In-gel SP3 processing

This document describes a protocol for processing of in-gel samples using a simplified SP3 protocol.

Quick links

1. Protein Sample Preparation

For all the solutions described below and throughout this protocol in general, you should do your best to use sterile glassware and reagents. As the goal here is to measure proteins, make an effort to minimize potential keratin contamination (e.g. wear a lab coat and don't touch your tubes with un-gloved hands).

1.1 Reagents and materials

- 1.5mL or 2.0mL Safe-Lock tubes (Thermo Scientific, CAT#05-402-25 or CAT#05-402-7)
- Benchtop centrifuge with holder for microcentrifuge tubes (multiple vendors)
- 1M TrisCl pH 7.5 (Thermo Scientific, CAT#J60636.K2)
- MgCl2 (Sigma, CAT#M8266)
- KCl (Sigma, CAT#P9333)
- NP-40 (Sigma, CAT#NP40)
- Deoxycholate (DOC) (Sigma, CAT#D6750)
- 20% SDS (Thermo Scientific, CAT#BP1311)
- cOmplete protease inhibitor tablets, EDTA free (Sigma, CAT#11836170001)
- TCEP (Sigma, CAT#580560)
- Chloroacetamide (Sigma, CAT#C0267)
- Ammonium bicarbonate (Sigma, CAT#A6141-500G)
- Urea (Sigma, CAT#U5128)
- Clean water (Thermo Scientific, CAT#10977023)
- Trypsin/rLysC mix (Promega, CAT#V5071)
- Thermomixer with 1.5mL tube block (Eppendorf)
- Thermoblock set to 60C (multiple vendors)
- Magnetic rack for 1.5mL or 2.0mL tubes (I like this rack: Promega, CAT#Z5342)
- Ethanol, absolute (whatever vendor sells this to your lab)
- SP3 beads (Sigma, CAT#65152105050250, CAT#45152105050250)
- Acetone (Sigma, CAT#179124)
- Water, HPLC grade (CAT#51140, Thermo Scientific)
- Trifluoroacetic acid, HPLC grade (CAT#85183, Thermo Scientific)

1.2 Solution recipes

- 100mM TrisCl pH 7.5 - 10uL of 1M stock for every 90uL of water (stock, stable at room temperature indefinitely)
- 2M KCl - 745mg in 5mL of water (stock, stable at room temperature indefinitely)
- 0.5M MgCl2 - 508mg in 5mL of water (stock, stable at room temperature indefinitely)
- 10% (v/v) NP-40 - 10uL for every 90uL of water (stock, stable at room temperature indefinitely)
- 10% (w/v) DOC - 500mg in 5mL of water (stock, stable at room temperature indefinitely)
- 10X cOmplete protease inhibitor stock - 1 tablet in 1mL of water (store at -80C)
- 2M urea (120mg for every 100uL of 100mM TrisCl pH 7.5, prepare just before use)
- Lysis buffer A (need 200uL per sample, recipe for 1mL)
    - 20mM TrisCl pH 7.5 (200uL of 100mM stock)
    - 150mM KCl (76uL of 2M stock)
    - 5mM MgCl2 (10uL of 0.5M stock)
    - 0.5% (v/v) NP-40 (50uL of 10% stock)
    - 0.5% (v/v) DOC (50uL of 10% stock)
    - 0.5X cOmplete protease inhibitor (50uL of 10X stock)
    - water to 1mL (565uL of clean water)
- Lysis buffer B (need 50uL per sample, recipe for 1mL)
    - 600mM TrisCl pH 7.5 (600uL of 100mM stock)
    - 8% (v/v) SDS (400uL of 20% stock)
- TC solution (need 40uL per sample, recipe for 500uL)
    - 6.2mg TCEP
    - 9.3mg of CAA
    - water to 500uL
- Ammonium bicarbonate solution (need 400uL per sample, recipe for 5mL)
    - 395mg ammonium bicarbonate
    - water to 5mL
- 80% (v/v) ethanol (prepare fresh)
- 10% (v/v) trifluoroacetic acid (TFA) in water

1.3 Protocol

This protocol assumes you have run an SDS-PAGE gel and have stained and cut out your bands of interest. Your gel bands should be in a 1.5mL tube to start this protocol.

1. If there is liquid in the tubes with the gel pieces, remove it to waste. Freeze the gel pieces in their 1.5mL tubes.
2. Using a plastic pestle, crush the gel pieces in the 1.5mL tube.
3. Add 75uL of Lysis buffer A to the tubes with the gel pieces followed by 25uL of Lysis buffer B.
4. Incubate at +60C for 5-minutes.
5. Add 20uL of TC solution and incubate at +30C for 30-minutes.
6. To perform clean-up prior to digestion, prepare the SP3 beads stock (if you don't have an existing stock, otherwise skip to Step 7):
    1. Vortex the two 15mL containers of bead stocks from the manufacturer to re-suspend the material.
    2. Take 500uL of each of the bead stocks and combine in a fresh 2mL tube.
    3. Place on a magnetic rack and wait for beads to settle. Discard the supernatant.

    4. Reconstitute the beads in 1mL of water with pipetting and place back on the magnetic rack. After the beads settle, discard the supernatant.
    5. Repeat the above rinse one additional time for a total of 2 rinses.
    6. Reconstitute the beads in 500uL of water. This is your bead stock and it can be stored at +4C indefinitely.
7. Add 10uL of SP3 bead stock to the 1.5mL tube with the gel pieces.
8. Add 400uL of acetone to each tube and gently shake the tube to mix. Incubate at +24C for 5-minutes.
9. During the above incubation, prepare your digestion mixture:
    1. Reconstitute urea powder with an appropriate amount of 1M ammonium bicarbonate solution. Vortex mix. NOTE - urea will increase the volume of the solution generally by the amount of powder you have weighed out. For example, to 120mg of urea, I would add 880uL of 1M ammonium bicarbonate solution to make 1mL of solution.
    2. Reconstitute a 20ug trypsin/rLysC vial using 100uL of the provided reconstitution solution.
    3. Transfer 4uL of trypsin per sample to a fresh tube, and add 100uL of prepared urea per sample. Vortex mix and keep on ice.
10. Centrifuge the protein lysate tubes at 5,000g for 5-minutes at room temperature.
11. Discard the supernatant from each tube and add 800uL of 80% ethanol. Pipette mix to reconstitute the beads.
12. Centrifuge the tubes at 5,000g for 5-minutes and discard the supernatant. Using a P200 or P20 micropipette, make sure you have removed as much leftover liquid from the rinse as possible from the tubes.
13. Add 100uL of the prepared trypsin/urea mix to each tube. Do not attempt to mix, vortex, or pipette.
14. Transfer the tubes to a shaking Thermomixer set at +30C and 800rpm, and digest for 2-4 hours.
15. Add 300uL of 1M ammonium bicarbonate solution to each tube and place back in the Thermomixer and incubate overnight at +30C and 1,000rpm mixing.
16. The next day, spin the tubes at 12,000g for 2-minutes and then place on a magnetic rack to recover the supernatant to a fresh 1.5mL tube.
17. Add 40uL of a 10% (v/v) TFA solution to acidify the peptide mixture.
18. At this point, the samples should be desalted using a standard protocol, such as StageTips.