STANDARD OPERATING PROCEDURE

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

Version #: 1 **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 Gold, (Promega, V5280)
- 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 1mL of 0.2M Tris pH 8. (see above).
  - **OR** thaw an aliquot @ 0.525ug/uL and dilute 5x in 0.2M Tris pH 8.
- Quench:
  - 4 parts HPLC water.
  - 1 part formic acid.

Procedure

Preparation of Samples

1. Cell lysate samples were prepared as described in SOP P-01 (Cell line lysis).
2. Dilute the cell lysate to 2mg/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 600rpm 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 for the 200ug total protein present.
10. Incubate with mixing at 600rpm 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 600rpm 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 preparation

1. The stock heavy SIS mix is at 100nM.
2. 10µL of stock is thawed and diluted with 390uL 0.1% FA in 3% ACN.
3. 10uL of the diluted spike sample is mixed into each sample.

Desalting Samples Offline by Positive Pressure

1. Set the system pressure to 80psi.
2. Condition cartridge with 3 x 400uL of 0.1% formic acid in 80% ACN at 12psi.
3. Equilibrate cartridge with 4 x 400uL of 0.1% formic acid in 100% water at 12psi.
4. Add sample to cartridge at 12psi.
5. Wash cartridge with 4 x 400uL of 0.1% formic acid in 100% water at 6psi.
6. Elute peptides with 3 x 400uL 0.1% formic acid in 80% acetonitrile at 3psi into a deep well plate.
7. Freeze eluates on dry ice or at -80°C for approximately 1 hour.
8. Lyophilize samples overnight to dryness.
9. Samples can be stored lyophilized at -80°C until ready for IMAC enrichment as described in SOP P-03 (Phosphopeptide enrichment).

Referenced Documents

SOP P-03 Phosphopeptide enrichment.pdf
SOP P-01 Cell line lysis.pdf