Protocol Name/Title: **Historic/Ancient Protein Extraction (SP3)**	
Based on: Hughes et al. 2019	Last Edited Date: 08/15/22

CONTACT: lclark@amnh.org

1P. ANCIENT PROTEIN EXTRACTION

PRINCIPLE:

This protocol is intended to isolate degraded (ancient or historic) proteins. Also called the SP3 protocol, the procedure outline below is based on Hughes et al. 2019 - "Single-pot, solid-phase-enhanced sample preparation for proteomics experiments". SP3 uses a hydrophilic bead-based approach that consists of nonselective protein binding and rinsing steps completed with a purification of proteinaceous material in a final elution step.

CONSUMABLE MATERIALS:

- 1.5 mL Safe-Lock tubes

REAGENTS -

Protein reconstitution -

- BSA
- Triton X-100
- Tween 20
- DTT
- NaCl
- HEPES sodium salt
- Sodium hydroxide
- Iodoacetamide
- SDS
- NP-40
- Deoxycholate
- EDTA
- Glycerol
- NaOH
- cOmplete, EDTA-free protease inhibitor

SP3 processing and protein digestion -

- Sera-Mag SpeedBeads
- Sera-Mag SpeedBeads

- 100% Ethanol
- Trypsin + rLysC mix
- Benzonase Nuclease
- Ammonium bicarbonate
- cOmplete protease inhibitor, EDTA-free

Benchtop Bleach:

For cleaning work surfaces. Approximately 1:5 dilution with distilled water.

TRAINING REQUIRED:

Please see Lauren Clark prior to starting any work detailed in this SOP if you have not completed all of the training listed above.

✋ Indicates gloves should be changed and/or that clean gloves are required.
🚦 Indicates a potential 'break point' in the protocol.

PROCEDURE:

1. Reagent Setup

- HEPES buffer
    - Prepare a 0.2 M HEPES stock solution in water. Adjust the pH to 8 using a 2 N stock solution of NaOH prepared in water. Once prepared, this solution can be stored at room temperature (24 °C) indefinitely.

- Prepare DTT stock solution at a concentration of 100 mM in water.
    - Note: DTT is oxygen sensitive and should be prepared directly before use.

- Prepare IAA stock solution at a concentration of 200 mM in water.
    - Note: IAA is light sensitive and should be prepared directly before use.

- Prepare SDS stock solution at a concentration of 10% (wt/vol) in water. Once prepared, this solution can be stored at room temperature indefinitely.

- Prepare Triton X-100 stock solution at a concentration of 10% (vol/vol) in water. Once prepared, this solution can be stored at room temperature indefinitely.

- Prepare NP-40 stock solution at a concentration of 10% (vol/vol) in water. Once prepared, this solution can be stored at room temperature indefinitely.

- Prepare deoxycholate stock solution at a concentration of 10% (wt/vol) in water. Once prepared, this solution can be stored at room temperature indefinitely.

- Prepare glycerol stock solution at a concentration of 10% (vol/vol) in water. Once prepared, this solution can be stored at room temperature indefinitely.

- Prepare NaCl stock solution at a concentration of 5 M in water. Once prepared, this solution can be stored at room temperature indefinitely.

- Prepare cOmplete protease inhibitor stock by dissolving one tablet in 1 mL of water. Once prepared, this solution can be stored at −20 °C indefinitely.

2. Buffer Preparation

- Reconstitution Solution
    - 50 mM HEPES, pH 8, 1% (wt/vol) SDS, 1% (vol/vol) Triton X-100, 1% (vol/vol) NP-40, 1% (vol/vol) Tween 20, 1% (wt/vol) deoxycholate, 5 mM EDTA, 50 mM NaCl, 1% (vol/vol) glycerol, 1× cOmplete protease inhibitor, and 5 mM DTT
        - This solution should be prepared fresh and stored on wet ice until use

- SP3 bead stock
    - See additional information and tips under **Notes**
    - SP3 is generally performed at a bead/protein ratio of 10:1 (wt/wt)
        i. Place the tube on a magnetic rack until the beads have settled to the tube wall and remove the supernatant.
        ii. Off the magnetic rack, reconstitute the beads in 200 μL of water and pipette-mix.
        iii. Place on the magnetic rack until the beads have settled to the tube wall and remove the supernatant as waste.
        iv. Reconstitute the beads at a suitable working concentration in water (e.g., 50 mg/mL). Prepared bead stocks can be stored at 4 °C for 1 month.

- SP3 rinse solution

- Prepare an 80% solution of ethanol in water. This solution should be prepared fresh weekly and stored at room temperature.

