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| STANDARD OPERATING PROCEDURE |
| |  |  | | --- | --- | | **Title: Mini-validation of Samples (Repeatability)** | | |  |  | | **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 according to its repeatability of measurement over 5 days. This is to estimate the performance of the assay measured in a complex sample over multiple days.

# Scope

This procedure covers overall preparation and running of samples for generating the validation samples with regards to CPTAC Assay Characterization Guidance experiment #2.

# 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

Mobile phase B: 0.1% FA in ACN

# 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. Pure heavy peptides were mixed together at three different concentrations, including low, median, and high and further stored in -80 ºC until use.

**Matrix:**

A background matrix consisting of ovarian cancer tumor cell digest (PA-1 and SKOV3 cell lines) was used for experiment 2. 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 sample). The cell digest was aliquoted and independent digestions were performed for each replicate of repeatability validation to make complete process replicates for each sample. Following digestion, cell digest was first diluted to a concentration of 0.1 µg/µl and prepared for future use.

# Procedure

**Determination of spike levels and preparation of samples**

1. Peptides were multiplexed according to the LLOQ and linear range determined from the response curves (experiment 1) in order to prepare validation samples at an appropriate concentration. Pure heavy peptides were spiked into cell digest matrix with the following spike levels:

Low: 7.5fmol; 30 fmol; 100fmol LOQ

Med: 1000fmol LOQ

High: 2500fmol LOQ

1. The stock heavy peptide mix is serially diluted with cell digest matrix (0.1 µg/µl) to create the required concentration above.
2. Each 200 µg cell sample digestions were spiked in 60 fmol light IS peptides and different amount heavy peptides, and lyophylization by speed-vacuum.
3. Pack TiO2 beads column (peptide: beads=1:12) by adding beads solution (10ug/µl beads in ACN solution) and centrifuge 3000g for 3 min.
4. Resuspend sample with 150 µL WB1 and Centrifuge at 3000xg for 10 min at 4°C.
5. Load the sample supernatants to column by centrifuging at 500xg for 15min at 4°C, save the Flow-through for next loading.
6. Wash column with 100uL WB1 by centrifuging at 1000xg for 15min at 4°.
7. Re-load the first flow-through sample to column column by centrifuging at 500xg for 15min at 4°C.
8. Wash column with 150 µl WB1 twice by centrifuging at 1000xg for 15min at 4°.
9. Wash column with 150 µl WB2 twice by centrifuging at 1000xg for 15min at 4°.
10. Elute column with 100 µl EB twice by centrifuging at 1000xg for 15min at 4°.
11. Elute solution was dried out by speed-vacuum.
12. Each samples were dissolved by 25 µl 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.
13. 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).
14. Analyze different samples in triplicates in randomized order on 5 consecutive days, 4 µl of sample is used for each run.

# 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