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Preparation of Tissue Sections for Proteomic Analysis V.3

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ABSTRACT

Scope:
To describe the procedure for the lysis, reduction/alkylation, trypsin digestion, and clean-up of protein extracts from tissue sections. Lysis will cover the lysing of tissue and protein concentration. Acetone precipitation will cover the precipitation of proteins. Digestion will cover the process for digesting 100 µg of protein using Promega Rapid Trypsin/LysC. Clean-up will cover the desalting and sample-loading process using EvoTips to prepare the samples for LC-MS/MS proteomics analysis.

Expected Outcome/Data:
Cell samples lysed, digested, and desalted for analysis on MS instrument. Samples to be analyzed within one or two days of desalting.

GUIDELINES

Definitions:
1. ACN is Acetonitrile
2. BCA is Bicinchoninic Acid Assay
3. IAA is Iodoacetamide
4. MeOH is Methyl Alcohol/Methanol
5. TCEP is Tris(2-carboxyethyl)phosphine
6. TFA is Trifluoroacetic Acid
7. TFE is Tetrafluoroethylene

MATERIALS

Reagents:

1. Water: (H_2O), Milli-Q System Water
2. Methyl Alcohol (Methanol), Fisher, A452
3. Acetone, Fisher A949
4. 1-propanol, Fisher
5. 2,2,2 Trifluoroethanol, Fisher, AC139750250
6. Iodoacetamide, Single Use, Fisher, PI90034
7. TCEP, Fisher, PI77720
8. Rapid Trypsin/LysC Digestion Kit, Promega, CS196901
9. Formic Acid, Sigma-Aldrich, F-0507
10. Trifluoroacetic Acid, 99.5%, Acros, AC29831
11. Trizma Base, minimum 99.9% titration, Sigma, T1503
12. Pierce Formic Acid Ampules, Fisher, PI28905
13. Optima Water, LCMS Grade, Fisher, W6-1
14. Acetonitrile, Fisher, A9984
15. NP-40 Detergent Surfactant Amps, Fisher PI28324
16. Ethylenediaminetetraacetic Acid, Sigma, EDS
17. Halt Protease Inhibitor Cocktails, Fisher, PI78430
18. Pierce BCA Protein Assay Kit, Fisher, PI23225

Equipment:

1. Ultrasonic Cleaner, Branson
2. Incubator, Thermo Scientific
3. Spectrophotometer, SpectraMax $M2^e$, Molecular Devices
4. EvoSep One, EvosepEvotip Pure, EvoSep, EV2011
5. Orbitrap Fusion, ThermoScientific

Reagent Preparation

1. Stock solution of 500mL Lysis Buffer:
   3.03g Trizma Base (50mM)
   4.39 Sodium Chloride (150mM)
   5mL Nonidet 40 (1%)
   0.146g EDTA (1
   Dissolve in 400mL Milli-Q H_2O and qs to 500mL
   Store at 4^oC

2. Working Lysis Buffer:
   Put 10mL stock lysis buffer in 15mL conical
   Add 100uL HALT inhibitor to conical
   Vortex and keep on ice until use

3. Stock of 75:25 Acetone: Methanol (to be kept at -20^oC)
   15mL Acetone + 5mL Methanol into scintillation vial

4. Stock of 100mM Tris pH 8.0
   6.057g Trizma Base into 500mL Milli-Q H_2O
   Completely dissolve Tris. Adjust to pH 8.0

5. Stock of 60% Formic Acid
   Add 12mL Formic Acid slowly to 8mL Milli-Q H_2O in a scintillation vial

6. Solvent A - Stock of 0.1% Formic Acid
   Add 1 Formic Acid Ampule to 1L bottle of Optima Water

7. Solvent B - Stock of 0.1% Formic Acid in Acetonitrile
   Add 1 Formic Acid Ampule to 1L bottle of Optima Acetonitrile

SAFETY WARNINGS

1. Safety glasses or goggles, proper gloves, and a lab coat required. The area should be adequately vented and a lab mat placed underneath all solutions

2. **Warning**: Trifluoroacetic Acid and Formic Acid: HARMFUL OR FATAL IF SWALLOWED. Vapor harmful. Affects the central nervous system. Causes severe eye irritation and respiratory tract irritation. May be harmful if absorbed through skin. Chronic exposure can cause adverse liver, kidney, and blood effects. Flammable liquid and vapor.

