from Johnston et al. (2022) https://doi.org/10.1021/acs.analchem.1c04200

SP4 (Solvent precipitation SP3) protocol

Glass bead preparation (optional):

- 9–13 *μ*m glass spheres/beads
  (e.g., https://www.sigmaaldrich.com/catalog/product/aldrich/440345)
  Glass beads broadly improved recovery, digestion efficiency and reproducibility, but are not required
- Suspend 100 mg in 1 mL of Ultrapure water, vortex until suspended fully, and pellet at > 500*g* for 1 min.
  Of note: approximately 50% of the beads are buoyant, and will not pellet, and should be removed over the course of these wash steps. Additionally, small amounts of metal in the beads can be removed by magnet or acid wash but had no effect on the performance of the beads. Larger scale preps are possible but may require additional washes due to buoyant beads.
- Resuspend, vortex and wash with ≥ 1 mL of: 100% acetonitrile (ACN) (1×), 100 mM ABC* (1×), and Ultrapure water (≥ 2×) ensuring no unpelleted beads remain. * or equivalent digestion buffer.

Then either:

- **A.** Resuspend beads in 0.9 mL of Ultrapure water to 50 mg/mL.
  given ~50% of beads are retained

or

- **B.** Resuspend beads in 0.9 mL acetonitrile to 50 mg/mL (recommended).
  - Avoids protein dilution from beads in water.
  - Ensures uniform bead dispersion
  - Dilute beads to at least 2.5× [protein] (so bead:protein is 10:1 from 4 volumes of bead–ACN suspension)

This will be sufficient to prepare 50 mg of protein—excess can be stored at 4 °C.
(with 0.2% sodium azide, if in water for an extended period)

Lysate/protein solution prep recommendations

- SP4 is broadly compatible with the majority of lysis buffers as for SP3 or acetone precipitation
  Tested with:
  - 5% total detergent ‘SP3 lysis buffer’
    - (50 mM HEPES pH 8, 1% SDS, 1% Triton X-100, 1% IGEPAL CA-630, 1% Tween 20, 1% sodium deoxycholate, 50 mM NaCl, 5 mM EDTA, 1% (v/v) glycerol, and 1× protease inhibitors)
  - 8 M urea (diluted to 2 M prior to ACN addition)
  - TFA/Tris diluted 1:1 with water as described for the ‘SPEED’ method
- For best results with SP4, protein concentration should be as high as possible (0.25–5 *μ*g/*μ*L).
  - For lower concentrations or where highest possible recovery is required, longer precipitation reactions, carboxylate-modified beads, pre-chilled ACN, and centrifugation at 4 °C may help yields.
- DNA shearing (e.g., by sonication), protease inhibitors, & lysate clearance are recommended.

SP4 protocol recommendations

- The use of the smallest possible tube will help create a denser pellet, e.g., 500 *μ*L tube for samples of less than 50 *μ*L.
- Liquids should be kept low in the tube, with losses/contamination possible from tube walls/lid.
- Set vortex to < 500 rpm for very gentle mixing.
- Pipette ACN directly into the sample to ensure rapid mixing, but do not touch the ACN–sample mix with the tip.
- Use the tube hinge to orientate the location of the pellet (fixed angle rotors).
  - Initially orientate the tube hinge inwards during the pellet precipitation and turn 180° after 2.5 min will give a denser pellet and less risk of loss from fragile wall adhesion.
- During wash removals, avoid touching the tube walls with the tip as precipitation may occur on them, pipette slowly and avoid agitating the pellet.
- If adding beads, ensure they maintain a uniform suspension in water/ACN by pipetting up and down at least once between additions.
- Organic solvent for aggregation/washes appears interchangeable between ethanol, ACN, IPA, & acetone (Ref. 2).

SP4 Protocol

1. Aliquot reduced/alkylated protein mixture/lysate into a fresh LoBind-type microcentrifuge tube.
    - Volumes and conditions are given for the example of 10 μg protein in 10 μL of 1 μg/μL lysate.
2. Options (choose one):

2a. Bead-free

- Add 4 volumes of ACN.
    - E.g., 40 μL for 10 μL sample

2b. Glass beads (in water)

- Add 50 μg/μL beads (water-suspended) at 10:1 beads:protein and vortex.
    - E.g., 100 μg (2 μL) beads
- Add 4 volumes of ACN
    - E.g., 48 μL to 12 μL sample:bead mix

2c. Glass beads (in ACN) (recommended)

- Adjust beads to 2.5× protein concentration.
    - E.g., 2.5 μg/μL for 1 μg/μL sample
- Add 4 volumes of this ACN:bead suspension.
    - E.g., 40 μL for 10 μL sample

3. Ensure complete mixing (without pipette mixing, e.g., by consistent ACN addition, or < 500 rpm vortex for 5 s).
4. Centrifuge for 5 min at 500–16,000*g*.
5. Remove supernatant by pipetting slowly and remove a consistent volume of 90–95%. Avoid disturbing beads/pellet.

    E.g., for a 50 μL total precipitation reaction remove 45 μL

6. Wash with 80% ethanol, volume ≥ 1.5× total precipitation volume (or at least 180 μL)
    - Pipette gently down the side opposite the hinge/pellet to avoid disturbance, do not vortex/resuspend.
7. Centrifuge for 2 min at 16,000*g*.
8. Remove 90–95% of wash.

    E.g., leaving ~5–10 μL during washes

9. Repeat wash steps for a total of 3 washes.
10. Remove >= 95% of final wash.
    - For larger volumes a final 2 min spin will help with removal of excess wash.

    E.g., leaving < 5 μL after final wash aspiration

11. Add preferred digestion buffer, e.g., 20–100 mM ABC or TEAB (pipette mixing will cause losses)
12. Add preferred digestion enzyme, e.g., trypsin/Lys-C at a 1:10 to 1:100 enzyme:protein ratio.
    - A digestion buffer/enzyme master mix will reduce variability and simplify pipetting—keep on ice.
    - Use a volume equivalent to ~0.5–2× the total precipitation volume.

      E.g., 25–100 μL for 50 μL precipitation reaction
    - In-bath sonication (5–10 min) can help to disrupt the pellet and increase surface area.
    - Larger bead-free pellets may require additional agitation to resuspend but keep sample low in tube.
    - 18 h digestion consistently worked without pellet resuspension for < 25 μg protein.
13. Incubate in a Thermomixer at 1000 rpm at desired conditions, e.g., for 18 h at 37 °C.
    - Beads were compatible with 2 h @ 47 °C using 1:10 trypsin or 2 h @ 70 °C (rapid digestion buffer), ensuring resuspension

Peptide collection

- Centrifuge the peptide mixture at 500–16,000*g* for 2 min & collect peptide supernatant.
- For maximum recovery, rinse pellet/tube in an equal volume of digestion buffer added above.
    - A final centrifugation step may be required to ensure no beads are carried over.
- Peptides solution at this stage is clean enough to be:
    - Acidified (e.g., by 0.1–1% formic acid or trifluoroacetic acid) for direct LC-MS injection.
    - Dried by vacuum concentration to provide near-pure peptides.

References

This protocol was originally published in our article:
Johnston HE, Yadav K, Kirkpatrick JM, Biggs GS, Oxley D, Kramer HB, Samant RS. Solvent Precipitation SP3 (SP4) enhances recovery for proteomics samples preparation without magnetic beads. *Anal Chem.* 2022; 94(29): 10230–8. doi:10.1016/j.mcpro.2022.100485

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