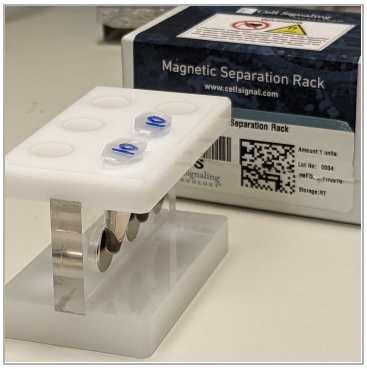

MAY 10, 2023

DOI:
dx.doi.org/10.17504/protocols.io.261ge3zeyl47/v1

Protocol Citation: ronan.ocualain 2023. Manual SP3 digestion and clean-up of protein lysates. **protocols.io** https://dx.doi.org/10.17504/protocols.io.261ge3zeyl47/v1

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

Created: May 05, 2023

Last Modified: May 10, 2023

PROTOCOL integer ID: 81454

Manual SP3 digestion and clean-up of protein lysates

ronan.ocualain[1]

[1]University of Manchester

protocols.io Ambassadors

BioMS CRF, UoM

ronan.ocualain

ABSTRACT

SP3 bead preparation of samples for MS analysis
Compatible with a wide range of buffer and detergent types.

GUIDELINES

pH: SP3 works in the pH range of pH 7.0 to pH 8.5.
Protein samples should be ultrasonicated to remove nucleic acid. DNA if in sample will coat the SP3 beads, causing their aggregation, and is best avoided. If ultrasonication is not available, use Benzonase to shear DNA.
Bead concentration during binding: A bead to protein ratio of 5:1 to 10:1 is recommended. For example, if processing 20 µg of protein, add
Sample concentration: The binding capacity of SP3 beads provides a flexible clean-up format across a range of protein and peptide concentrations (10 µg/mL to 5 mg/mL), as long as the concentration of beads is adjusted as described above.
Sample handling: The use of 200 µL gel loading pipette tips for sample aspiration is recommended. Care should be taken to gently aspirate supernatant to avoid dislodging protein or peptide-bound beads from the magnet.
QC: Aliquots of the load, flow-through, washes, and bound bead: sample may be processed by SDS PAGE and visualised with Colloidal Coomassie or SIlver staining to determine binding efficiency of the sample to beads, and if wash steps are sufficient.

MATERIALS

- Protein lysates, reduced and alkylated, and protein concentration estimated, in 5% SDS lysis buffer in 50mM TEAB.
- SDS lysis buffer - Sodium dodecyl sulfate (SDS) (Sigma-Aldrich Cat. #L6026) in 100 mM TEAB (SIgma-LAdrich Cat. # T7408), pH 7.5 – prepare 10% stock solution and store at room temperature for up to 2 years.

SP3 protein cleanup and enzymatic digestion
·Cytiva SpeedBeads (Cat. #GE45152105050250
and
Cytiva SpeedBeads (Cat. #GE65152105050250).
·Ethanol (EtOH) HPLC/Spectrophotomeric grade 200 proof (Sigma-Aldrich Cat. #459828).
·Trypsin (Sequencing Grade, Promega Cat. #V5113).
·Ammonium Bicarbonate (AmBic) (Sigma Aldrich BioUltra - > 99.5% Cat. #09830) – prepare 100 mM buffer fresh.
·Ultrapure filtered water, or LC-MS grade water.

Equipment
·Magnetic stand such as the Cell Signalling 7017S 6-tube magnetic separation rack for 1.5 mL microcentrifuge tubes.
·Bath sonicator.
·Microcentrifuge tubes that demonstrate low binding of proteins and peptides and that can also tolerate high organic solvent concentrations without leaching plasticizers. Sarstedt™ 1.5 mL screw cap microcentrifuge tubes (Cat. #72.692) were used for all steps in this protocol.
·Heater / Mixer platform (e.g. Eppendorf™ thermomixer).
·Centrifuge for pelleting cellular debris in the range of 14000 x g e.g. Eppendorf 5425 or equivalent.
·pH paper strips e.g. GE healthcare Whatman pH strips pH 0–14 (#11375254) for checking sample pH throughout the protocol.

SAFETY WARNINGS

Please refer to the copies of Risk Assessment Forms held in both B2071 and B2075 for hazards to health, and other identified hazards and risks, associated with the use of this protocol before starting.

