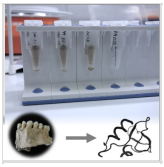

SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus

Nature Communications

Jan 27, 2021

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1 Works for me dx.doi.org/10.17504/protocols.io.bfgrjjv6

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ABSTRACT

Dental calculus from archaeoloigcal samples is now commonly extracted for palaeoproteomic analysis. This protocol combines an EDTA based pretreatment and a SP3 (single-pot, solid-phase-enhanced) protein extraction to prepare samples for LC-MS/MS analysis. This protocol is for moderate to well preserved archaeological calculus samples with a starting weight of 5-15 mg of calculus. We recommend batch sizes of 2-10 calculus samples, 1 extraction control (we use a powdered bone sample from an achaeological sheep bone as this should not be modern material), and 1 extraction blank. For the first time doing the protocol in a lab, we recommend using flanking blanks to identify any contaminants as from handling or from solutions.

This protocol is based upon the original SP3 protocol published in Hughes et al. (2014) and the developments for palaeoproteomics of bone (Cleland et al., 2018). When using please cite the following three publications the protocol is based upon as well as the DOI for this protocol.

Hughes, C. S., Foehr, S., Garfield, D. A., Furlong, E. E., Steinmetz, L. M., & Krijgsveld, J. (2014). Ultrasensitive proteome analysis using paramagnetic bead technology. *Molecular Systems Biology*, *10*, 757. https://doi.org/10.15252/msb.20145625

Hughes, C. S., Moggridge, S., Müller, T., Sorensen, P. H., Morin, G. B., & Krijgsveld, J. (2019). Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. *Nature Protocols*, *14*(1), 68–85. https://doi.org/10.1038/s41596-018-0082-x

Cleland, T. P. (2018). Human Bone Paleoproteomics Utilizing the Single-Pot, Solid-Phase-Enhanced Sample Preparation Method to Maximize Detected Proteins and Reduce Humics. *Journal of Proteome Research*, *17*(11), 3976–3983. https://doi.org/10.1021/acs.jproteome.8b00637

EXTERNAL LINK

https://www.nature.com/articles/s41467-020-20682-3#citeas

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Bleasdale, M; Richter, K.K; Janzen, A; Brown, S; Scott, A; Zech, J; Wilkin, S; Wang, K; Schiffels, S; Desideri, J; Besse, M; Reinold, J; Saad, M; Babiker, H; Power, R.C; Ndiema, E; Ogola, C; Manthi, F.K; Zahir, M; Petraglia, M; Trachsel, C; Nanni, P; Grossmann, J; Hendy, J; Crowther, A; Roberts, P; Goldstein, S.T; Bovin, N. (2020). Ancient proteins provide evidence of dairy consumption in eastern Africa. Under review.

ATTACHMENTS

DA-ProteomicsProtocol_102_DentalCalculus_InternalVersion.pdf

DOI

dx.doi.org/10.17504/protocols.io.bfgrjjv6

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6

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PROTOCOL CITATION

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Bleasdale, M; Richter, K.K; Janzen, A; Brown, S; Scott, A; Zech, J; Wilkin, S; Wang, K; Schiffels, S; Desideri, J; Besse, M; Reinold, J; Saad, M; Babiker, H; Power, R.C; Ndiema, E; Ogola, C; Manthi, F.K; Zahir, M; Petraglia, M; Trachsel, C; Nanni, P; Grossmann, J; Hendy, J; Crowther, A; Roberts, P; Goldstein, S.T; Bovin, N. (2020). Ancient proteins provide evidence of dairy consumption in eastern Africa. Under review.

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KEYWORDS

mass spectrometry, palaeoproteomics, dental calculus, LC-MS/MS, SP3, proteomics, archaeology

LICENSE

IMAGE ATTRIBUTION

Image by Kristine Korzow Richter. Photo of SP3 tubes by Shevan Wilkin. Photo of dental calculus by Ayushi Nayak.

CREATED

Apr 23, 2020

LAST MODIFIED

Jun 29, 2020

PROTOCOL INTEGER ID

36081

GUIDELINES

Perform this protocol only in a clean room laboratory setting with dedicated dead air work stations for:
1. Chemical and buffer preperation
2. Sample preperation and demineralization
3. Sample extraction and purification

Wear clean room clothes, 2 pairs of solvent resistent gloves, a face mask, safety glasses, a hair net and clean room shoes.
Be aware of your specific lab guidelines regarding sample handling and storage.
Be aware of your country and facility specific guidelines regarding the dispose of chemical waste.

