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SP3 protein cleanup - digestion w/ rapizyme and ACN - SP3 peptide cleanup

Forked from a private protocol

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Protocol status: Working
Main workhorse protocol used in our proteomics lab since 2023.

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Abstract

This procedure takes as input samples (cells, tissue, tumor piece, biopsy, etc) previously lysed in SDS-based lysis buffer (4% (w/v) SDS, 25 mM HEPES pH 7.6, 1mM DTT) and with DNA already sheared, and it delivers tryptic peptides in water. It consists of protein clean-up with sp3-beads, denaturation, reduction and alkylation of cysteines, followed by digestion of proteins to peptides by trypsin (rapizyme from Waters), while using 10% ACN instead of urea during digestion, and ends with peptide level sp3 cleanup.

Materials

- **Reagents and solvents:**

DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D0632 or

DTT (DL-Dithiothreitol) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**43819

DL-Dithiothreitol, threo-1,4-Dimercapto-2,3-butanediol
CAS No.: 3483-12-3
Molecular Weight: 154.25 g/mol

- Use powder, molecular biology grade or similar (e.g., Sigma-Aldrich Cat. No. **43819**, ≥99% (RT)) to prepare 1M aqueous stock solution; aliquots can be prepared and frozen

2-Chloroacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**C0267

CAS No.: 79-07-2
Molecular Weight: 93.51 g/mol

- Use crystalline powder (e.g., Sigma-Aldrich Cat. No. **C0267**, ≥98%) to prepare fresh aqueous solution immediately before use

Gibco™ HEPES (1M) **Fisher Scientific Catalog #**15-630-080

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
CAS No.: 7365-45-9
Molecular Weight: 238.30 g/mol

- Use powder, molecular biology grade or similar (≥98.5%) to prepare 1M aqueous stock solution (pH 7.8)

or

- Use commercially available 1M aqueous stock solution (e.g., Gibco, Fisher Scientific Cat No. **15-630-080**; 100 mL)

Water, Optima LC/MS Grade, Fisher Chemical **Fisher Scientific Catalog #**W6212

- Use ultra-pure water - we recommend Fisher Optima LCMS grade (Fisher Scientific P/N **10505904**)

Acetonitrile, Optima LC/MS grade, Fisher Chemical **Fisher Scientific Catalog #**A955212

CAS No.: 75-05-8

- Use LCMS-grade, e.g. Acetonitrile Optima LCMS grade (Fisher Scientific P/N **10055454**) or HPLC grade S (Rathburn)

Cytiva Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophobic, 65152105050250 **Fisher Scientific Catalog #**11819912

(Sigma-Aldrich P/N: GE65152105050250)

Cytiva Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophilic, 45152105050250 **Fisher Scientific Catalog #**11548692

(Sigma-Aldrich P/N: GE45152105050250)

RapiZyme Trypsin, MS Grade, 4/pk (100ug) **Waters Catalog #**186010108

Ethanol, 99.8%, for HPLC, absolute, Thermo Scientific Chemicals **Fisher Scientific Catalog #** 12347163

Sodium dodecyl sulfate solution, BioUltra, for molecular biology, 20% in H2O **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**05030-500ML-F

- **Plastics and equipment:**

Eppendorf Safe-Lock micro test tubes 1.5mL **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP0030123328

Eppendorf Safe-Lock micro test tubes 2mL **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP0030123344

Vortex-Genie® 2 mixer, AC/DC input 220 V AC, Schuko plug, Merck, cat Number: **Z258423-1EA**.