BEFORE START INSTRUCTIONS

Bead preparation:
Both types of Cytiva Carboxylate SpeedBeads are shipped at 50 mg/mL concentration (5% solids) in water with 0.05% sodium azide. It is a good idea to combine the beads and aliquot them for long term storage at 4 °C . Preparing aliquots of stock beads avoids excess handling of the main bottles and minimizes the risk of contamination.

To do this: Let both stock beads equilibrate to room temperature for 30 minutes. If the beads have settled during storage they should be resuspended by inversion or gentle vortexing until no solid bead mass is visible at the bottom of the bottle. Combine both types of beads into a 1:1 ratio, and aliquot into 10 mg , 50 mg , and 100 mg volumes, store at 4 °C until further use.

SP3 bead preparation

2m 10s

1 Briefly vortex the **1:1 bead** mixture and place the tube on a magnetic stand for two minutes to collect the beads.

2 Add ultrapure water at a volume corresponding to **5 to 10 times** the initial volume of mixed beads. 2m 10s
Vortex the beads for 00:00:10 and place on a magnetic stand for 00:02:00 to collect the beads. Carefully aspirate and discard the wash buffer with a gel loading tip.

3 Repeat the wash steps a further two times.

4 Resuspend the beads with ultrapure water at a final concentration of [M] 10 µg/µL .

5 Washed beads may be stored at 4 °C for up to one month.

SP3 protein clean-up

2m 10s

6 Check the pH of the sample is in the range of pH 7.0 to pH 8.5 for optimal binding by measuring an aliquot on pH paper.

7 Add washed beads (prepared as above) to the samples in a ratio of 5-10 µg of beads to 1 µg of protein and briefly vortex.

8 Immediately add a volume of [M] 100 % (v/v) ethanol to the samples to obtain a **50%** final concentration to initiate protein binding to the beads.

9 Vortex the samples to mix but ensure that beads are not stuck on the sides of the tube.

> Note
>
> The protein-bead mixture will be sticky at this stage. Avoid touching the beads to minimize sample loss.

10 Incubate the samples on a room temperature mixer platform for 00:10:00 at 1000 rpm 10m

11 Remove the samples from the mixer, centrifuge them for 00:00:02 and place them on the magnetic stand for 00:02:00 . 2m 2s

12 Transfer the supernatants to a clean Sarstedt tube. This is the **"flow-through"** fraction.

13 Wash the beads by adding a volume of [M] 80 % (v/v) ethanol corresponding to at least twice the initial sample volume and vortex for 00:00:30 — 30s

14 Centrifuge the samples briefly for two seconds on a mini centrifuge and place back on the magnetic stand for 00:02:00 — 2m

15 Remove the supernatants and save in a separate Sarstedt tubes. Label these **wash 01.**

16 Repeat the wash steps a further **three** times, and transfer the supernatants to clean Sarstedt tubes, labelled **wash 02**, **wash 03**, and wash 04. — 2m
For the final wash (wash 04) - perform this by transferring the resuspended beads in [M] 80 % (v/v) ethanol to a new, labelled tube. Stand for 00:02:00 on a magnetic rack, and remove the supernatant, the beads are now ready for digestion.
(This is a critical step, because residual detergent on the sides of the tube and even beads may be transferred to the downstream steps).

17 After the final wash, air dry the beads for 00:00:30 to remove as much ethanol as possible. — 30s

SP3 digestion — 2m 10s

18 Resuspend the beads in 25 µL of [M] 100 millimolar (mM) ammonium bicarbonate buffer
(This is a critical step, because residual detergent on the sides of the tube and even beads may be transferred to the downstream steps).

19 Do not vortex the beads at this stage. Instead, place the tubes on a floating rack and sonicate in a water batch for 00:02:00 to resuspend them. — 2m

20 Add Promega™ sequencing grade trypsin in a 1:20 enzyme-to substrate ratio to each sample, and digest at 37 °C Overnight on an Eppendorf thermomixer. 2m

21 After 16 to 18 hours, add the tubes to a magnetic stand for 00:02:00 . Carefully remove the supernatant, and transfer to a clean, labelled tube. Add an additional 60 µL volume of [M] 100 millimolar (mM) ammonium bicarbonate buffer to the beads, briefly vortex, and allow to stand for 00:02:00 on a magnetic rack. Carefully transfer the supernatant to the same labelled sample tube, to create a pooled sample. 4m

22 Centrifuge the peptide for 10 minutes at 14000 x g, and proceed to R3 desalting (see https://www.protocols.io/view/96-well-plate-r3-desalt-and-clean-up-protocol-for-dm6gpbnqdlzp/v1)