- SP3 digestion solution
  - Digestion solution is 100 mM ammonium bicarbonate, pH 8, in water. This solution should be prepared fresh and kept on wet ice until use.

3. Extraction

Protein Preparation

1. Preheat a ThermoMixer to 60°C

2. Prepare a 1 mL of a stock solution of BSA in a 1.5-mL tube at 1 mg/mL in a reconstitution solution.

3. Heat the prepared BSA stock tube in a ThermoMixer at 60°C for 30 min while mixing at 1,000 rpm

4. Remove the tube from the ThermoMixer and allow it to cool to room temperature in a rack on the lab bench.

5. Alkylate reduced disulfides by adding 100 µL of IAA stock to the BSA tube, and then incubate at room temperature in the dark for 30 min.
   a. IAA is light sensitive and incubations should take place in the dark.

6. Quench the alkylation reaction by adding 50 µL of DTT stock to the BSA tube, and then incubate at room temperature for 15 min in a rack on the lab bench.
   a. The prepared sample can be stored at –80°C indefinitely.

SP3 protein cleanup and digestion

7. Precool the ThermoMixer to 24 °C.

8. Dilute 10 µg (10 µL of the prepared 1 mg/mL stock) of the prepared BSA to a final volume of 48 µL in reconstitution solution.

9. Add 100 µg of prepared SP3 beads and pipette-mix to homogenize the solution. This will be 2 µL of a prepared 50 µg/µL SP3 bead stock, giving a final total volume of 50 µL of protein solution.

a. Ensure mixing of beads with sample by gently pipetting the mixture up and down.

10. To induce binding of the proteins to the beads, add 50 µL of ethanol to the BSA mixture containing the SP3 beads. Briefly shake the tube to homogenize.
    a. Do NOT finger flick/vortex the sample and avoid any excessive shaking after adding ethanol.

11. Incubate the binding mixture in a ThermoMixer at 24 °C for 5 min at 1,000 rpm.
    a. Avoid mixing at >1,000 rpm.
    b. Look for 'clumping' of the beads which indicates that proteins are binding.

12. After the binding is complete, place the tube in a magnetic rack and incubate it until the beads have migrated to the tube wall.
    a. Look for beads binding to the wall of the tube.

13. Remove and discard the unbound supernatant in an appropriate waste container.
    a. Be careful not to disturb the magnetic beads when removing the supernatant.

14. Remove the tube from the magnetic rack, and add 180 µL of 80% ethanol SP3 rinse solution and GENTLY pipette-mix three to four times to reconstitute and rinse the beads

15. Place the tube on the magnetic rack and incubate until the beads have migrated to the tube wall.

16. Remove the supernatant, taking care not to disrupt the beads.
    a. An SDS-page assay can be used to visualize and troubleshoot lack of protein binding.

17. Repeat Steps 12–16 two further times to completely rinse the proteins bound to the SP3 beads.
    a. Upon the final rinse, be sure to remove as much liquid as possible in order to avoid carryover to enzyme digestion.

18. Remove the tube from the magnetic rack and add 100 µL of digestion solution containing 0.4 µg of trypsin + rLysC mix.
    a. Do NOT pipette to mix
    b. Amount of trypsin can vary depending on sample type.

19. Using a micropipette with a 200-µL tip, gently push the beads that are not covered by liquid along the tube wall into the digestion solution. Do not attempt to pipette the mixture.
    a. If necessary, very GENTLY shake the tube, but do NOT flick.

20. If available, sonicate for 30 s in a water bath to fully disaggregate the beads. If a sonicating water bath is not available, incubate the tube at 37 °C in a ThermoMixer for 10 min at 1,000 rpm mixing.

21. Pipette-mix to ensure proper reconstitution of the beads and incubate for 18 h at 37 °C in a ThermoMixer at 1,000 rpm mixing.
    a. Incubation can be shorter than 18h depending on sample type.

22. After the digestion is complete, centrifuge the tube at 20,000*g* at 24 °C for 1 min.

23. Place the tube on a magnetic rack until the beads have settled onto the tube wall and remove the supernatant to a fresh tube.
    a. Be sure not to remove the beads when discarding the supernatant.
    b. Do NOT freeze the sample if it contains beads.

24. Proceed with MS analysis of the peptide sample.

4. Notes

- In this protocol example, we are going to process 10 µg of protein and therefore need 100 µg of beads (2 µL from each of the 50 mg/mL vendor stocks).
- SP3 is generally performed at a bead/protein ratio of 10:1 (wt/wt), with a minimum volumetric concentration of 0.5 µg/µL.
- Based on the concentration of your bead solution, take the required amount of the two vendor bead stocks and combine them in a single tube.