MATERIALS TEXT

MATERIALS

Seq Grade Modified Trypsin, 100ug (5 x 20ug) **Promega Catalog #V5111**

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #10977035**

UltraPure 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher Catalog #15575020**

Trifluoracetic acid for HPLC ACROS organics **Fisher Scientific Catalog # Product Code. 11904951**

Acetonitrile ROTISOLV® HPLC Gradient **Carl Roth Catalog #HN44.1**

Methanol ROTIPURAN® ≥999 % p.a. ACS ISO **Carl Roth Catalog #4627.1**

TCEP HCl Biotechnology Grade **VWR international Ltd Catalog #K831-10G**

2-Chloroacetamide for synthesis **Merck Millipore Catalog #802412**

Ammonium hydrogen carbonate 98 % pure ACROS Organics **Fisher Scientific Catalog #10364072**

Ethanol absolute for HPLC Fisher Chemical **Fisher Scientific Catalog #10542382**

Guanidine hydrochloride for molecular biology ≥99% **Sigma Aldrich Catalog #G3272-100G**

Natriumhypochlorit 10 - 15% available chlorine Acros Organics **VWR international Ltd Catalog #ACRO219255000**

AttractSPE™ Disks Bio - C18 - 47mm - 20/pk **Affinisep Catalog #SPE-Disks-Bio-C18-100.47.20**

SpeedBeads™ magnetic carboxylate-modified particles 15 ml **Sigma Aldrich Catalog #GE45152105050250**

SpeedBeads™ magnetic carboxylate-modified particles 15 ml **Sigma Aldrich Catalog #GE65152105050250**

- Standard glass bottles in different sizes (500 ml, 250 ml, 100 ml, 50 ml, 20 ml) made of borosilicate glass, e.g. Laboratory bottles, round, clear, with PP-screw cap and pouring ring, Borosilicate glass 3.3 from VWR + 20 ml brown glass bottle made from borosilicate glass, e.g. DURAN®, borosilicate glass 3.3, brown
- Sterile filter pipette tips for different volumina (0.5 µl - 5 ml) e.g. from STARLAB or Eppendorf
- Pipettes with different volumina ranges (0.5- 10 µl, 2-20 µl, 20-200 µl, 100-1000 µl, 0.5-5 ml), e.g. Eppendorf Research® plus
- 200 µl sterile pipette tips for StageTip preperation e.g. epT.I.P.S. Biopur, sterile
- Homemade tool for StageTip preperation (Punch-Out syringe)
- Microcentrifuge tubes e.g 1.5 ml and/or 2.0 ml, safe lock, Eppendorf
- Black 1.5 ml microcentrifgue tubes, e.g. LightSafe micro centrifuge tubes, conical black polypropylene, Sigma-Aldrich
- 5 ml tubes e.g. Eppendorf Tubes® 5.0 ml with snap cap, sterile
- 15 ml & 50 ml centrifuge tubes: e.g PP-screw cap 50 ml or 15 ml, sterile, SARSTEDT
- Standard tube racks for microcentrifuge, 15 ml and 50 ml tubes
- Dead air working station, e.g. AirClean® Systems dead air box with *UVTect™ Controller*
- Vortexer with an adapter for microcentrifuge tubes, e.g. Vortex Genie 2, neoLab
- pH strips, e.g. MColorpHast (ranges from 0-6, 4-7, 0-14), Merck Millpore
- Magnetic stands for microcentrifuge tubes, e.g MagneSphere® Technology Magnetic Separation Stands, Promega
- Rotators for microcentrifuge tubes, e.g. Mini LabRoller rotator, Sigma-Aldrich

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6

- Mini incubator with a cooling function, e.g. Cooling Incubator INCU-Line® Standard from VWR
- Thermoshaker with heating function incl. heat block adapters for 1.5 ml and/or 2.0 ml microcentrifuge tubes, e.g. Thermoshaker Pro Cell Media + adapter block C (for1.5 ml tubes) and/or heat block D (for 2.0 ml tubes)
- StageTip inserts/holders for 1.5 ml/2.0 ml microcentrifuge tubes e.g. Centrifuge adapters for 10 µl and 200 µl tips, GLscience
- Scale for labarotory use, e.g. Fisherbrand™ analytical scale, FisherScientific
- Centrifuge with a rotor for 1.5 ml/2.0 microcentrifuge tubes, e.g. Eppendorf 5424 with rotor FA-45-24-11
- Cleaning agents: denatured EtOH (e.g. ROTIPURAN® ≥99,8 %, p.a., denatured, Carl Roth) and 5% bleach (e.g. DANKLORIX cleaner/desinfectant).
- Ultrapure water system, e.g. MilliQ Adavantage A10, MerckMillipore

SAFETY WARNINGS

Be aware of your country and facility specific 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/guiedelines.