Protocol materials

- SDS 20% solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**05030-500ML-F
- HEPES **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3375-500G
- DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**43815
- HEPES **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3375-500G
- RapiZyme Trypsin, MS Grade, 4/pk (100ug) **Waters Catalog #**186010108
- CaCl2 hexahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**21108-500G
- Water Optima™ LC/MS Grade Fisher Chemical™ **Fisher Scientific Catalog #**W6-4
- Ethanol, 99.8%, for HPLC, absolute, Thermo Scientific Chemicals **Fisher Scientific Catalog #** 12347163
- Eppendorf Safe-Lock micro test tubes 2mL **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP0030123344
- Sodium dodecyl sulfate solution, BioUltra, for molecular biology, 20% in H2O **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**05030-500ML-F
- Eppendorf Safe-Lock micro test tubes 1.5mL **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP0030123328
- Water, Optima LC/MS Grade, Fisher Chemical **Fisher Scientific Catalog #**W6212
- DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D0632
- 2-Chloroacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**C0267
- Gibco™ HEPES (1M) **Fisher Scientific Catalog #**15-630-080
- Acetonitrile, Optima LC/MS grade, Fisher Chemical **Fisher Scientific Catalog #**A955212
- DTT (DL-Dithiothreitol) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**43819
- Cytiva Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophobic, 65152105050250 **Fisher Scientific Catalog #**11819912
- Cytiva Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophilic, 45152105050250 **Fisher Scientific Catalog #**11548692
- RapiZyme Trypsin, MS Grade, 4/pk (100ug) **Waters Catalog #**186010108

Troubleshooting

Safety warnings

SP3 Beads can handle low and high pH, and can be safely heated up to 60ºC. However:
!!! Never freeze the beads.
!!! Never sonicate the beads.

Before start

This protocol assumes the cell lysis has been done and as such lysis buffer is already available. If not, prepare lysis buffer (4% (w/v) SDS, 25 mM HEPES 7.8±0.2, 1mM DTT) before starting.
All water used throughout is

Water Optima™ LC/MS Grade Fisher Chemical™ **Fisher Scientific** **Catalog #**W6-4

Reagent preparation

36m

1 **Reducing reagent preparation**

5m

Freshly prepare at least 100 µL of 100 millimolar (mM) aqueous solution of dithiothreitol (DTT).

- weigh approximately 1-2 mg or one spoonful of a microspatula of DTT powder.
- add the exact volume of water so as to reach a final concentration of 100 millimolar (mM) .
- mix thoroughly (vortex) until dissolved.
- pulse-spin in a microcentrifuge.

Note

Example:
$n = m/M = 1.5\ mg/154.253\ mg/mmol \approx 10\ \mu mol$
dissolving in 100 µl of water gives the desired 100 mM concentration.

Note

This may not be needed if lysis buffer already contains 1 mM DTT.

2 **Alkylation reagent preparation**

5m

Prepare 400 µL of 200 millimolar (mM) aqueous solution of chloroacetamide (CAA).

- weigh approximately 7-8 mg or four spoonfuls of the microspatula of CAA powder
- add the exact volume of water to a final concentration of 200 millimolar (mM)
- mix thoroughly (vortex) until dissolved
- pulse-spin in a microcentrifuge

Note

Example
$n = 7.5\ mg/93.51\ mg/mmol \approx 80\ \mu mol$
dissolving in 400 µl of water gives the desired 200 mM concentration.

Note

the volumes of reagents can be adjusted to the number of samples

3 **SP3-bead slurry preparation**
(sufficient for 20 samples)

3.1 Take out the two bottles with Sera-Mag speed beads from the fridge and keep them at room temperature for 10 minutes. 00:10:00 10m

3.2 Shake the two SP3 bead bottles gently (without vortex, until all beads are suspended in liquid, takes about 5 minutes). 00:05:00 5m

3.3 Combine 50 µL of each bead type in a clean Safe-lock Eppendorf tube (1.5mL or 2mL). 1m

3.4 Wash the beads: 10m

- Place the tube with the bead mixture on a magnetic rack and let the beads settle for 2 minutes. 00:02:00
- Remove and discard the supernatant.
- Rinse the beads with 500 µL water by gentle pipette mixing (off the magnetic rack).
- Repeat wash steps two more times.
- Re-suspend and store the beads in 500 µL water in 4 °C

Note

The washed beads can be stored in +4°C up to two weeks. Beads can handle low and high pH, and can be safely heated up to 60°C.
!!! Never freeze the beads.
!!! Never sonicate the beads.

Protein denaturation, reduction and alkylation of thiol groups

35m

4 Take an aliquot of protein extract (sample lysate) containing 200 µg of proteins and transfer to a clean tube (Eppendorf safelock **2mL**). Takes about 10min 00:10:00 to do 20 samples. 10m

> Note
>
> Make sure to use a 2mL Eppendorf tube, because the 2mL capacity will be needed later.

