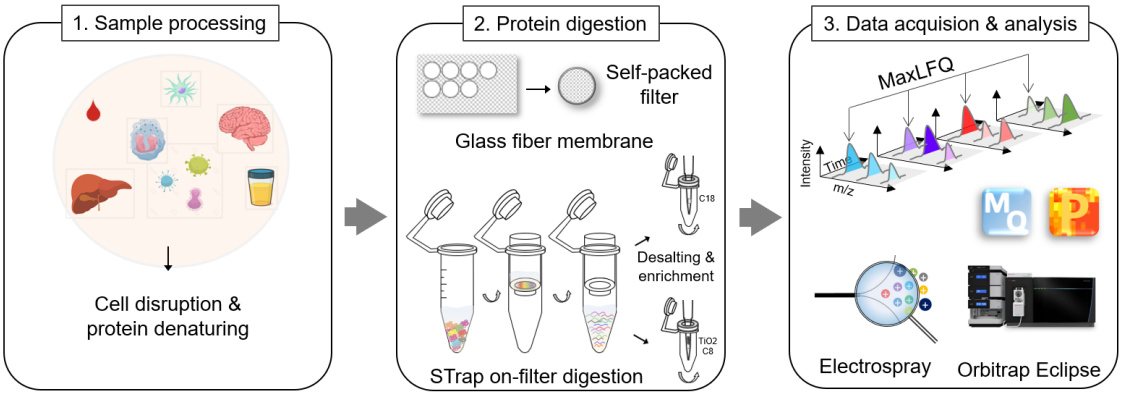
UNIVERSITY OF DELAWARE MASS SPECTROMETRY FACILITY

Sample Preparation for Shotgun Proteomics

Summary

This standard operating procedure pertains to processing cells (including bacterial and mammalian cells) and tissue samples for proteomics analysis. There are three major modules in this SOP, including cell lysis, protein digestion, and peptide desalting. Cell lysis can be accomplished using physical disruption procedures along with detergents, such as homogenization, probe-based sonication, bead beating, or vortex. It can also be done with non-detergents such as trifluoroacetic acid (**TFA**). Suspension Trapping (**STrap**) approach is based on organics-induced protein precipitation onto glass fiber membrane. The approach is rapid and efficient in the context of detergents depletion and protein digestion. **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. The below shows a typical workflow for shotgun (or bottom-up) proteomics.



Cell Lysis and Protein Extraction

1. Materials and Equipment.

(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.

	Description	Vendor	Catalog No.
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) SED buffer: 4% SDS, 10 mM EDTA, 0.05% Tween-20, 100 mM Tris-HCl, pH 8.0. Add fresh DTT (20 mM) before use.
Stock solutions:
1 M solution of Tris/Tris-HCl, pH = 8 (Fisher Cat #: AAJ22638AP).
1 M dithiothreitol (DTT) in water, frozen at -20°C (Fisher Cat #: FERR0861).
500 mM Na-EDTA in water (Fisher Cat #: 15575020).
1% Tween-20 in water.
10% SDS (Fisher Cat #: BP2436200).
(5) Cell Disruptor: BioSpec beadbeater (Mini-Beadbeater-16).
PowerBead Tubes, Ceramic 1.4 mm (Qiagen Cat #: 13113-50).
(6) Trifluoroacetic Acid (TFA; Fisher Cat #: 60-017-61)

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 and tissue, add 50-500ul of SED lysis buffer and 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 and tissue, add 5-10X volume of pure TFA, incubate at RT for 3-5 min. Gentle vortex will facilitate lysis.
b). Add 10X volume TrisBase (2M, pH=10) to neutralize TFA.
c). Add 50mM IAA (iodoacetamide), incubate at RT for 30min.
c). Add 4X volume of cold acetone to precipitate proteins. The sample is now ready for STrap digestion.

Protein Digestion with Suspension Trapping (STrap)

1. Materials and Equipment.

(1) Sequencing Grade Modified Trypsin (Promega), stock solution 0.1 µg/µl in 50mM ammonium bicarbonate.
(2) Triethylammonium bicarbonate (TEAB): 1.0 M stock solution.
(3) Solutions:
Binding/Wash solution: 90% methanol in 100 mM TEAB, pH 7.1.
Neutralization solution: 12% phosphoric acid.
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 Use commercial S-Trap filters and follow manufacture's procedure (Protifi, Cat #: CO2-micro-80). Or pack you own filters.

2.2 Pack STrap Filters.

(1) Use the 10/32" hole puncher to cut discs from the glass microfiber membrane disc (Whatman GF/F membrane, pore size 0.7 µm). Use a tweezer to pack two layers of glass fiber discs in the filter base, assemble the O-ring, and snap on the sample tube.

(2) Transfer the filter device onto a collection tube and flush the filter disc with 300 µl binding solution by centrifugation at 2000 rpm for 1 min, discard the flow-through.

2.3 Sample Processing.

(1) <u>From detergent-based cell lysis:</u>

a). Aliquot desired amount of proteins (20~30ug), alkylate free cysteines by adding IAA (final concentration 50mM) to samples, and incubate in dark for 20-30 min.

b). Add phosphoric acid (10:1 ratio; for instance, with 100 uL sample, add 10 uL acid) to the protein sample, mix and incubate for 1-2 min at room temperature.

c). Add six volumes of binding/wash solution, mix well and incubate for 1-2 min.
For example, if you have 100ul protein solution, add 600ul binding/wash solution.

d). Load the sample onto the STrap filter device, 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 collection tube. Add 200ul TEAB solution, and trypsin at a 50:1 (wt/wt) ratio.
For example, if you have an estimated amount of 30ug protein, add 0.6ug trypsin.

g). Incubate the tube at 37°C overnight.

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

l). Pool all eluents together. Dry samples on a SpeedVac. Store in -20ºC until further experiments.

(2) <u>From detergent-based cell lysis:</u>

a). Transfer acetone-precipitated proteins to STrap filters.

b). Go to step (e) 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 (The Nest Group, Inc., Southborough, MA).
(4) Solutions:
<u>Activation buffer</u>: 100% methanol.
<u>Wash and equilibration buffer</u>: 0.5% acetic acid in water.
<u>Elution buffer I:</u> 0.5% acetic acid, 60% acetonitrile and 40% water.
<u>Elution buffer II:</u> 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 @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 (❶, ❷…) 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 H2O/0.5% HAc, spin 4000 rpm for 2~3min; this step may repeat 2~3 times. Also, depending on the salt amount, the spin time may vary (2~4 min).
(10) Transfer tips to new collection tubes, label tubes (❶, ❷…).
(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.