Please pay attention to TFA and GuHCl (used in this protocol)!

1. Never mix or clean up GuHCl with bleach (Sodium hypochloride). This will create chlorine gas which is highly toxic. and would lead to the need to evacuate your lab immediately.
2. Triflouroacetic acid (TFA) is very hazardous (corrosive)! Handling the stock TFA should only be performed under a fume hood. Always pipette water first, then slowely add the acid! Glass pipettes or syringes, not plastic, should be used to pippette fully concentrated TFA to avoid plastic contamination. Working solutions with TFA for this protocol are 5% of under and can be handled outside of a fumehood and with plastic pippettes.

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

Ethanol:

- 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)

Methanol:

- H225 (Highly Flammable liquid and vapor), H301+H311+H331 (Toxic if swallowed, in contact with skin or if inhaled), H370 (Causes damage to organs)
- P210 (Keep away from heat, hot surface, sparks, open flames and other ignition sources. - No smoking), P270 (Do not eat, drink or smoke when using this product), 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), P304+P340 (IF INHALED: Remove person to fresh air and keep comfortable for breathing), P308+P311 (IF exposed or concerned: Call a POISON CENTER/doctor/...)

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

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rinsing), P310 (Immediately call a POISON CENTER or doctor/physician)

0.5 M Ethylendiaminetetraacetic acid (EDTA):

- Statements not applicable

Tris(2-carboxyethyl)phosphin (TCEP):

- H314 (Causes severe skin burns and eye damage)
- 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)

Chloroacetamide (CAA):

- H361f (Suspected of damaging fertility), H301 (Toxic if swallowed), H317 (May cause an allergic skin reaction)
- P281/Use personal protective equipment as required), P302 + P352 (IF ON SKIN: wash with plenty of water), P308+P310 (IF exposed or concerned: Immediately call a POISON CENTER or doctor/physician), P405 (Store locked up), P501 (Dispose of contents/container to an approved waste management facility)

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)

Guandine hydrocholoride (GuHCl):

- H302+ H332 (Harmful if swallowed or if inhaled), H315 (Causes skin irritation), H319 (Causes serious eye irritation)
- P261 (Avoid breathing dust/fume/gas/mist/vapors/spray), P280 (Wear protective gloves/protective clothing/eye protection/face protection), P301+ P312+ P330 (IF SWALLOWED: call a POISON CENTER/doctor IF you feel unwell and rinse your mouth), P304+ P340+ P312 (IF INHALED: Remove person to fresh air and keep comfortable for breathing and call a POISON CENTER or doctor if you feel unwell), 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)

Sodium hypochlorite (Bleach):

- H290 (May be corrosive to metals), H314 (Causes severe skin burns and eye damage), H400 (Very toxic to aquatic life), H411 (Toxic to aquatic life with long lasting effects)
- 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 or 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), P310 (Immediately call a POISON CENTER or doctor/physician)

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)
- P264 (Wash thoroughly after handling), P280 (Wear protective gloves/protective clothing/eye protection/face protection), P284 ([In case of inadequate ventilation] Wear respiratory protection), P302 + P352 (IF ON SKIN: wash with plenty of water), P305+ P351+ P338 (IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing), P312 (Call a POISON CENTER or doctor/... if you feel unwell)

BEFORE STARTING

We recommend the preperation and storage of chemicals in combusted glass bottles only (e.g. the usage of boron silicate glassware with combustion for 5 hrs at 500 °C) and no long term storage of chemicals and buffers in plastic tubes/containers.
Preperation of buffers and chemicals at least 24 hrs prior to start (please find detailed instrutctions on solution preperation in section 1 of this protocol).
StageTips in this protocol are homemade! Prepare them prior to start your experiment.

