|  |
| --- |
| **STANDARD OPERATING PROCEDURE** |
| |  |  | | --- | --- | | **Title: Trypsin Digestion of Cell Lysate** | | | **SOP#: D-01** |  | | **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
2. Materials

Urea: Sigma Ultra Cat# U0631

1 M Tris (pH8.0): Sigma Cat# T2194

EDTA: Sigma Cat# E7889

EGTA: Sigma Cat# E0396

TCEP: Sigma Cat# C4706

Trypsin Gold: Promega Cat# V5280

HPLC water: Fisher Cat# W6-4

Formic Acid: EDM Cat# 11670-1

Acetonitrile: Fisher Cat# A955-4

Oasis desalting cartridge.

Sigma Phosphatase Cocktail 1 Cat#P2850

Sigma Phosphatase Cocktail 2 Cat#P5726

1. Reagents

**Solutions**:

7.5 M Urea. Must be made fresh daily:

* 1. Add 4.50g Urea to a 15mL Falcon tube.
  2. Add 6mL HPLC water and mix until Urea is in solution.
  3. Add HPLC water to a final volume of 10mL.

0.2M Tris, pH8.0:

* 1. 4 parts HPLC water.
  2. 1 part 1M Tris, pH8.0.

0.5M Iodoacetamide (IAM) Stock Solution. Prepare immediately before use and keep out of light:

* 1. To one 56mg vial of iodoacetamide, add 605L of 0.2M Tris pH 8. (see above).
  2. Mix until dissolved.

Trypsin Gold (Promega):

* 1. 100μg resuspended in 1mL of 0.2M Tris pH 8. (see above).

Quench:

* 1. 4 parts HPLC water.
  2. 1 part formic acid.

1. Procedure

**A. Preparation of Samples**

1. Dilute the cell lysate to 2mg/mL with lysis buffer.
2. Move 50L of cell lysate to a deep well plate.
3. Add 33L of lysis buffer containing 50mM TCEP.
4. Incubate with mixing at 600rpm for 30 min at 37 °C.
5. Add 7μL of 0.5 M IAM, to yield an IAM concentration

of ~40 mM.

1. Alkylate at room temperature for 30 min in the dark.
2. Add 740L of 100mM Tris, pH 8.0 to each tube to decrease the urea concentration to 0.6M.
3. Add 20L trypsin to each digest to achieve a 1:50 enzyme-to-substrate ratio for the 50g total protein present.
4. Incubate with mixing at 600rpm for 2 h at 37 °C.
5. Add 10L trypsin to each digest to achieve a 1:100 enzyme-to-substrate ratio.
6. Incubate with mixing at 600rpm for 16 h at 37 °C.
7. Add 45μL of 20% FA to each digest to quench the digestion for a final acid concentration of 1%.

**B. Spiking Samples with 13C/15N Peptide Internal Standards**

1. Thaw the 400nM 13C/15N IS peptide mixtures from the three sites.
2. Combine the three mixtures with 0.1% FA in 3% ACN at a ratio of 1:1:1:1 for a final IS stock concentration of 100nM.
3. To each sample, add 10L of IS peptide mixture, for a final concentration of 10fmol IS per 1ug of digested cell lysate.

**C. Desalting Samples Offline by Positive Pressure**

1. Set the system pressure to 80psi.
2. Condition cartridge with 3 x 400L of 0.1% formic acid in 80% ACN at 12psi.
3. Equilibrate cartridge with 4 x 400L of 0.1% formic acid in 100% water at 12psi.
4. Add sample to cartridge at 12psi.
5. Wash cartridge with 4 x 400L of 0.1% formic acid in 100% water at 6psi.
6. Elute peptides with3 x 400L 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.

Samples can be stored lyophilized at -80°C until ready for SRM analysis.

**D. Reconstituting Samples**

**(To be performed just prior to executing LC-SRM)**

1. Reconstitute dried and desalted digests with 100L of 3% acetonitrile, 0.1% formic acid to each sample to achieve 1μg/μL digest solution for the cell lysates.
2. Vortex sample, spin down, and transfer ~95μL to an autosampler vial.
3. Referenced Documents