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| **CPTAC STANDARD OPERATING PROCEDURE** |
| |  |  | | --- | --- | | **Title: Preparation of Purified Tryptic Peptides from Cell lysates** | | |  |  | | **Version #: 1** | **Authors: Townsend Lab** | | **Date: March, 2021** |  | |

# Purpose

The purpose of this document is to describe preparation of tryptic peptides from proteins that are extracted from tissue culture cells. The preparation of desalted complex peptide mixture for utilization as a matrix for MS-based assay development is detailed.

# Scope

This procedure is used to reduce, alkylate and digest proteins as a complex mixture for LC-MS analysis. A method for trypsin digestion and peptide desalting is provided.

# 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.

# Materials

# Reagents

* BCA kit (Pierce, cat. no.23225)
* Urea (Millipore Sigma, cat. no. U4884)
* tris(hydroxymethyl)aminomethane (Tris) (Thermo Fisher Scientific, cat. no. BP152)
* Dithiothreitol (DTT) (Pierce, cat. no. A39255)
* Iodoacetamide (Pierce, cat. no. A39271)
* LysC (Waco, cat. no. 129-02541)
* Trypsin (Promega, cat. no. V5113)
* Acetonitrile (MeCN), LC-MS grade (JT Baker, cat. no. 9829-03)
* Methanol (MeOH) (Honeywell, cat. no. 34966 4x4L)
* Water, HPLC grade (JT Baker, cat. no. 909302)
* Formic Acid (FA) (Honeywell Fluka, cat. no. 94318)
* Trifluoroacetic acid (TFA) (Pierce, cat. no. 28904)

# Equipment

* Express Speedvac Concentrator SC250 (Thermo Fisher Scientific)
* Sorval RC 6 Plus Centrifuge (Thermo Fisher Scientific)
* Vantage Lyophilizer (SP Scientific)
* Thermomixer F (Eppendorf)
* 1.7 mL microcentrifuge tubes (Axygen, cat. no. MC-175-C)
* Autosampler vials, 250 μL polypropylene crimptop (VWR, cat. no. 200 046)
* Snap caps for autosampler vials, 11 mm, PTFE/DK blue silicone (SUN SRI, cat. no. 501 390)
* 15 mL conical centrifuge tubes (Fisher Scientific, cat. no. 05-539-12)
* 50 mL conical centrifuge tubes (Fisher Scientific, cat. no. 06-443-19)
* Sep-Pak Vac 3 cc (200 mg) tC18 Cartridges (Waters, cat. no. WAT054925)
* Glass vial, 4 mL (Fisher Scientific, cat. no. 03-339-22B)
* 100mL glass bottle (Fisher Scientific, cat. no. FB800100)

# Reagent setup

Lysis buffer containing DTT (500 mM)

Add 100 µL lysis buffer (Lysis buffer is described in SOP WU-SOP-CL-01)

to a vial of DTT. Vortex to dissolve.

Digestion buffer (50 mM Tris-HCl, pH 8)

Weigh 0.303 g Tris into 50 mL conical centrifuge tube. Add 40 mL water; stir to dissolve. Check pH and adjust to pH 8 if necessary. Add water to 50 mL.

Iodoacetamide (150 mM)

Add 335.5 µL DI water to vial of iodoacetamide. Vortex to dissolve.

Solid phase extraction elution buffer (50% MeCN, 0.1% (vol/vol) FA)

Add 50mL MeCN, 49.9 mL LC-MS grade water and 100µL of concentrated FA to a 100 mL glass bottle. Mix.

Solid phase extraction equilibration buffer (0.1% TFA)

Add 100µL of concentrated TFA to 99.9 mL LC-MS grade water in a 100 mL glass bottle. Mix.

Solid phase extraction wash buffer (1%(vol/vol) FA)

Add 1mL concentrated FA to 99mL LC-MS grade water in a 100mL glass bottle. Mix.

Resuspension buffer for peptide assay (2% (vol/vol) MeCN)

Add 2 mL MeCN to 98 mL LC-MS grade water in a 100 mL glass bottle. Mix.

# Procedure

# Digestion of Cell lysates

1. Digest 1 mg aliquots in a 1.7 mL microcentrifuge tube; protein concentration: 8mg/mL. The protein concentration is adjusted with lysis buffer to 8 mg/mL before freezing 1 mg aliquots.
2. Reduce protein with 5 mM DTT for 45 minutes at room temperature with mixing on the thermomixer by adding 1.25 µL of lysis buffer containing 500 mM DTT.
3. Alkylate proteins with 10 mM Iodoacetamide for 45 min in the dark at room temperature with mixing on the thermomixer by adding 8.4 µL 150 mM Iodoacetamide
4. Dilute 1:4 with 50 mM Tris-HCl, pH 8, to reduce the urea concentration to below 2 M (375 µL).
5. Add LysC (5 mU) and incubate for 2 hours at room temperature in the thermomixer.
6. Add trypsin at a ratio of 1:50 trypsin to protein and digest overnight at room temperature in the thermomixer.
7. Quench the reaction by adding 50% TFA to a final concentration of 1% TFA.
8. Desalt peptides using SepPak.

# Solid phase extraction of peptides

1. Label 15 mL conical tubes with the following labels: MeOH, wash, equilibrate, desalt,. One of each is required per SepPak.
2. Wet SepPak with 3 mL of 100% MeOH. Collect flow-through in conical tube marked “MeOH” and discard. Wash SepPak with 3 mL of 50% (vol/vol) MeCN, 0.1% (vol/vol) FA. Collect flow-through in conical tube and discard.
3. Equilibrate SepPak with 4x3 mL of 0.1% (vol/vol) TFA. Collect flow-through in conical tube and discard.
4. Load sample on SepPak using gravity flow. Collect flow through in a 15mL conical and re-load the sample onto the same SepPak.
5. Desalt SepPak with 3x3 mL 0.1% (vol/vol) TFA. Collect flow-through and discard.
6. Wash cartridge with 1x3 mL 1% FA.
7. Elute peptides with 2x 1.5 mL 50% (vol/vol) MeCN in 0.1% (vol/vol) FA and collect flow-through in 4 mL glass vial for lyophilization.
8. Freeze eluates at -80 °C for 30 min or until the lyophilizer shelf temperature has reached -60 °C.
9. Turn on Vantage lyophilizer and set shelf temperature to -60 °C. It will take 30-45 minutes to reach temperature.
10. Place frozen samples on the pre-chilled shelf with the cap half a turn open.
11. Turn on the condenser and set temperature to -70 °C. Once temperature is reached, turn on the vacuum. Vacuum is set to 200 mTor.
12. Once vacuum is reached, bring shelf temperature to 0 °C and let the samples lyophilize overnight (approximately 16 hours).
13. Next day, break vacuum, turn off condenser and take lyophilized samples out when atmospheric pressure has been reached.
14. Solubilize lyophilized peptides in 2 % (vol/vol) MeCN to a theoretical concentration of 10 µg/µL based on total protein input.
15. Freeze 100 µg aliquots at -80 °C and lyophilize to dryness. Use as a matrix for peptide assay development.

# Referenced Documents

SOP WU-SOP-CL-01 - Preparation of Soluble lysates from Cell pellets

# Abbreviations

Tris, tris(hydroxymethyl)aminomethane

DTT, Dithiothreitol

MeCN, Acetonitrile

MeOH, Methanol

FA, Formic acid

TFA, Trifluoroacetic acid