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We recommend the following cleaning protocol for surfaces and equipment:

1. 5% bleach (Sodium hypochlorite) (Tools (like saples) should be cleaned with 50% (v/v) technical bleach (10-15% free chlorine available) after their usage)
2. Ultrapure water (e.g. MilliQ water)
3. 70% (v/v) (denatured) Ethanol

Reagent Preparation 4h

1

General Information

1. *We recommend to use combusted glassware for buffers and solutions.*
2. *Solutions should be prepared under a dedicated dead air hood for reagent preparation.*
3. *TFA should be handled only under a fume hood.*
4. *Prepare the Guanidine Hydrochloride solution last!*
5. *All bottle and tubes containing stock solutions shall be labelled with name of the chemical, concentration and the date.*
6. *For buffers and solution preparation we recommend the usage of UltraPure DNase/RNase-Free Distilled Water (see materials list, in this protocol referred to as "molecular grade water").*

2 **Bead Solution** (**20 µg/µl** ; Shelf life: 1 month at **4 °C** , mix before use)

2.1 Carefully invert the bottles of bead stock solutions and knock (!) them firmly on the counter to gain an equal distribution of the beads in the stock solution.

Stock concentration of the bead stock solutions: 50 mg/ml.
Beads tend to be very sticky! Make sure you can visibly see that the beads are no longer stuck to the walls of the bottle before pipetting.

2.2 

Pipette **200 µl** of the hydrophobic and hydrophilic beads each into one microcentrifuge tube (combine them).

2.3 Place the tube on the magnetic rack, remove the stock supernatant and discard.

2.4 

Replace with **1000 µl** molecular grade water and resuspend the beads through up and down pipetting (first wash).

2.5 

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Repeat the wash 2x.

2.6 

After the final wash, add **1000 µl** molecular grade water to the beads and resuspend to mix.

Bead working concentration is now **20 µg/µl**

3 **Reduction and Alkylation solution** (Shelf life of weighed out powder: 6 months at **4 °C**)

3.1 Reduction and alkylation solution is **1 mL** of **100 mM** CAA (2-Chloracetamide), **100 mM** TCEP (Tris(2-carboxyethyl)phosphine) prepared during extraction.To facilitate this, weigh out:

1. **28.66 mg** of TCEP into a 1.5 or 2.0 ml tube
2. **9.35 mg** of CAA into a 1.5 ml black tube as it is slightly light sensitive

4 

6 M Guanidine Hydrochloride (GuHCl) (Shelf life: 3 months at **4 °C**)

Prepare the GuHCl soltution last .

Never mix or clean up GuHCl with bleach (Sodium hypochloride). This will create chlorine gas which is highly toxic and would necessitate the immediate evacuation of your lab.
After preparing the GuHCl solution, wipe down all surfaces with ultrapure water before cleaning the surfaces with bleach.

4.1 Weigh out **28.66 g** GuHCl powder into a 50 ml tube.

4.2 Aliquot out around **40 mL** of water into a 50 ml tube.

4.3 Now mix the GuHCl with **25 mL** molecular grade water and check the volume.

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4.4 Fill up to 50 ml with molecular grade water and transfer the solution to a 50 ml glass bottle for long-term storage.

5 **[M]100 % (v/v) and [M]80 % (v/v) Ethanol** (Shelf Life: 1 month at **4 °C**)

5.1
1. **[M]100 % (v/v)** Ethanol: Aliquot out **50 mL** of HPLC grade Ethanol into a 50 ml bottle.
2. **[M]80 % (v/v)** Ethanol: Add **40 mL** of HPLC grade Ethanol into a 50 ml bottle. Add **10 mL** of molecular grade water.

6 **[M]100 mM Ammonium bicarbonate (AmBic,** pH**8** , Shelf life: 6 months at **4 °C**)

6.1 Weight out **395 mg** of AmBic powder.

6.2 Dissolve AmBiC powder in **50 mL** molecular grade water and mix by shaking gently.

7 

[M]5 % (v/v) Trifluoroacetic acid (TFA) (Shelf Life: 3 months at **Room temperature**)

Triflouroacetic acid (TFA) is very hazardous (corrosive)! Handling should only be performed under a fume hood.
Prepare the solution in a brown glass bottle as TFA is light sensitive.
Always pipette water first, then add the acid slowly!
Repeated used of plastic pipettes in TFA stock solutions causes plastics to build up in the TFA stock solution and can interfere with mass spectrometry, therefore ALWAYS use a glass pipette/syringe for TFA stock solution. Working solutions with TFA for this protocol are 5% and can be handled outside of a fume hood and with plastic pipettes.

7.1 

Dilute **1 mL** TFA stock solution using a glass syringe/pipette (no plastic) in **19 mL** molecular grade water and mix by shaking carefully.

