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| STANDARD OPERATING PROCEDURE |
| |  |  | | --- | --- | | **Title: Cell Lysis, Tryptic Digestion, and Phosphopeptide Enrichment by Automated Immobilized Metal Affinity Chromatography (IMAC)** | | |  |  | | **Version #: 1** | **Author: Broad Inst Proteomics Platform** | | **Date: May 25, 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 injested.

# 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 96AM cartridges packed with Ni-NTA superflow agarose beads (Qiagen, #1018611)

**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. sodium butyrate
10. iodoacetamide (IAA) (Sigma, A3221)
11. Phosphatase Inhibitor Cocktail 2 (Sigma, P5726)
12. Phosphatase Inhibitor Cocktail 3 (Sigma, P0044)
13. 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. trypsin (Promega, V511X)
2. formic acid (Fluka, 56302)
3. 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

**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. Estimate protein concentration of supernatant by A660 Protein Assay.
   1. dilute samples 1:5 in water (5 uL sample + 20uL water)
   2. add 150 uL reagent to 10 uL diluted sample
   3. make BSA curve with 1:5 diluted lysis buffer
   4. measure absorbance at 660 nm in duplicate

**Sample Digestion**

1. Reduce denatured proteins with 5 mM DTT for 45 min at 37°C.
2. Alkylate proteins with 10 mM IAA for 45 min in the dark at room temperature.
3. Dilute sample 1:4 with 50 mM Tris pH 8.0 to decrease urea concentration below 2 M.
4. Add trypsin (enzyme to substrate ratio (1:50) and incubate at 800 RPM and 37°C overnight.
5. Acidify digest by adding 0.5% TFA to pH < 2.
6. Add methanol to 3% to improve loading on C18.
7. Urea may precipitate at this step, centrifuge peptide solution for 5 min at 10,000 x g to remove precipitate.
8. Transfer supernatant to a fresh tube.

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

1. Condition cartridge with 1 mL methanol, followed by 1 mL 50% methanol/0.1% FA.
2. Equilibrate with 4 x 1 mL 0.1% TFA.
3. Load sample.
4. Wash cartridge with 3 x 1 mL 0.1% TFA.
5. Wash cartridge with 1 x 1mL of 1% FA (to remove TFA).
6. Elute peptides from cartridge with 2 x 750 uL of 50 % methanol/0.1 % FA.
7. Based on protein concentration, aliquot approximately 500 ug/well into a 96 well plate.
8. Freeze plate at -80°C, cover with Breathe-EASIERTM (Diversified Biotech No. BERM-2000) and lyophilize or speed-vac to dryness.

**Sample Plate Preparation**

1. Resuspend dried pellets by adding 88 uL 100%ACN/0.1% TFA to each well.
2. Sonicate for 10 min, vortex thoroughly and centrifuge briefly (1 min) at 1500 x g.
3. Add 132 uL 50%ACN/ 0.1%TFA.
4. Repeat sonication, vortexing and centrifugation.

*Samples are now in 80%ACN/0.1%TFA and ready for IMAC enrichment*

1. Add 10 uL of stable isotope standard peptides to reach the designated concentrations (fmol/ug) as specified in the procedures for Experiment 1 (curves) and Experiment 2 (repeatability).

**Automated IMAC Enrichment using AssayMAP Nickel NTA (NiNTA) cartridges and Desalt using AssayMAP RPS cartridges**

**Preparation of AssayMAP IMAC NiNTA Cartridges**

1. Add sufficient cartridges onto AssayMAP Bravo for number of samples being enriched.
2. Use dummy cartridges (cartridges without resin) to fill in the remaining wells in each column that are not being used (AssayMAP Bravo pressures are applied based on number of complete columns).
3. Wash AssayMAP cartridges 3 times with 25 uL water at 25 uL/min.
4. Strip nickel from cartridges 2 times with 25 uL 100mM EDTA at 2 uL/min.
5. Wash cartridges 3 times with 25 uL water at 25 uL/min.
6. Load iron onto stripped cartridges 2 times with 25 uL 10mM FeCl3 at 2uL/min.
7. Wash unbound FeCl3 from cartridges with 25 uL water at 25 uL/min.

