed out of the workplace
- P280 Wear protective gloves/protective clothing/eye protection/face protection

- P302 + P352 IF ON SKIN: wash with plenty of water
- P304 + P340 IF INHALED: Remove person to fresh air and keep comfortable for breathing
- P305+ P351+ P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing
- P332+P313 IF SKIN irritation occurs: Get medical advice/attention
- P310 Immediately call a POISON CENTER or doctor/physician
- P403+P233 Store in a well-ventilated place. Keep container tightly closed
- P501 Dispose of contents/container to an authorised landfill

Before start

1. Reagents should be prepared and stored in glass labware only. We recommend using the same glass labware for every analysis to avoid external contamination by detergents or other chemicals
2. We recommend preparing reagents just before starting the protocol.
3. Handle reagents under a fume hood only
4. Use ultrapure water for the preparation of reagents
5. Wipe down all working benches with 70% isopropyl alcohol or 70% Ethanol prior to begin working
6. All steps should be performed in a dedicated wet chemistry laboratory. If the presence of human remains is suspected, we recommend sampling in a DNA-free/P3 laboratory

Initial sampling

15m

1 **Sample extraction**

Use a pair of plyers to break apart a bone chip from bone sample or select a small bone fragment

Note

It is recommended to avoid drilling the specimens to obtain bone powder as the high speed of the drill head produces heat that can damage already degraded proteins

Note

To avoid unnecessary damage of archaeological specimens with no preserved proteins, a screening step using ATR-FTIR has been developed and will be published separately

1.1 **Sample cleaning**

Wipe the external surface of the bone with bleach 10% Bleach

Remove the outer layer of the bone sample, either by polishing or manual removal

2 **Sample homogenising**

Grind manually with mortar and pestle until sample is homogenised into a fine powder

3 **Sample weighing**

Weigh out 20 mg - 30 mg bone powder for each technical/biological duplicate

Add bone powder to 1.5 mL LoBind eppendorf tube

Note

It is recommended to process technical duplicates (ideally triplicates) for each of the extracted samples

4 **Decontamination**

15m

Place open tubes under UV light for at least 00:15:00

Acid demineralisation

4d 0h 15m

5 Add 500 µL of freshly made [M] 0.6 Molarity (M) Hydrochloric acid **Merck MilliporeSigma (Sigma-Aldrich)** to each sample

4d

6 Store samples at 4 °C until demineralisation is completed

Note

Demineralisation can take several days. When demineralised, bone becomes spongy and translucent
Monitor the samples to avoid protein loss

Note

If demineralisation stops (no bubbling is visible) but the samples are not fully demineralised, exchange supernatant after centrifugation at 10000 x g, Room temperature, 00:10:00

Note

Prepare laboratory blanks at this step. Blanks should follow the same steps as the archaeological samples

7 When samples are demineralised, centrifuge samples at 10000 x g, Room temperature, 00:10:00 to precipitate the extracted proteins to the base of the tube
Extract supernatant and discard. If the acid soluble fraction wants to be analysed, store supernatant in a new tube at -20 °C

10m

Note

Do not disturb bone pellet during pipetting

8 **Acid washing**

5m

Wash bone pellet with 500 µL of pH 8 [M] 50 millimolar (mM)

Ammonium bicarbonate (NH4HCO3) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**AC393212500

Centrifuge at 10000 x g, Room temperature, 00:05:00 Discard buffer

Repeat the washing step until samples reach neutral pH

Equipment

Centrifuge

NAME	
TYPE	Benchtop Centrifuge
BRAND	Eppendorf
SKU	5405000441
LINK	https://online-shop.eppendorf.us/US-en/Centrifugation-44533/Centrifuges-44534/Centrifuge-5425-PF-243560.html
SPECIFICATIONS	Any benchtop centrifuge will suffice

