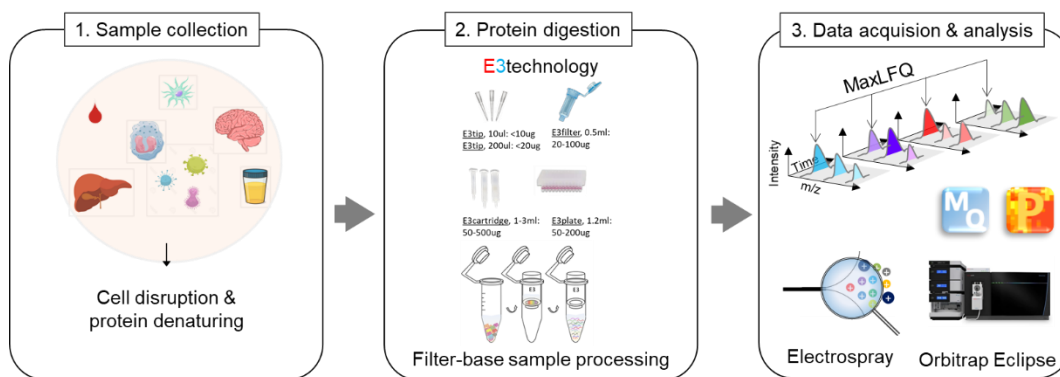


UNIVERSITY OF DELAWARE MASS SPECTROMETRY FACILITY

Sample Preparation for Shotgun Proteomics

Summary

This standard operating procedure pertains to processing cells (e.g., bacterial, fungi, mammalian, etc.) and tissue samples for mass spectrometry (MS)-based proteomics analysis. There are three major modules in this SOP, including cell lysis, protein digestion, and peptide desalting. Cell lysis can be accomplished by detergent-based lysis buffer along with physical disruption procedures, such as homogenization, probe-based sonication, bead beating, or vortex. It can also be done with non-detergents such as trifluoroacetic acid (TFA). **E3technology** is based on protein precipitation induced by organic solvents and glass beads membrane. The approach is more efficient, effective, and economical in comparison to many existing methods, such as S-Trap, iST, SP3/SP4, and FASP. StageTips are C18 based peptide desalting processing. When using self-packed tips with C18 Empore membrane, the approach is highly flexible and cost-effective. These methods will be described in this SOP. Here shows a typical workflow for shotgun (or bottom-up) proteomics.



Cell Lysis and Protein Extraction

1. Materials.

- (1) Please **ALWAYS** use low binding tubes and tips for all proteomics related preparations.
- (2) Please **ALWAYS** use glass containers for buffer storage.
- (3) **LCMS grade solvents** are recommended. Please see "[Recommended consumables and supplies for proteomics experiments](#)" separately.

	Description	Vendor	Catalog No.
Buffer container	Qorpak™ Glass Bottle Beakers with PTFE Caps	Fisher	2992588
Solvents	Optima™ LC/MS Grade, Water	Fisher	W6-4
	Optima™ LC/MS Grade, Methanol	Fisher	A456-4
	Optima™ LC/MS Grade, Acetonitrile	Fisher	A955-4
Tubes	Axygen™ MaxyClear Snaplock Microtubes, 1.5 ml	Fisher	14-222-158
	Axygen™ MaxyClear Snaplock Microtubes, 2.0 ml	Fisher	14-222-183
Tips	Thomas Scientific Woodpecker Reloads™ Tip, 20ul	Fisher	NC1589074
	Thomas Scientific Woodpecker Reloads™ Tip, 200ul	Fisher	NC1741132
	Thomas Scientific Woodpecker Reloads™ Tip, 1.0ml	Fisher	NC1741131

- (4) Common chemicals:
SDS-based lysis buffer: 4% SDS, 100 mM Tris-HCl, pH 8.0. Add fresh TCEP (10mM)/ and CAA (40mM) before use.

Stock solutions:

10% SDS (Fisher Cat #: BP2436200).

1 M solution of Tris/Tris-HCl, pH = 8 (Fisher Cat #: AAJ22638AP).

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Fisher, Cat #: AA4058704)

2-Chloroacetamide (CAA) (Fisher, Cat #: AAA1523830)

2. Experimental Procedures.

- (1) For cells from fresh cultures, collect cell pellets by removing culturing media and 2-3 washes with cold PBS.
For mouse tissue samples, animals should be first euthanized with CO₂, and then perfused with 50 ml of precooled PBS supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche) and a mixture of phosphatase inhibitor cocktail 1, 2 and 3 (Sigma-Aldrich). Tissues and organs should be dissected, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis.
- (2) Detergent-based cell lysis:
a). Depending on the size of cell pellet and tissue, add 50-500ul of SDS lysis buffer, vortex (or transfer to PowerBead tubes, disrupt for 5-15 sec in beadbeater).
b). Boil at 95°C for 5-10min, spin at max speed for 10-20min, collect supernatant and store in -80C until further experiments.
- (3) Non-detergent-based cell lysis:
a). Depending on the size of cell pellet and tissue, add 5-10x volume of pure TFA, incubate at RT for 3-5 min. Gentle vortex will facilitate lysis.
b). Add 4x volume of cold acetone to precipitate proteins. The sample is now ready for E3 digestion.

