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| STANDARD OPERATING PROCEDURE |
| |  |  | | --- | --- | | **Title: Cell Lysis, Tryptic Digestion, and Phosphopeptide Enrichment by Automated Immobilized Metal Affinity Chromatography (IMAC)** | | |  |  | | **Version #: 3** | **Author: Broad Institute Proteomics Platform – Carr Lab** | | **Date: August 1, 2016** | **BRD-001** | |

# Purpose

The purpose of this document is to describe the procedures for cell lysis, tryptic digestion, and automated phosphorylated peptide enrichment by immobilized metal affinity chromatography (IMAC) of samples for analysis by mass spectrometry.

# Scope

This procedure may be used to make a cell lysate, prepare a tryptic digest, and enrich for phosphorylated peptides.

# 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.

# Safety

* Use proper safety precautions when handling Iron (III) chloride. It is extremely reactive. It is extremely sensitive to air movement and static electricity and will cause skin and eye burns and other chronic damage to internal organs if inhaled or ingested.

# Equipment

1. Microcentrifuge
2. Benchtop vortex mixer
3. Incubator
4. Agilent Bravo Automated Liquid-Handling Robot with AssayMAP head

# Materials

1. tC18 SepPak cartridges(Waters, 500 mg WAT036790)
2. Agilent Bravo AssayMAP Fe(III)-NTA cartridges (Agilent #G5496-60085)
3. Agilent Bravo AssayMAP Reversed Phase (RP-S) cartridges (Agilent # G5496-60033)

# Reagents

**Cell Lysis Reagents**

1. urea
2. Sodium Chloride (NaCl)
3. Tris
4. EDTA
5. aprotinin (Sigma, A6103)
6. leupeptin (Roche, #11017101001)
7. PMSF (Sigma, 78830)
8. Sodium fluoride (NaF)
9. iodoacetamide (IAA) (Sigma, A3221)
10. Phosphatase Inhibitor Cocktail 2 (Sigma, P5726)
11. Phosphatase Inhibitor Cocktail 3 (Sigma, P0044)
12. HPLC grade water

**BCA Assay**

1. BCA assay (Pierce, 23227)

**Reduction, Alkylation, Digestion**

1. dithiothreitol (DTT) (Thermo Scientific, 20291)
2. iodoacetamide (IAA)
3. Tris HCl, pH 8.0

**Digest Solutions**

1. LysC (Wako, 125-05061)
2. Trypsin (Promega, V511X)
3. formic acid (Fluka, 56302)
4. HPLC grade water

**Desalt**

1. acetonitrile (ACN)
2. formic acid (FA)
3. trifluoroacetic acid (TFA) (Fluka, TX1276-6)
4. HPLC grade water

**Automated IMAC Enrichment – Tip Prep Reagents**

1. HPLC grade water
2. EDTA
3. Iron chloride (FeCl3) (Sigma, 451649)

**Automated IMAC Enrichment – pSTY Enrichment Solutions**

1. acetonitrile
2. methanol
3. acetic acid
4. trifluoroacetic acid (Fluka, TX1276-6)
5. potassium phosphate dibasic (K2HPO4)
6. HPLC grade water

**Automated IMAC Enrichment – Desalt Solutions**

1. acetonitrile (ACN)
2. trifluoroacetic acid (TFA) (Fluka, TX1276-6)
3. HPLC grade water

# Solutions

**Cell Lysis Solutions**

1. Lysis Buffer – For lysis of 1E6 suspension cells use at least 1 mL lysis buffer (to obtain a protein concentration < 5 mg/mL)

8 M Urea

75 mM NaCl

50 mM Tris pH 8.0

1 mM EDTA pH 8.0

*Add immediately before use the following additives:*

2 µg/ml Aprotinin (1:500 of 1 mg/mL in water)

10 µg/ml Leupeptin (1:200 of 2mg/mL in water)

10 mM NaF (1:100 of 1 M stock in water

PIC3 (1:100 Phosphatase inhibitor cocktail 1)

PIC2 (1:100 Phosphatase inhibitor cocktail 2)

1 mM PMSF (1:100 of 100 mM stock in Ethanol)

Ex. to make 5 mL lysis buffer, add:

2.4 g urea

750 uL 1M NaCl

500 uL 1M Tris pH 8.0

20 uL 500mM EDTA pH 8.0

add water to make total volume 4.5 mL

*Add the following additives* ***immediately*** *before use*:

20 uL 2 µg/mL Aprotinin (1:500 of 1 mg/mL in water)

50 uL 10 µg/mL Leupeptin (1:200 of 2mg/mL in water)

100 uL 10 mM NaF (1:100 of 1 M stock in water)

100 uL PIC3 (1:100 Phosphatase inhibitor cocktail 1)

100 uL PIC2 (1:100 Phosphatase inhibitor cocktail 2)

100 uL 1 mM PMSF (1:100 of 100 mM stock in Ethanol)

**Digest Solutions**

1. 5 mM DTT
2. 10 mM IAA
3. 10% Formic Acid

**Desalt Solutions**

1. 50mM Tris HCl, pH 8.0
2. 50% acetonitrile/0.1% formic acid
3. 1% formic acid
4. 0.1% trifluoroacetic acid