Protein denaturation

1h 10m

9 **Buffer exchange**

Add 100 µL of [M] 50 millimolar (mM) AmBic to bone pellet

10 **Gelatinization**

1h

Incubate samples for 01:00:00 at 65 °C

Note

Samples will be hot! Take care when handling the samples

Equipment

ThermoMixer	NAME
Benchtop Incubator	TYPE
Eppendorf	BRAND
5382000023	SKU
https://online-shop.eppendorf.us/US-en/Temperature-Control-and-Mixing-44518/Instruments-44519/Eppendorf-ThermoMixerC-PF-19703.html	LINK
Any heat block will suffice	SPECIFICATIONS

11 Centrifuge samples at 10000 x g, Room temperature, 00:10:00 10m

12 Prepare two 1.5 mL LoBind Eppendorf tubes for each sample

Note

Label tubes with sample name/number followed by 'A' and 'B'

13 Transfer 50 µL of supernatant to each of the labelled 1.5 mL LoBind Eppendorf tubes

Note

If required, store samples that will not be use at -20 °C

Protein content quantification

14 To calculate the amount of protease required to successfully digest your sample into peptides, use the protein assay method more suited for your samples and your plate reader

Note

Commercial protein assay kits provide user guides which describe the working procedures specific for each kit. In general, and based in our experience, BCA kits perform well for archaeological samples with low collagen/protein concentrations

Protein digestion (trypsin)

1h

15

Note

There are several available Mass Spectrometry Grade Proteases, each following specific digestion procedures. Here we describe the common protocol used for
Pierce Trypsin Protease **Thermo Fisher Scientific Catalog #**90057

16 Reconstitute lyophilised trypsin with [M] 50 millimolar (mM)
Glacial acetic acid **Fisher Scientific Catalog #**A38-500 to a concentration of
[M] 1 mg/mL

17 Add protease to a ratio of 1:20-1:100

18 Incubate samples Overnight at 400 rpm, 37°C 1h

19 Stop digestion by freezing at -20 °C

20 Concentrate samples to dryness

> Note
>
> Samples can be concentrated in a Speedy Vacuum
> Concentration can take around 06:00:00 , depending on equipment, temperature, volume and other factors. Monitor samples carefully

Peptide purification (C18 tips, Agilent OMIX) 10m

21 **Preparation**
Prepare conditioning, washing and elution solution (see Materials for details on reagents)
Label three 0.5 mL tubes per sample. Each tube will contain conditioning solution (40 µL), washing solution (70 µL) and elution solution (30 µL). Add an additional tube per sample for solvent waste

22 **Pretreat sample** 10m
Resuspend samples in 20 µL [M] 0.1 % (v/v) Trifluoroacetic acid (TFA) **Bio Basic Inc. Catalog #**TC8960.SIZE.100mL .
Vortex thoroughly and sonicate for 00:10:00 to ensure resuspension

23 **Condition tip**
Set pipettor at 10 µL and securely attach the tip
Aspirate 10 µL of conditioning solution and discard solvent. Repeat. Keep pipette plunger depressed.

24 **Equilibrate tip**

Aspirate 10 µL washing solution and discard. Repeat. Keep pipette plunger depressed.

25 **Bind sample**

Aspirate up to 10 µL of pre-treated sample into tip. Dispense and aspirate for up to 10 cycles to improve binding. Dispense sample into a waste tube. Keep pipette plunger depressed

26 **Purify/desalt**

Aspirate 10 µL of washing solution and discard solvent. Repeat 3 times. Keep pipette plunger depressed.

27 **Elute**

Elute with 10 µL [M] 0.1 % (v/v) Formic acid, LC-MS grade **Thermo Fisher Scientific Catalog #**28905 in [M] 75 % (v/v) Acetonitrile (ACN) **Fischer Scientific Catalog #**10660131 .
Dispense in glass vial LC/MS-grade

28 Concentrate samples in Speedy Vacuum until dry

Peptide injection into LC MS/MS

10m

29 Resuspend samples in 30 µL of [M] 5 % (v/v) ACN in [M] 0.1 % (v/v) Formic Acid

30 Sonicate for 00:10:00 and mix thoroughly making sure there are no bubbles on the bottom of the vial insert

10m

Note

Samples are now ready to be injected into the LC MS/MS instrument. If not analysing immediately, store samples in 4 °C (1-2 days) or -20 °C (long storage)