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Automated, Rapid Preparation of Tissue Sections for Proteomic Analysis V.1

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Protocol status: Working
We use this protocol and it's working

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Abstract

Scope:
To describe the procedure for the lysis, reduction/alkylation, trypsin digestion, and clean-up of tissue. 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 the Agilent AssayMap Bravo Robot and Promega Rapid Trypsin/LysC. The clean-up of the cells will cover the desalting process on the Agilent AssayMap Bravo and the subsequent preparation of the samples for LC/MS peptide 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. 2,2,2 Trifluoroethanol, Fisher, AC139750250
5. Iodoacetamide, Single Use, Fisher, PI90034
6. TCEP, Fisher, PI77720
7. Rapid Trypsin/LysC Digestion Kit, Promega, CS196901
8. Formic Acid, Sigma-Aldrich, F-0507
9. Trifluoroacetic Acid, 99.5%, Acros, AC29831
10. Trizma Base, minimum 99.9% titration, Sigma, T1503
11. Pierce Formic Acid Ampules, Fisher, PI28905
12. Optima Water, LCMS Grade, Fisher, W6-1
13. Acetonitrile, Fisher, A9984
14. NP-40 Detergent Surfactant Amps, Fisher, PI28324
15. Ethylenediaminetetraacetic Acid (EDTA), Sigma, EDS
16. Halt Protease Inhibitor Cocktails, Fisher, PI78430
17. Pierce BCA Protein Assay Kit, Fisher, PI23225

Equipment:

1. Ultrasonic Cleaner, Branson
2. Incubator, Thermo Scientific
3. Spectrophotometer, SpectraMax $M2^e$, Molecular Devices
4. AssayMap Bravo Robot, Agilent
5. PlateLoc, Agilent
6. C18 Cartridges, Agilent 5190-6532
7. 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^{O}C)
   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. Stock of 0.1% Formic Acid
   Add 1 Formic Acid Ampule to 1L bottle of Optima Water

7. Stock of Equilibration Buffer: 0.1% TFA
   Add 1mL Trifluoroacetic Acid to 999mL Milli-Q H_2O

8. Stock of Priming and Syringe Wash Solution: 100% ACN, 0.1%TFA
   Add 1mL Trifluoroacetic Acid to 999mL Milli-Q H_2O

9. Stock of Elution Buffer: 70% ACN, 0.1% TFA
   Add 1mL Trifluoroacetic Acid to 700mL Acetonitrile and 299mL Milli-Q H_2O

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.

Lysis/Concentration Assay

1 Place 5-10 sections of tissue in an Eppendorf tube and keep on ice.

2 Add 200 µL lysis buffer to tubes and vortex for 30-60 seconds.

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

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

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

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

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

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

9 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

10 Add 100 µg of protein sample to 1.2mL square Abgene plate.

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

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

13 Seal plate with sealing mat and agitate gently by hand. Incubate for 02:00:00 at -80 °C . Alternatively, incubate overnight at -20 °C . Place plate rotor in cold centrifuge at 4 °C .

14 Remove plate 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.

15 Carefully remove and discard supernatent.

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

17 Remove and discard supernatent. Briefly allow residual acetone to evaporate from the plate 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.

100ug Digestion

18 Prepare (items) for use on Bravo:

1. Prepare 100nM TCEP (Make the following for each column used on Bravo)
   Add 42 µL of 0.5M TCEP + 168 µL of Rapid Trypsin Digestion Buffer
   Add 25 µL to each well in a Greiner clear U bottom plate

2. Prepare 200mM IAA (Make the following for each column used on Bravo)
   Dilute 1 vial of pre-weighed IAA with 100 µL of Rapid Trypsin Digestion Buffer
   Add 84 µL of 0.5M IAA + 126 µL of Rapid Trypsin Digestion Buffer
   Add 25 µL to each well in a Greiner clear U bottom plate