Tissue Lysis

1 Begin with tissue cryosections in eppendorf tubes. Add 100µL-500µL lysis buffer to tissue.

2 Place tubes in dry ice for 00:05:00

3 Defrost tubes on wet ice for 00:05:00 and then vortex briefly.

4 Add ice to water in the sonicator to make an icy slurry.

5 Sonicate samples in ice bath for 00:10:00 and vortex.

6 Spin tubes in microcentrifuge for 00:05:00 at 14000 rpm.

7 Pipet supernatant into new labeled Eppendorf tube. Discard pelleted tissue.

8 Determine protein concentration of samples via Pierce BCA Protein Assay kit:

1. Prepare BSA standard curve with lysis buffer following BCA kit instructions.
2. Pipette 25 µL of standards into the “curve” wells in a clear flat bottom plate.
3. Pipette 20 µL of lysis buffer into the sample wells.
4. Pipette 5 µL of sample into each sample well and mix 5x.
5. Prepare working reagent as instructed in BCA protocol.
6. Add 200 µL working reagent to each curve/sample well.
7. Incubate samples for 00:30:00 at 37 °C.
8. Add template to Softmax Pro during 00:30:00 incubation, with 5x dilution for samples.
9. Read plate at an absorbance of 562 nm.
10. Export results into BCA excel workbook to determine volume for 100ug of protein for the precipitation.

Acetone Precipitation

9 Add 100 µg of protein sample to a 1.5mL labeled eppendorf tube.

10 Add lysis buffer to the sample to equal 100 µL.

11 Add 300 µL ice cold 75:25 acetone:methanol to the sample.

12 Vortex sample and incubate for 02:00:00 at -80 °C.
Alternatively, incubate overnight at -20 °C.

Place tube rotor in cold centrifuge at 4 °C.

13 Remove tubes from freezer and centrifuge samples for 00:15:00 at 4000 RPM. When removing from centrifuge, place on ice or cold block to prevent pellet from dislodging.

14 Carefully remove and discard supernatent.

15 Add 300 µL of ice cold acetone to all samples and spin for 00:15:00 at 4000 RPM.

16 Remove and discard supernatent. Briefly allow residual acetone to evaporate from the tubes at room temperature. The drying should only be as long as it takes to get TFE and Tris ready to add. Do not over-dry the pellet, or it may not dissolve properly.

Protein Digestion

17 Resuspend the 100 µg pellet in 10 µL of neat TFE and 10 µL of 100mM Tris (pH 8.0). Vortex to dissolve pellet.

18 Reduce samples with 1 µL premade TCEP (0.5M) at room temperature for 00:30:00. 30m

19 Prepare 0.5M IAA:
Dilute 1 vial of pre-weighed IAA with 100 µL of Rapid Trypsin Digestion Buffer.
Keep IAA in a drawer (light sensitive).

20 Alkylate samples with by adding 2 µL of 0.5M IAA to each sample. 30m

Incubate in a dark drawer at room temperature for 00:30:00 .

21 Add 73 µL of Rapid Trypsin Buffer to each sample.

22 Prepare Promega Rapid Trypsin:
Add 100 µL of Promega Resuspension Buffer to 1 bottle of rapid trypsin.

23 Add 4 µL prepared 1ug/ul Rapid Trypsin to each sample (1:25 enzyme:protein).

24 Gently vortex each sample.
Incubate plate at 55 °C for 00:45:00 .

25 Remove samples from incubator and pulse in centrifuge to to push any condensation out of the cap.

26 Add 5 µL 60% Formic Acid to each well sample to stop the digestion.

27 Place samples on ice to prepare for desalting or place them in -80 °C for future use.

Desalting Samples and Preparing for LCMS

28 Prepare tip soaking plate by placing 100 µL of 1-propanol into the wells of a 96 well plate with MTP adaptor. Only fill the wells that tips will be placed in.

Prepare tip rack by only placing the amount you need for digested samples and quality control samples.

29 **Wash** 1m
Add 20 µL of Solvent B to each tip.
Place the Evotips box in a centrifuge with appropriate counter balance.
Centrifuge at 700g for 700 x g for 00:01:00 .

30 **Condition** 1m
Place Evotip adaptor rack on top of the MTP plate with 1-propanol.
Soak for 00:01:00 .
Inspect the tips to make sure that all tips are pale white. If not, soak longer.

31 **Equilibrate** 1m
Add 20 µL of Solvent A to each tip.
Place the Evotips box in a centrifuge with appropriate counter balance.
Centrifuge at 700g for 700 x g for 00:01:00 .

32 **Sample Load** 1m
Add of sample or control to each tip, for a total of 20 µL .
For samples, add 500ng of digested sample.
For controls, add 100ng of HeLa digest.

Place the Evotips box in a centrifuge with appropriate counter balance.

Centrifuge at 700g for 700 x g for 00:01:00 .

33 **Wash** 1m

Add 20 µL of Solvent A to each tip.

Place the Evotips box in a centrifuge with appropriate counter balance.

Centrifuge at 700g for 700 x g for 00:01:00 .

34 **Preservation** 10s

Add 100 µL of Solvent A to each tip.

Place the Evotips box in a centrifuge with appropriate counter balance.

Pulse the centrifuge at 700g for 700 x g for 00:00:10 .

This will keep the tips wet.

35 Samples are now ready to for LCMS analysis.