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8 [M]100 % (v/v) MeOH (Shelf life: 1 month at 4 °C)

8.1 Aliquot out 50 mL analysis grade MeOH into a 50 ml bottle.

9 

[M]3 % (v/v) Acetonitrile (ACN), [M]0.1 % (v/v) Trifluoracetic acid (TFA) (Shelf life: 3 Months at 4 °C)

Triflouroacetic acid (TFA) is very hazardous (corrosive)! Handling should only be performed under a fume hood.
Repeated used of plastic pipettes in TFA stock solutions causes plastics to build up in the TFA stock solution and can interfere with mass spectrometry therefore ALWAYS use the glass pipette for the TFA.

9.1 First pour roughly 80 mL molecular grade water into a 100 ml glass bottle.

9.2 

Add 3 mL ACN by pipetting and fill up to 100 mL with molecular grade water.

9.3 

Add 100 µl TFA with a glass syringe/pipette.

10 

[M]60 % (v/v) Acetonitrile (ACN), [M]0.1 % (v/v) TFA (Shelf life: 3 Months at 4 °C)

Triflouroacetic acid (TFA) is very hazardous (corrosive)! Handling should only be performed under a fume hood.
Repeated used of plastic pipettes in TFA stock solutions causes plastics to build up in the TFA stock solution and can interfere with mass spectrometry therefore ALWAYS use the glass pipette for the TFA.

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10.1 First pour **20 mL** molecular grade water in a 50 ml glass bottle.

10.2 Add **30 mL** ACN.

10.3 

Add **50 µl** TFA with a glass syringe/pipette.

11 **Trypsin solution** (Stable once resuspended for 1 month at **-20 °C**)

11.1 

Freeze dried Trypsin (**20 µg** , stored at **-20 °C**) is resuspended in **100 µl** of trypsin resuspension buffer (included in Trypsin Kit, see materials list). The final concentration is now **[M]0.2 µg/µl** .

11.2 Transmit the solution into a labelled 0.5 ml tube and store at **-20 °C** .

Sample Preparation 2h

12 **Weigh out the samples and positive control**

General Information and preparation:
1. *All steps should take place in a dedicated sample preparation hood.*
2. *Wipe down all surfaces (1. 5% (v/v) bleach, 2. ultrapure water, 3. 70% (v/v) Ethanol).*
3. *Alliquot out the reagents you will need (EDTA).*

12.1 Weigh out **5 mg** - **10 mg** calculus in a microcentrifuge tube and record the exact weight.

Calculus needs to be removed from the teeth using a dental scaler prior to analysis.

12.2 Weigh out **2 mg** - **3 mg** of positive control.

We use a powdered archeological sheep bone as positive control. Do not use modern material.

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13

Demineralization

Add **500 µl** of **0.5 M** EDTA (from your aliquot) to each sample and place on the rotator.

Do not forget to prepare an extraction blank tube with EDTA only at this step.

13.1

Samples (including the positive control and extraction blank) should be left on the rotator for 4-7 days at **Room temperature** until the calculus (and bone) samples look visibly demineralised. Your blank should remain clear.

The demineralized calculus can range from being completely invisible to feathery bits floating around in the EDTA. There should be no mineralized chunks remaining. If there are, leave the samples to further demineralize for an extra couple of days.

If the room temperature is > 30 °C or fluxuates greatly, demineralization can be performed at **4 °C** *or* **Room temperature** *in an incubator (see materials list).*

If not immediately proceeding to Day 1 of SP3 (see step 14), samples can be stored in the freezer at **-20 °C** *. Thaw samples completely after freezing before continuing.*

SP3 Protocol Day 1 5h

14

General Information and preperation:

!Start this section only when your samples are adequately demineralized!

1. *All preparations and pretreatment steps should take place in a dedicated extraction hood.*
2. *Wipe down all surfaces (1.Bleach, 2. ultrapure water, 3. 70% (v/v) Ethanol).*
3. *Alliquot out the reagents that you need (6 M GuHCl, 100 mM AmBiC, molecular gerade water, 100% (v/v) Ethanol, 80% (v/v) Ethanol)*

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6

4. *Check if you have prepared Trypsin solution (0.2 µg/µl), working bead solution (20 µg/µl), weighed out CAA/TCEP , StageTips, and clean magnetic racks.*

15 Turn the Thermoshaker to **99 °C** in preparation for the heating step.

16

Remove all samples from the rotator and centrifuge **20.000 x g, Room temperature , 00:10:00** (or max speed) to separate the supernatant from the pellet.

17 During centrifugation, label a new set of microcentrifuge tubes.

These tubes are to collect the EDTA supernatant (see Step 19) to keep as backup. Use the suffix "EDTA sup" for labeling.