**Phosphorylated peptide (pSTY) Enrichment on AssayMAP FeNTA Cartridges**

1. Prime cartridges with 100 uL of ACN:methanol:0.01% acetic acid (1:1:1) at 300 uL/min.
2. Load sample in dispense mode at 5 uL/min.
3. Load sample in dispense mode at 2 uL/min)
4. Wash cup 2 times with 50 uL 80% ACN/0.1% TFA at 5 uL/min
5. Wash sample once with 50 uL 80% ACN/0.1% TFA at 5 uL/min.
6. Wash syringe 2 times with 50 uL 500mM K2HPO4 at 5 uL/min.
7. Elute peptides from cartridges with 50 uL of 500 mM K2HPO4 at 5 uL/min.
8. ***(Manual)*** Freeze flow through at -80°C.
9. ***(Manual)*** Remove the IMAC tips and place in a labeled box, store dry at room temperature.

**Desalt of IMAC enriched peptides on AssayMAP RPS Cartridges**

1. Prime desalting RPS cartridges with 150 uL 50% ACN/0.1% TFA at 300 uL/min.
2. Equilibrate cartridges with 50uL 0.1% TFA at 25 uL/min.
3. Load sample in aspirate mode at 2 uL/min.
4. Wash cup 2 times with 50 uL 0.1% TFA.
5. Wash sample with 50 uL 0.1% TFA at 25 uL/min.
6. Wash syringe 2 times with 50 uL 50% ACN/0.1% TFA at 5 uL/min.
7. Elute peptides 2 times with 25 uL 50% ACN/0.1% TFA at 5 uL/min.
8. ***(Manual)*** At the end of the Bravo protocol, cover the flow through plate with a foil seal mat and freeze at -80°C.
9. ***(Manual)*** Transfer samples into HPLC autosampler vials and freeze at -80°C.
10. ***(Manual)*** Rotoevaporate HPLC autosampler vials and speedvac to dryness.

# Referenced Documents

For complete details of protocols for cell lysis, digestion and automated IMAC enrichment:

[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

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

[Abelin JG](http://www.ncbi.nlm.nih.gov/pubmed/?term=Abelin%20JG%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Patel J](http://www.ncbi.nlm.nih.gov/pubmed/?term=Patel%20J%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Lu X](http://www.ncbi.nlm.nih.gov/pubmed/?term=Lu%20X%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Feeney CM](http://www.ncbi.nlm.nih.gov/pubmed/?term=Feeney%20CM%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Fagbami L](http://www.ncbi.nlm.nih.gov/pubmed/?term=Fagbami%20L%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Creech AL](http://www.ncbi.nlm.nih.gov/pubmed/?term=Creech%20AL%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Hu R](http://www.ncbi.nlm.nih.gov/pubmed/?term=Hu%20R%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Lam D](http://www.ncbi.nlm.nih.gov/pubmed/?term=Lam%20D%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Davison D](http://www.ncbi.nlm.nih.gov/pubmed/?term=Davison%20D%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Pino L](http://www.ncbi.nlm.nih.gov/pubmed/?term=Pino%20L%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Qiao JW](http://www.ncbi.nlm.nih.gov/pubmed/?term=Qiao%20JW%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Kuhn E](http://www.ncbi.nlm.nih.gov/pubmed/?term=Kuhn%20E%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Officer A](http://www.ncbi.nlm.nih.gov/pubmed/?term=Officer%20A%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Li J](http://www.ncbi.nlm.nih.gov/pubmed/?term=Li%20J%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)2, [Abbatiello S](http://www.ncbi.nlm.nih.gov/pubmed/?term=Abbatiello%20S%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1,[Subramanian A](http://www.ncbi.nlm.nih.gov/pubmed/?term=Subramanian%20A%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Sidman R](http://www.ncbi.nlm.nih.gov/pubmed/?term=Sidman%20R%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)2, [Snyder E](http://www.ncbi.nlm.nih.gov/pubmed/?term=Snyder%20E%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)3, [Carr SA](http://www.ncbi.nlm.nih.gov/pubmed/?term=Carr%20SA%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)1, [Jaffe JD](http://www.ncbi.nlm.nih.gov/pubmed/?term=Jaffe%20JD%5BAuthor%5D&cauthor=true&cauthor_uid=26912667)4.