5 Dilute the samples with lysis buffer ([M] 4 % (m/v) SDS, [M] 50 millimolar (mM) HEPES pH 7.8 7.8±0.2, [M] 1 millimolar (mM) DTT) 10m

SDS 20% solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**05030-500ML-F

HEPES **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3375-500G

DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**43815

to get the same volume in all samples (200 µL). Final protein concentration is [M] 1 µg/µL .

6 Add 10 µL of [M] 200 millimolar (mM) chloroacetamide, so as to reach a final concentration of [M] 10 millimolar (mM) chloroacetamide. 5m

7 Vortex to mix, then spin down briefly. Incubate for 10 min. 00:10:00 15m

Protein level SP3 cleanup

45m

8 Add SP3 bead solution to the protein sample using a ratio of 1:10 of bead solution : protein solution. 10m

E. g. add 20 µL of SP3 beads solution to 200 µL of protein solution (containing 200 µg protein). Mix gently by pipetting. Takes about 10min 00:10:00 to do 20 samples.

9 Add acetonitrile (ACN) to obtain a final ACN composition of ≥ [M] 70 % (v/v) (e. g. 700 µL). 2m

10 Incubate for 20min 00:20:00 in the continuously (and gentle) rotating rack. 20m

11 Place the tubes in the magnetic rack and incubate for 2 minutes 00:02:00 . 2m

12 Remove and discard the supernatant. 2m

13 Add 200 µL of [M] 70 % (v/v) EtOH and incubate for 30s 00:00:30 in magnetic rack. Remove and discard supernatant. 3m

14 Repeat previous step. 3m

15 Add 200 µl of neat ACN and incubate for 15s 00:00:15 in magnetic rack. Remove and discard supernatant, and air dry the beads for 30s 00:00:30 . Make sure you close the lid to avoid over-drying of the beads. 3m

Protein digestion 17h 7m

16 Reconstitute the beads in 100 µL of digestion solution ([M] 50 millimolar (mM) HEPES pH 7.8 pH7.8±0.2, [M] 10 millimolar (mM) $CaCl_2$, [M] 10 % (v/v) ACN, 4 µg Rapizyme (Waters)). Mix gently by pipetting. 10m

HEPES **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3375-500G

CaCl2 hexahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**21108-500G

RapiZyme Trypsin, MS Grade, 4/pk (100ug) **Waters Catalog #**186010108

17 Incubate for 16h 16:00:00 at 37 °C with mild shaking, using an oven type incubator (i.e. avoid temperature gradient between the bottom and the top of the tube). 16h

Day 2 - peptide level SP3 cleanup

1h 24m 15s

18 Take the tubes from the incubator, and allow to cool down to room temperature. Spin down briefly.

19 Add 1900 µL of ACN to the samples to reach final content of > [M] 95 % (v/v) .

Note

In order guarantee the binding of all peptides to the SP3 beads, the organic content composition of the liquid must be >95%.

20 Incubate for 20min 00:20:00 in the continuously (and gentle) rotating rack. 20m

21 Place the tubes in the magnetic rack and incubate for 2 minutes 00:02:00 . 2m

22 Remove and discard the supernatant.

23 Add 200 µl of neat ACN and incubate for 15s 00:00:15 in magnetic rack. Remove and discard supernatant. 15s

24 Repeat previous step.

25 Add 200 µL of water and mix gently by brief vortex and spin down.

26 Place on magnetic rack for 2 min 00:02:00 . Transfer supernatant to new tubes (1.5mL). 2m

Note

If sample is to proceed directly to LCMS vial, repeat this step. The point is to avoid any remaining residual magnetic beads to be carried over into the LC system.

27 Determine peptide concentration with Bio-Rad Dcc. The BSA standard should be prepared in water. 1h

Protocol references

1. Hughes, C.S., et al., *Ultrasensitive proteome analysis using paramagnetic bead technology.* Mol Syst Biol, 2014. **10**(10): p. 757.
2. Hughes, C.S., et al., *Single-pot, solid-phase-enhanced sample preparation for proteomics experiments.* Nat Protoc, 2019. **14**(1): p. 68-85.