18 Prepare the **100 mM** CAA/ **100 mM** TCEP solution.

18.1

Add **1 mL** molecular grade water to the TCEP preweighed tube and vortex until dissolved.

18.2

Transfer the TCEP solution to a black CAA preweighed tube and vortex for **00:01:00** .

19

Remove **400 µl** of the original samples' supernatant and transfer to the tubes labelled with "EDTA Sup." Store at **-20 °C** for backup. You should have **100 µl** and the pellet remaining in the original sample tube.

20

Denaturing and Lysis

Add **200 µl** of the **6 M** GuHCl to each sample and resuspend to mix.

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6

You should see most, if not all, of the pellet disappear. If you have humics, such as dirt or other non-mineralized materials in your sample, these may still be in the pellet and they will be removed in later rinse steps.

21 

Briefly centrifuge the samples to get the liquid to the bottom of the tube.

22 

Reduction and Alkylation

Add **30 µl** of the prepared **100 mM** CAA/ **100 mM** TCEP solution to each tube

Final working concentration should be **10 mM** *CAA/* **10 mM** *TCEP. Adjust if you use a different starting volume.*

23 

Vortex each sample quickly (~ 2 sec) and place the samples in the Thermoshaker for **0 rpm, 99°C, 00:10:00** .

24 While waiting for your samples on the Thermoshaker, label a new set of microcentrifuge tubes.

These tubes are to collect a further supernatant (see Step 32) to keep as backup. Use the suffix "SP3 sup" for labeling.

25 After 10 min of incubation, remove your tubes and give your samples approximately **00:05:00** to cool and turn the Thermoshaker to **24 °C** .

If necessary, you can speed up the cooling process of the Thermoshaker with prepared microcentrifuge tubes of ice.

26 

Briefly centrifuge the samples to get the liquid to the bottom of the tube.

27 

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6

Protein Purification

Add **20 µl** of the working bead solution (**20 µg/µl**) to each tube to begin SP3 clean-up and mix by gentle pipetting.

Ensure complete homogenization of the SP3 beads in the solution. Improper mixing will reduce the protein recovery.

Final bead concentration at this point is **1.14 µg/µl** *. It is recommended that during binding (after the addition of Ethanol) the ratio of beads to protein should be 1:10, but no lower than* **0.5 µg/µl** *. Adjust the amount of bead solution used if you use a different volume or know you have an extremely high protein concentration.*

Once the beads are added, the sample cannot be stored or frozen until after the beads are removed.

27.1

Add **350 µl** of **100 % (v/v)** Ethanol to your sample and briefly shake the tube by hand to mix.

Avoid excessive shaking and do not flick or invert the tube. Both can cause protein loss due to beads sticking to the upper areas of the tube. Complete homogenization is not essential as it will be achieved during the incubation on the Thermoshaker.

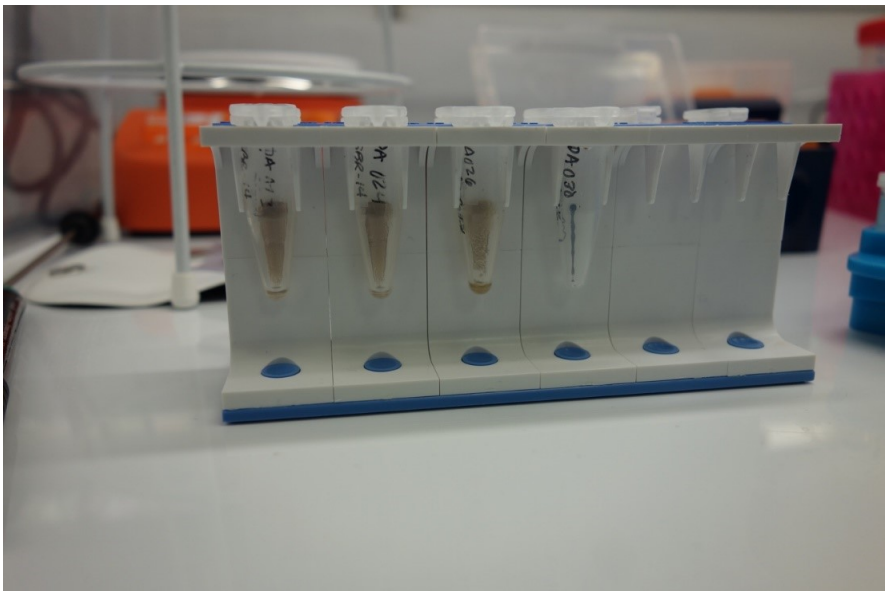
Volume of **100 % (v/v)** *Ethanol should be the same as the volume of solution in the tube. Adjust if you use a different volume.*

27.2

Incubate your samples on the Thermoshaker at **1000 rpm, 24°C, 00:05:00** .

