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| **STANDARD OPERATING PROCEDURE** |
| |  |  | | --- | --- | | **Title: Automated Trypsin Digestion of Plasma** | | | **SOP#: D-02** |  | | **Version #: 1** | **Author: Paulovich Lab** | | **Date Approved:** | **Date Modified:** | |

1. PURPOSE

The purpose of this document is to describe enzymatic digestion of a cell lysate for protein analysis compatible with mass spectrometry.

1. SCOPE

This procedure may be used to reduce, alkylate, and proteolyze samples.

1. RESPONSIBILITIES

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

1. Equipment

Automated Pipetting System (e.g. EP Motion, TECAN, Bravo)

Waters Positive Pressure-96 Manifold

1. Materials

1.1mL round bottom polypropylene 96 well plates, Thermo.

KingFisher 250uL plates, VWR 970-02-540

Oasis HLB 96-plate, 30mg (30um), Waters.

1. Reagents

**Required Reagents:**

1. Urea, SigmaUltra.
2. BondBreaker Neutral TCEP Solution, Pierce.
3. Trizma Preset Crystals, pH 8.1, Sigma.
4. Iodoacetamide, Fluka.
5. Water, LC/MS Chromasolv, Fluka.
6. Acetonitrile, LC/MS Chromasolv, Fluka
7. HCl, Fluka.
8. TPCK-treated Trypsin, Worthington.
9. 15N protein mix (if available)
10. 13C/15N peptide mix (prepared for desired set of targets)

**Preparations for 3 x 96-well plates, 30uL plasma per well:**

1. 0.2M Trizma pH 8.1 ~200mL
2. 0.8M Trizma pH 8.1 ~ 5mL
3. 9M Urea ~30mL
4. 500mM Iodoacetamide ~10mL
5. TCEP:0.8M Trizma pH 8.1 (1:1) ~6mL
6. Trypsin at 0.6mg/mL in 10mM HCl ~ 35mL
7. 15N protein mix, stock = 2.5pmoL/uL, working stock = 25fmol/uL (diluted in 1X PBS, 0.01% CHAPS)
8. 13C/15N peptide mix, stock = 1pmol/uL, working stock = 10fmol/uL (diluted in 30% ACN, 0.1%FA)
9. Procedure
   * 30µL per replicate
   * pipet 30µL of plasma in triplicate for each subject
   * All reagents prepared fresh. IAA was kept at 4C in the dark, trypsin was kept at 4C at < pH 2.5 and 20% formic acid was directly before use to prevent evaporation.

***The following steps are performed using the liquid handling robot:***

* 1. Pipet 84µL of 9M Urea into each well (final conc. 6M)
  2. Pipet 10.8µL of Bondbreaker neutral TCEP + Trizma solution (dilute 0.5M stock solution 1:1 with 0.8M Trizma preset crystals pH 8.1 for a working stock= 0.25M TCEP, 31mM Trizma)
  3. Pipet 10µL of 15N QC protein mix working stock into each well (working stock= 25fmol/ul , total 250fmol per well)
  4. Pipette 30uL patient plasma into each well (repeat in horizontal triplicates)
  5. Transfer plate to thermoshaker and mix at 700rpm for 30sec
  6. Manually seal plate with Teflon plate seal and transfer to 37C air incubator for 30min
  7. Centrifuge for 1min at 2000rpm and manually transfer back to robot
  8. Pipet 20µL of iodoacetamide into each well (final conc. 80mM).
  9. Transfer plate to thermoshaker and mix at 700rpm for 30sec
  10. Transfer plate to parking garage (dark) and incubate for 10min (accounts for individual addition of iodoacetamide that takes ~20min)
  11. Pipet 600µL of 0.2M Trizma pH 8.1 (added individually to keep incubation times equal)
  12. Pipet 50µL of trypsin into each well (total amount 30µg, 1:20 E:S assuming starting plasma protein concentration of 60 mg/mL).
  13. Transfer plate to thermoshaker and mix at 700rpm for 30sec
  14. Manually transfer plate to 37C air incubator and incubate for 4 hr
  15. Centrifuge for 1min at 2000rpm and manually transfer plates back to robot
  16. Pipet 50µL of trypsin into each well (total amount 30µg, 1:20 E:S assuming starting plasma protein concentration of 60 mg/mL).
  17. Transfer plate to thermoshaker and mix at 700rpm for 30sec
  18. Centrifuge for 1min at 2000rpm and manually transfer plates back to robot
  19. Pipet 40µL of 20% formic acid into each well (final conc. 1%)
  20. Manually seal with Teflon plate seal and store at -80C until desalt

(Desalt)

***The following steps are performed manually using Waters Positive Pressure Vacuum Manifold:***

1. Thaw plates at room temp (ambient) keep on wet ice (4 oC)
2. Wash cartridge with 2 mL of 80% acetonitrile: 0.1% formic acid (do not collect effluent)
3. Equilibrate with 2 mL of 0.1% formic acid (do not collect effluent)
4. Add 0.5 mL of 0.1% formic acid
5. Add 10 µL of 13C/15N heavy peptide mastermix into each well (working stock= 10fmol/uL therefore 100 fmol per well)
6. Pipet sample onto cartridge, apply positive pressure (do not collect effluent)
7. Wash tube with 0.5mL and add rinseate to cartridge (do not collect effluent)
8. Wash cartridge with 4 mL 0.1% formic acid (do not collect effluent)
9. Elute with 2 volumes of 0.4 mL of 50% acetonitrile:0.1% formic acid into the same deepwell plate (total volume 800 µL)
10. Seal plate with Breathe-Easy adhesive membrane and freeze eluates at -80 °C
11. Place deepwell plate in drum of lyophilizer and bring eluate to dryness
12. Remove Breathe-Easy membrane and seal plate with silicone sealing mat then foil adhesive
13. Store at -80oC until shipment or analysis
14. Referenced Documents