3. Add 100 µL of Rapid Trypsin Digestion Buffer to each well in a 1.2mL Abgene plate.

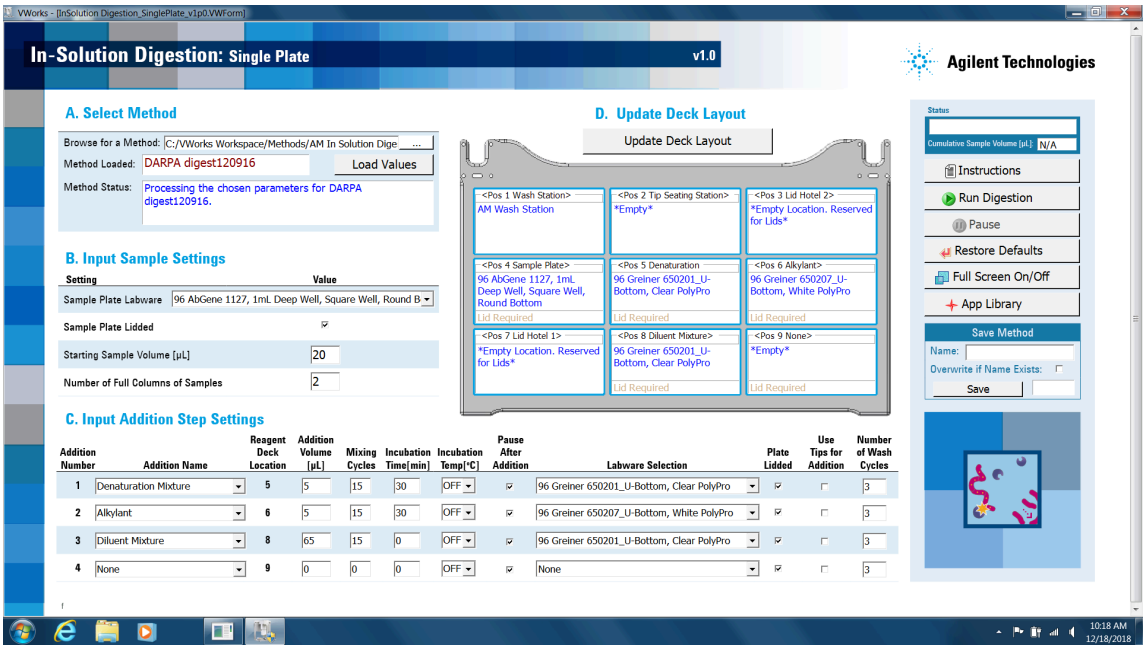
19 Resuspend the 100ug pellet in 10 µL of neat TFE and 10 µL of 100mM Tris (pH 8.0). Vortex gently or use T-shake on Bravo at max speed for 00:02:00 .

20 Set up automation with Bravo In-Solution Digestion: Single Plate v1.0:

1. Browse for a Method:
   VWorks Workspace→Methods→AM In Solution Digestion Single Plate v1.0→DARPA in Solution Digestion
   (or most current digestion method)

2. Load Values

3. Place plates/reservoirs with lids on appropriate spots on robot deck.



21 Reduce with 5 µL of 100mM TCEP at room temperature for 00:30:00. Bravo will pause after addition. Place IAA plate on Bravo deck and restart.

22 Akylate with 5 µL of 200mM IAA in the dark at room temperature for 00:30:00 (place in drawer below Bravo). Bravo will pause after addition.

23 Add 65 µL of Rapid Trypsin Digestion Buffer to sample with Bravo.

24 While Bravo is adding diluent, prepare Promega Rapid Trypsin by adding 100 µL of Promega Resuspension Buffer to 1 bottle of rapid trypsin.

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

26 Replace sealing mat or seal plate with PlateLoc and gently shake.

27 Incubate plate at 55 °C for 00:45:00 .

28 Remove plate from incubator and pulse in centrifuge for 00:00:20 to move any condensation back into the wells.

29 Add 5 µL 60% Formic Acid manually to each well.

30 Seal plate with sealing mat or aluminum seal using PlateLoc and either prepare for desalting or place plate in -80 °C for future use.

Desalting Samples

31 Prepare items for use on Bravo and place on plate deck.
* Columns filled in 12 well reservoir plates will correspond to sample columns filled in PCR plate:

1. Prepare Equilibration plate by adding 4.2 mL Equilibration buffer to wells
2. Prepare Priming and Syringe plate by adding 4.2 mL Priming and Syringe wash buffer to wells.
3. Prepare Elution plate by adding 4.2 mL Elution buffer to each column.
4. Make sure that water wash bottles are full to the top.
5. Place C18 cartridges on tip deck corresponding to number of samples.
6. Add 30 µL of sample to Eppendorf PCR plate (this includes 10 uL overage).
7. Centrifuge plate at 4000g for 00:02:00 to pellet any debris and remove bubbles.

32 Open Peptide Cleanup v.2.0 in App Library:

1. Set columns for the amount of samples.

2. Load 21 µL of sample.
3. Elute in 20 µL of sample.
4. Run Program.

33 Place plate in SpeedVac for approximately 00:30:00. Continue checking until samples are completely dried.

34 If finished, seal plate with aluminum seal or PCR cap strips and place in -80 °C for future use.

Preparing Samples for Instrument - Reconstitute Dried Sample

35 Label Eppendorf tubes and place vial inserts into each one.

36 Label LC autosampler vials and set aside.

37 Add 20 µL of 0.1% Formic Acid to each well, pipetting up and down 10x. This will make a 1ug/uL solution for analysis.

38 Pipette sample into respective vial insert/tube.

39 Briefly spin tube to remove any air bubbles in insert.

40 Use forceps to move into labeled LC vial and cap.

41 Store in -20 °C freezer until ready for instrument.