Protein Digestion with E3technology

1. Materials and Equipment.

- (1) E3technology filters ([CDS Analytical LLC](#)):

Item Description	Part Number	Format	Size	Package
Empore 6601 E3tips	70-2019-3001-1	Pipette Tips	200 µl	96/Case
Empore 6601 E3tips	70-2019-3002-3	Pipette Tips	10 µl	96/Case
Empore 6701 E3filter	70-2019-3101-0	Spin Column	0.5 mL	100/Case
Empore 6801 E3plate	70-2019-3201-9	96-Well Plate	1.2 mL	12/Case

- (2) Sequencing Grade Modified Trypsin (Promega; cat #: V5111), stock solution 0.1 µg/µl in 50mM ammonium bicarbonate.
- (3) Triethylammonium bicarbonate (TEAB; Fisher cat #: 60-044-973): 1.0 M stock solution.
- (4) Solutions:
Binding/Wash solution: 80% acetonitrile.
Digestion buffer: 50 mM TEAB.
Elution solution I: 0.2% formic acid in H₂O.
Elution solution II: 0.2% formic acid in 50% acetonitrile/50% H₂O.

2. Experimental Procedures.

2.1 Sample Processing.

(1) From detergent-based cell lysis:

a). Aliquot desired amount of proteins (20~100ug), add 4x volumes of binding/wash solution, gentle mix and incubate at room temperature for 1-2 min.

Note: you should be able to see instant protein precipitation in the tube, if you have enough proteins. For some cases such as pulldown samples, the precipitates may not be clear/obvious, but please feel free to continue the procedures.

d). Load the sample onto the E3filters, centrifuge at 2000 rpm for 2 min. Discard flow-through.

e). Add 200 µl binding/wash solution and centrifuge at 2000 rpm for 2 min. Repeat this step two more times.

f). Transfer the filter device to a clean 2.0-ml collection tube. Add 200ul digestion solution, and trypsin at a 50:1 (wt/wt) ratio.

For example, if you have an estimated amount of 50ug of proteins, add 1.0ug of trypsin.

g). Incubate at 37°C overnight with gentle shaking.

h). Next day, without opening the cap, centrifuge the tube at 2000 rpm for 2 min to collect digested peptides in the collection tube.

i). Add 200 µL Elution solution I and centrifuge, and transfer the eluent to a clean tube.

j). Add 200 µL Elution II and centrifuge at 2000 rpm for 2 min.

k). Dry elution samples in a SpeedVac.

l). Store in -20°C until further experiments.

(2) From detergent-free cell lysis:

a). Transfer acetone-precipitated proteins to E3 filters, centrifuge at 2000 rpm for 2 min, then discard flow-through.

b). Add 200 µl binding/wash solution and spin at 2000 rpm for 2 min.

c). Add 200ul 50mM TEAB, spin at 2000 rpm for 2 min. Discard flow through.

d). Add 100ul 50mM TEAB, plus 10mM TCEP and 40mM CAA, incubate at 45°C for 5min. Spin then discard flow thought.

e). Add 200ul 50mM TEAB, spin at 2000 rpm for 2 min. Discard flow through. Repeat this step two more times.

f). go to step f) above.

Peptide Desalting with StageTip

1. Materials and Equipment.

(1) Empore C18 Extraction disk (Fisher, cat # 13-110-018).

(2) Empore C18 StageTips (Fisher, cat # 13-110-055).

(3) Pipette tip adaptors (CDS Analytical, cat #: 70-2019-1021-3).

(4) Solutions:

Activation buffer: 100% methanol.

Wash and equilibration buffer: 0.5% acetic acid in water.

Elution buffer I: 0.5% acetic acid, 60% acetonitrile and 40% water.

Elution buffer II: 0.5% acetic acid, 80% acetonitrile and 20% water.



2. Experimental Procedures.

(1) Sample pre-treatment:

Re-dissolve dried peptide sample with 200uL Wash and equilibration buffer (H₂O/0.5%HAc),

- vortex for 10~15min, then spin @ 13,000 rpm for 3-5 min.
- (2) Use commercial StageTips, or pack you own tips.
Pack two layers of C18 membrane into 200uL pipette tips. Put adaptor onto tip, and put the whole set into 2-ml tube; Please label tips (①, ②...) at this step (no need to label tubes).
 - (3) Activation I: Load 200uL Methanol, spin @ 4000 rpm for 1~2min.
 - (4) Activation II: Load 200uL 80% ACN/0.5% HAc, spin @ 4000 rpm for 1~2min.
 - (5) Discard all the liquid in the collection tube.
 - (6) Equilibration: Load 200uL H₂O/0.5% HAc, spin @ 4000 rpm for 1~2min.
 - (7) Discard all the liquid in the collection tube.
 - (8) Loading: Load 200uL sample into tip, spin @ 1500-2000 rpm for 10~15min. Discard flow through.
 - (9) Washing: Load 200ul Wash and equilibration buffer, spin 4000 rpm for 1-2min; this step may repeat 2~3 times. Also, depending on the salt amount, the spin time may vary (2~5 min), and speed may go up to 8,000 rpm.
 - (10) Transfer tips to new collection tubes, label tubes (①, ②...).
 - (11) Elution I: load 200uL 60% ACN/0.5% HAc, spin 4000 rpm for ~2min;
 - (12) Elution II: load 200uL 80% ACN/0.5% HAc, spin 4000 rpm for ~2min; repeat this time one more times.
 - (15) Discard tips. Dry samples on a SpeedVac.