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| 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 605L 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. 10L 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