27.3 Place the tubes on a magnetic rack and wait for the beads to migrate to the wall (approx. **00:02:00**).

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6



Samples containing protein will have a fan shaped appearance against the wall of the tube (first three tubes from the left). Blanks and samples with very low protein will appear as a thin line of beads along the wall of the tube (right tube). Photo credit: Shevan Wilkin.

27.4

Remove the sample/Ethanol solution, without disturbing the beads, and place in the tubes labelled “SP3 Sup”. Store at **-20 °C** as a backup.

Set the Thermoshaker to **37 °C** *in preparation for the tryptic digestion (see step 28).*

27.5

Remove the tubes from the magnetic rack and add **200 µl** of **80 % (v/v)** Ethanol and resuspend the beads in the solution to rinse.

Aggressive or excessive pipetting during washing can result in protein loss.

27.6 Put the tubes back on the magnetic rack and wait for the beads to migrate to the magnetic wall. Remove and discard the supernatant without disturbing the beads.

27.7 Repeat **80 % (v/v)** Ethanol wash two additional times for a total of three washes (see step 27.5 and 27.6).

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6

Calculus samples can be extremely "dirty" and the beads may become sticky. If the beads stick to the walls, gently push them with a pipette tip back into the liquid so they can fully migrate to the tube wall which is against the magnetic rack. Each rinse should reduce this "stickiness" and the beads should easily migrate to the wall by the end. There is no harm in performing an extra rinse if needed!

27.8 After the final rinse, be sure to remove as much of the Ethanol supernatant as possible without disturbing the beads.

You should not leave more than **5 µl** *in the tube.*

28

Digestion

Remove the tubes from the magnetic rack and add **75 µl** of **100 mM** AmBic solution to each sample.

Use the tip of a pipette to push any beads down into the AmBic solution, but do not actually pipette/resuspend the beads at this point. Also do not shake, flick or invert the tube.

28.1 Remove the trypsin solution from the freezer for thawing.

Trypsin should be removed just prior to use (leaving enough time to thaw) and then immediately returned to the freezer.

28.2

Incubate the samples in the Thermoshaker for 10 min **750 rpm, 37°C** **OR** sonicate your samples for **00:00:30** at **Room temperature** .

This is to disaggregate the beads and make them more amenable to pipetting.

28.3

Add **1 µl** of the **0.2 µg/µl** trypsin solution to each tube.

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6

This will end up with **0.2 µg** *of trypsin to each sample. To use a different amount, adjust the amount of trypsin accordingly.*

28.4 Mix your sample again through up and down pipetting to ensure an equal distribution of the beads and the trypsin.

28.5

Incubate your samples at the Thermoshaker at **750 rpm, 37°C, 18:00:00** .

Reminder: The samples cannot be stored or frozen until the beads have been removed.

SP3 Protocol Day 2 3h

29

General Information and preperation:

1. *All preparations and pretreatment steps should take place in a dedicated extraction hood.*
2. *Wipe down all surfaces (1. 5% (v/v) bleach, 2. ultrapure water, 3. 70% (v/v) Ethanol).*
3. *Alliquot out the reagents that you need (5% (v/v) TFA, Methanol, 60% (v/v) ACN 0,1% (v/v) TFA, 3% (v/v) ACN 0,1% (v/v) TFA).*
4. *Check if you have prepared StageTips, clean magnetic racks, and clean StageTip holders.*

30

Acidification

Remove the samples from the Thermoshaker and centrifuge them briefly.

30.1 Place the tubes on the magnetic rack and wait until all beads have migrated to the wall.

30.2 As you wait, Label a new set of microcentrifuge tubes with your sample IDs.

These tubes will not be retained after use.

30.3 Transfer the entire sample supernatants to the labelled tubes and discard the tubes with the remaining beads.

30.4

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6

Acidify the samples by adding **10 µl** of **5 % (v/v)** TFA to bring the pH below **2.5**.

