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| STANDARD OPERATING PROCEDURE |
| |  |  | | --- | --- | | **Title: Response Curve** | | |  |  | | **Version #: 3** | **Author: PNNL Lab** | | **Date: 06/01/2016** |  | |

# Purpose

The purpose of this document is to describe the characterization of a set of assays by response curve.

# Scope

This procedure covers overall preparation and running of samples for generating the response curve.

# 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

Microcentrifuge

Eppendoff Thermomixer

# Materials

Waters glass vial

# Solutions

Mobile phase A: 0.1% FA in H2O

# Reagents

Water, HPLC grade (H2O)

Acetonitrile, HPLC grade (ACN) (Fisher Scientific, A955-4)

Formic Acid (0.1%)/Acetonitrile (EMD, FX0437P-1)

Formic Acid (FA) (Agilent Technologies, G2453-85060)

WB1:0.3M Phthalic acid in 80% ACN/5%TFA

WB2: 80% ACN/5%TFA

EB: 5% NH4OH in 50% ACN, pH  10.5

**Peptide Standards:**

Both pure heavy stable isotope-labeled peptides and sequence matched pure light versions were synthesized. Heavy peptides incorporated a fully atom labeled 13C and 15N isotope at the C-terminal lysine (K) or arginine (R) position of each (tryptic) peptide, resulting in a mass shift of +8 or +10 Da, respectively. Those pure peptides were purified to >95% purity by HPLC from the vendor. They were quantified by amino acid analysis and aliquots were stored in 5% acetonitrile/0.1% formic acid at -80°C until use. Pure light peptides are spiked in as internal standards (IS). The stock of light internal standard was stored in -80 ºC freezer. Different heavy peptides were spiked in at different concentration level depending on the response of peptides.

**Matrix:**

A background matrix consisting of ovarian cancer tumor cell digest was freshly prepared. Cell sample was processed as described in SOP TP-1 (Cell sample Preparation). Digestion was performed according to SOP TD-1 (Trypsin Digestion of cell lysis sample). The cell digest was aliquoted and stored in -80 for the response experiment.

# Procedure:

**Preparation of Phosphopeptide Samples for LC-MRM by TiO2 Enrichment**

1. The following is designed to create 9-13 points of varying concentrations of analyte (pure heavy labelled peptide) depending on the peptide response and 1 blank.
2. The pure heavy peptide stock is serially diluted with cell digest matrix (0.1 µg/µl).
3. 4 µl of each concentration point of heavy stock is added to 34 µL of the digested cell lysis matrix. (By doing this, heavy peptide standard only account for less than 10% of final total volume).
4. Each 200 µg cell sample digestions were spiked in 60 fmol light IS peptides and different amount heavy peptides, and speed-vacuum to dry.
5. Pack TiO2 beads column (peptide: beads=1:12) by adding beads solution (10ug/µl beads in ACN solution) and centrifuge 3000g for 3 min.
6. Resuspend sample with 150 uL WB1 and Centrifuge at 3000xg for 10 min at 4°C.
7. Load the sample supernatants to column by centrifuging at 500xg for 15min at 4°C, save the Flow-through for next loading.
8. Wash column with 100uL WB1 by centrifuging at 1000xg for 15min at 4°.
9. Re-load the first flow-through sample to column column by centrifuging at 500xg for 15min at 4°C.
10. Wash column with 150 µl WB1 twice by centrifuging at 1000xg for 15min at 4°.
11. Wash column with 150 µl WB2 twice by centrifuging at 1000xg for 15min at 4°.
12. Elute column with 100 µl EB twice by centrifuging at 1000xg for 15min at 4°.
13. Elute solution was dried out by speed-vacuum.
14. Each samples were dissolved by 25 ul 0.1% FA solution. Shake the vial on thermomixer with 800 rpm, 4 ºC for 10 min. All samples are prepared in Waters glass vial.
15. Put all samples into LC autosampler and get ready for LC-MRM detection (See SOP LC-1 for Liquid Chromatography and SOP PM-1 for Peptide MRM on TSQ Vantage).
16. 4 µl of sample is used for each run with the run order of blank, low concentration to high concentration as a batch, and acquire the data in three replicates at each concentration point.

# Referenced Documents

SOP TD-1 for Trypsin Digestion of cell sample.pdf

SOP TP-1 for cell Sample Preparation.pdf

SOP LC-1 for Liquid Chromatography.pdf  
SOP PM-1 for Peptide MRM on TSQ Vantage.pdf