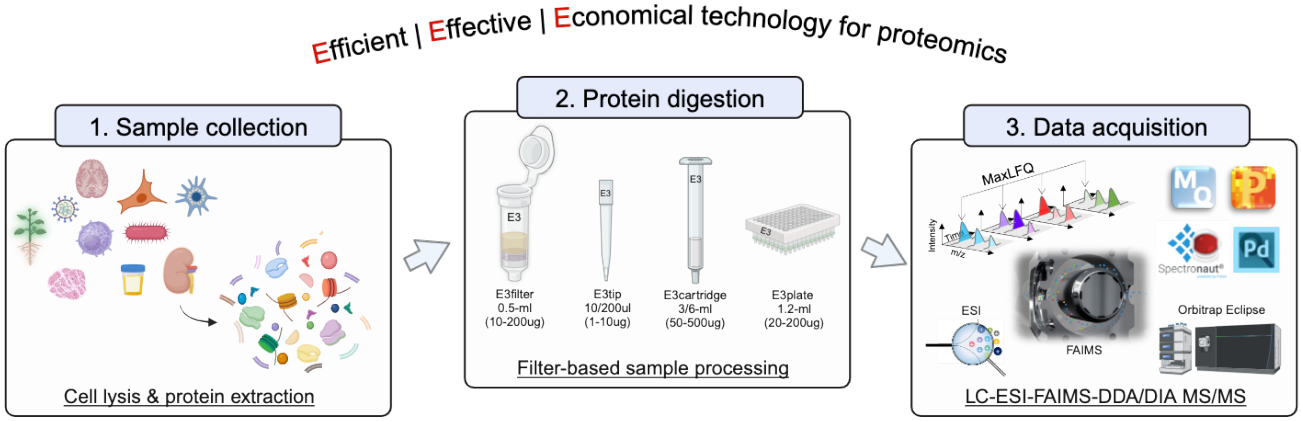
UNIVERSITY OF DELAWARE MASS SPECTROMETRY FACILITY

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 CO_2, 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 or tissue, add 50-500ul of SDS lysis buffer, vortex (or transfer to PowerBead tubes, disrupt for 5-15 sec in beadbeater).
b). Boil at 95ºC 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 or 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/Elution solution I: 50 mM TEAB.

Elution solution II: 0.2% formic acid in H_2O.
Elution solution III: 0.2% formic acid in 50% acetonitrile/50% H_2O.

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 400 x g for 2 min. Discard flow-through.
e). Add 200 µl binding/wash solution and centrifuge at 400 x g 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 400 x g for 2 min to collect digested peptides in the collection tube.
i). Add 200 µL Elution solution II and centrifuge, and transfer the eluent to a clean tube.
j). Add 200 µL Elution III and centrifuge at 400 x g for 2 min.
k). Dry elution samples in a SpeedVac.
l). Store in -20ºC until further experiments.

(2) From detergent-free cell lysis:

a). Transfer acetone-precipitated proteins to E3 filters, centrifuge at 400 x g for 2 min, then discard flow-through.
b). Add 200 µl binding/wash solution and spin at 400 x g for 2 min.
c). Add 200ul 50mM TEAB, spin at 400 x g for 2 min. Discard flow through.
d). Add 100ul 50mM TEAB, plus 10mM TCEP and 40mM CAA, incubate at 45ºC for 5min. Spin then discard flow thought.
e). Add 200ul 50mM TEAB, spin at 400 x g 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 (H_2O/0.5%HAc), vortex for 10~15min, then spin @16,000 x g 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 (❶, ❷…) at this step.
(3) Activation I: Load 200uL Methanol, spin @ 1,500 x g for 1~2min.
(4) Activation II: Load 200uL 80% ACN/0.5% HAc, spin @ 1,500 x g for 1~2min.
(5) Discard all the liquid in the collection tube.
(6) Equilibration: Load 200uL H_2O/0.5% HAc, spin @ 1,500 x g for 1~2min.
(7) Discard all the liquid in the collection tube.
(8) Loading: Load 200uL sample into tip, spin @ 400 x g for 10~15min. Discard flow through.
(9) Washing: Load 200ul Wash and equilibration buffer, spin 1,500 x g 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 7,000 x g.
(10) Transfer tips to new collection tubes, label tubes (❶, ❷…).
(11) Elution I: load 200uL 60% ACN/0.5% HAc, spin 1,500 x g for ~2min;
Note: please be cautions to **NOT** rinse the labeling off the tip.
(12) Elution II: load 200uL 80% ACN/0.5% HAc, spin 1,500 x g for ~2min; repeat this time one more time (optional).
(15) Discard tips. Dry samples on a SpeedVac.