**Automated IMAC Enrichment – Tip Prep Solutions**

1. HPLC grade water
2. 100mM EDTA
3. 10mM FeCl3

**Automated IMAC Enrichment – pSTY Enrichment Solutions**

1. 1:1:1 ACN:MeOH:0.01%AceticAcid
2. 80% ACN / 0.1% TFA
3. 500mM K2HPO4, pH 7

**Automated IMAC Enrichment – Desalt Solutions:**

1. 50% ACN/ 0.1% TFA
2. 0.1% TFA

# Procedure

**Cell Lysis**

1. Add 1mL cold (4°C ) lysis buffer to cell pellet containing approximately 1E6 cells (target protein concentration < 5 mg/mL)
2. Incubate on wet ice with occasional vortexing for 30 min.
3. Centrifuge at 20,000 x g to remove cell debris at 15°C for 15 min.
4. Transfer supernatant to a fresh tube
5. Measure protein concentration using BCA.

**Sample Digestion**

1. Reduce denatured proteins with 5 mM DTT for 30 min at 37°C .
2. Alkylate proteins with 10 mM IAA for 45 min at room temperature in the dark.
3. Dilute sample 1:4 with 200 mM Tris pH 8.0 to decrease urea concentration below 2 M.
4. Add endoproteinase Lys-C (Wako) to an enzyme to substrate ratio of 1:50 and incubate at 800 RPM at 37°C for 2h.
5. Add trypsin (Promega) to an enzyme to substrate ratio of 1:50 and incubate at 800 PRM at 37°C overnight (~ 16h).
6. Add formic acid to 10% to acidify the digest to pH 1.5 – 2 to quench enzymatic activity.

**Desalt via tC18 SepPak (Waters, 500 mg WAT036790)**

1. Condition cartridge with 5 mL 100% acetonitrile followed by 5 mL 50% acetonitrile / 0.1% FA.
2. Equilibrate with 4 x 5 mL of 0.1% TFA.
3. Load sample.
4. Wash/desalt with 3 x 5 mL of 0.1% TFA.
5. Wash/desalt with 1 x 5mL of 1% FA (to remove TFA).
6. Elute with 2 x 3mL of 50 % acetonitrile / 0.1 % FA.
7. Aliquot to 5 mg/tube in 2 mL tubes (Sarstedt).
8. Freeze eluate with liquid N2 (or at -80°C) and lyophilize (or speed-vac) to dryness and store in -80°C freezer.

**Sample Plate Preparation**

1. Thaw and resuspend a pellet of digested desalted cell lysate (5 mg/tube) in 1 mL 50 %ACN/ 0.1% TFA. Vortex thoroughly and centrifuge briefly (20 s at 2000 x g).
2. Add 1.5 mL 100% ACN/ 0.1% TFA. Transfer to 15 mL tube (Falcon). Repeat vortexing and centrifugation. Samples are now at 80% ACN/ 0.1% TFA.

**Automated IMAC Enrichment using Fe(III)-NTA AssayMAP cartridges and Desalt using AssayMAP Reversed Phase (RP-S) cartridges**

**Automated IMAC Enrichment Steps (Agilent Workbench Protocol: Phosphopeptide\_enrichment\_v2.0)**

1. Prime Fe(III)-NTA cartridges with 100 uL of 1:1:1 (*100 uL/min*).
2. Equilibrate cartridges with 80% ACN/0.1% TFA.
3. Load sample in dispense mode (*5 uL/min).*
4. Wash cup with 25 uL 80% ACN/0.1% TFA .
5. Wash sample with 50 uL 80% ACN /0.1% TFA (*10 uL/min*).
6. Stringent syringe wash with 50 uL 500 mM K2HPO4.
7. Elute peptides from cartridges with 50 uL of 500 mM K2HPO4 (*5 uL/min*).
8. *(Manual)* Freeze flowthrough at -80°C. Remove the IMAC cartridges, place in a labeled box and store dry at room temp.

**Automated Desalt Protocol**

1. Prime RP-S cartridges with 50 uL 80% ACN/0.1% TFA (*10 uL/min*).
2. Equilibrate RP-S cartridges with 50 uL 0.1% TFA (*10 uL/min*).
3. Load sample in dispense mode (*2 uL/min*).
4. Wash cup with 25 uL 0.1% TFA.
5. Wash sample with 50 uL 0.1% TFA (*10 uL/min*).
6. Wash syringe with 50 uL 50% ACN/0.1% TFA.
7. Elute peptides with 50 uL 50% ACN/0.1% TFA (*2 uL/min*).
8. *(Manual)* After protocol completes, cover flowthrough plate with a foil sealmat. Freeze at -80°C.
9. *(Manual)* Transfer each sample into autosampler vials. Freeze at -80°C.
10. *(Manual)* Speedvac autosampler vials to dryness.

# Referenced Documents

Protocol for automated IMAC enrichment:

Abelin et al Reduced-representation Phosphosignatures Measured by Quantitative Targeted MS Capture Cellular States and Enable Large-scale Comparison of Drug-induced Phenotypes.

[Mol Cell Proteomics.](http://www.ncbi.nlm.nih.gov/pubmed/26912667) 2016 May;15(5):1622-41. doi: 10.1074/mcp.M116.058354. Epub 2016 Feb 24. PMID:26912667