30.5 Quickly vortex the sample and test the pH using **1 µl** - **4 µl** of sample on the pH strips.

*If **10 µl** does not lower the pH enough, add an additional **5 µl** of **5 % (v/v)** TFA until the desired pH is reached (< 2,5).*
It is essential to acidify the samples in order to stop the tryptic digestion and to enable the peptides to adhere to the stage tip C18 material.
*Record the total volume of **5 % (v/v)** TFA added to each sample and the resulting pH to keep track of any anomalies.*

31 Peptide Purification/StageTipping

Prepare 2.0 ml tubes by placing stage tip holders into them to allow the StageTips to be inserted. Label a set of stage tips with your sample IDs.

It is necessary to clean the StageTips before loading the samples. Since the waste from cleaning the StageTips will be discarded prior to peptide collection, it is not necessary to label this set of 2.0 ml tubes with your sample IDs.

Be careful when handling the stage tips. Discard any stage tips if the filters are not properly situated in the tips or are disrupted during handling.

31.1 Prepare a second set of 2.0 ml tubes with your samples IDs.

These tubes are to collect the the load and wash flow-throughs during peptide purification (see Step 32) to keep as backup. Use the suffix "L+W" for labeling.

31.2 Add **150 µl** of Methanol (MeOH) to the StageTip. Centrifuge at **2000 x g, Room temperature , 00:01:00** .

Check your StageTips after every centrifugation step to see if all the liquid went through and that the and that the filter is still properly situated in the StageTip. If the filter has become dislodged, discard the StageTip and prepare a new one.

31.3 Add **150 µl** of **60 % (v/v)** ACN, **0.1 % (v/v)** TFA to the tips and centrifuge **2000 x g, Room temperature , 00:01:00** .

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6

Check your StageTips after centrifugation to see if all the liquid went through and that the StageTip is still intact.

31.4 To equilibrate the StageTips, add **150 µl** of **3 % (v/v)** ACN, **0.1 % (v/v)** TFA and centrifuge at **2000 x g, Room temperature , 00:01:00** .

Check your StageTips after centrifugation to see if all the liquid went through and that the StageTip is still intact.

31.5 Repeat the above step (add **150 µl** of **3 % (v/v)** ACN, **0.1 % (v/v)** TFA and centrifuge at **2000 x g, Room temperature , 00:01:00**).

Check your StageTips after centrifugation to see if all the liquid went through and that the StageTip is still intact.

31.6 Carefully transfer the StageTip in its holder to the 2.0 ml tube labelled “Load/Wash” and discard the waste accumulated in the tubes.

31.7 

Centrifuge the tubes containing your acidified samples for a short spin.

If samples contain particles or undigested parts of the pellet at this point, you can spin longer or at higher speeds in order to avoid transferring accumulated particles to the StageTip as these could clog it.

31.8 Place the acidified sample tubes back on the magnetic rack to make sure no remaining beads are transferred to the StageTip.

Avoid transferring any beads or any particulates to the StageTip as these could clog it.

31.9 

Transfer the sample to the StageTip and centrifuge at **2000 x g, Room temperature , 00:03:00** .

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6

Check each StageTip to see if all the liquid has completely passed through. If not, continue centrifugation.

31.10 

Add **150 µl** of **3 % (v/v)** ACN, **0.1 % (v/v)** TFA to the StageTip and centrifuge at **2000 x g, Room temperature , 00:02:00** .

Check each StageTip to see if all the liquid has completely passed through. If not, continue centrifugation.

31.11 Repeat the above step (add **150 µl** of **3 % (v/v)** ACN, **0.1 % (v/v)** TFA to the StageTip and centrifuge at **2000 x g, Room temperature** for **00:02:00**).

Check each Stage tip to see if the liquid has completely passed through! If not, continue centrifugation until all samples have passed the tip.

31.12 Store the "Load/Wash" tubes at **-20 °C** as backup.

31.13 Store the stage tips containing the peptides **-20 °C** until they undergo elution for mass-spetrometric analysis.

If eluting: Transfer StageTip with the holder to a new tube and add **60 % (v/v)** *ACN,* **0.1 % (v/v)** *TFA to the StageTip and centrifuge at* **2000 x g, Room temperature , 00:02:00** .

Citation: Shevan Wilkin, Richard Hagan, Sandra Hebestreit, Madeleine Bleasdale, Ayushi Nayak, Li Tang, Traci N Billings, Nicole Boivin, Kristine Korzow Richter (01/27/2021). SP3 (Single-Pot, Solid-Phase, Sample-Preperation) Protein Extraction for Dental Calculus. https://dx.doi.org/10.17504/protocols.io.bfgrjjv6