STANDARD OPERATING PROCEDURE

Title: Trypsin Digestion of Cell Lysate, using automated liquid handler

Version #: 2 (500µg input) **Author: Paulovich lab**

Date: 8/17/2015

Purpose

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

Scope

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

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.

Equipment

- EpMotion Automated Pipetting System (Eppendorf).

Materials

- Trypsin, sequencing grade, (Promega, V5111)
- Urea, (Sigma, U0631)
- 0.5M TCEP, (Pierce, 77720)
- Iodoacetamide (IAM), (Sigma, A3221-10VL)
- 1M Tris (pH8.0), (Sigma, T2694)
- EDTA (Sigma, E7889)
- EGTA (Sigma, E0396)
- Water, HPLC grade (Fisher, W5-1)
- Acetonitrile, HPLC grade (Fisher, A998-1)
- Formic Acid (EDM, 11670-1)

- Sigma Phosphatase Inhibitor Cocktail 1 (Sigma, P2850)
- Sigma Phosphatase Inhibitor Cocktail 2 (Sigma, P5726)
- Deep-well 96-well plate (Eppendorf, 0030 502.248)
- Oasis HLB 96-well plate 30µm (5mg) (Waters, 186000309)
- Square well deep-well V-bottom 96-well plate (Thermo, 95040450).

Solutions

- Lysis Buffer. **Must be made fresh daily:**
  - 4 Parts 7.5M Urea (see below).
  - 1 Part 5x Lysis Buffer Stock Solution (see below).
  - Add 1% Sigma phosphatase cocktail 1.
  - Add 1% Sigma phosphatase cocktail 2.
  - Mix well.
- 5x Lysis Buffer Stock Solution. May be made in advance and stored at room temp.
  - 12.5mL 1M Tris (pH8.0).
  - 1.0mL 0.5M EDTA.
  - 1.0mL 0.5M EGTA.
  - Add HPLC water to 100mL.
  - Sterilize with 0.22µm filter.
- 7.5 M Urea. **Must be made fresh daily:**
  - Add 4.50g Urea to a 15mL Falcon tube.
  - Add 6mL HPLC water and mix until Urea is in solution.
  - Add HPLC water to a final volume of 10mL.
- 0.2M Tris, pH8.0:
  - 4 parts HPLC water.
  - 1 part 1M Tris, pH8.0.
- 0.5M Iodoacetamide (IAM) Stock Solution. **Prepare immediately before use and keep out of light:**
  - To one 56mg vial of iodoacetamide, add 605µL of 0.2M Tris pH 8.
  - Mix until dissolved.
- Trypsin, Sequence Grade (Promega):
  - 100µg resuspended in 200µL of 0.2M Tris pH 8. (see above).
  - **OR** thaw an aliquot @ 0.525ug/uL.
- Quench:
  - 4 parts HPLC water.
  - 1 part formic acid.
- Heavy peptide stock solutions: Heavy peptides may be obtained from synthetic peptide vendors. Prepare stock solutions of individual heavy peptides at 100-500 µM (pmol/µL) in Peptide storage solution (30% acetonitrile / 0.1% formic acid) in sealed screw-top microcentrifuge tubes. Store aliquots at -80 °C for 6-12 months.

- Heavy peptide working solution (SIS mix): Generate a master mix of the standard heavy peptides to yield an equal molar mix at 2 pmol/µL in 3% acetonitrile / 0.1% formic acid. Store aliquots at -80 °C for 3 months.

Procedure

Preparation of Samples

1. Cell lysate samples were prepared as described in SOP P-02 (Cell line lysis).
2. Adjust the cell lysate to 5mg/mL with lysis buffer.
3. Move 100uL of cell lysate to a deep well plate.
4. Add 6uL of 500mM TCEP.
5. Incubate with mixing at 700rpm for 30 min at 37 °C.
6. Add 14µL of 0.5 M IAM, to yield an IAM concentration of ~40 mM.
7. Alkylate at room temperature for 30 min in the dark.
8. Add 880uL of 200mM Tris, pH 8.0 to each tube to decrease the urea concentration to ~0.6M.
9. Add 20uL trypsin to each digest to achieve a 1:50 enzyme-to-substrate ratio.
10. Incubate with mixing at 700rpm for 2 h at 37 °C.
11. Add 10uL trypsin to each digest to achieve a 1:100 enzyme-to-substrate ratio.
12. Incubate with mixing at 700rpm for 16 h at 37 °C.
13. Add 54µL of 20% FA to each digest to quench the digestion for a final acid concentration of 1%.

Heavy SIS spike addition

1. The stock heavy SIS mix is at 2uM.
2. Dilute the stock SIS mix to 10 fmol/uL with 3% acetonitrile/0.1% formic acid.
3. 10uL of the diluted spike sample is mixed into each sample.

Desalting Samples Offline by Positive Pressure

1. Desalt the digested peptides on an Oasis HLB 96 well plate, or equivalent solid phase extraction medium.
    a. Condition the wells with 4 × 0.4 mL of 0.1% formic acid in 50% acetonitrile.
    b. Equilibrate the wells using 4 × 0.4 mL of 0.1% formic acid in water.
    c. Apply the digests to the wells.
    d. Wash with 4 × 0.4 mL of 0.1% formic acid in water.
    e. Elute peptides with 3 × 0.4 mL of 0.1% formic acid in 50% acetonitrile. Collect eluates in a 2 mL polypropylene 96 deep well plate.
2. Lyophilize the digests and store frozen (-70 °C) as dried powder until the next step.

Referenced Documents

SOP P-02 Cell line lysis.pdf