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| STANDARD OPERATING PROCEDURE |
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| **Title: Mini-validation of Samples (Repeatability)** |
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| **Version #: 1** | **Author: PNNL Lab** |
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# 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)

**Peptide Standards:**

Crude heavy stable isotope-labeled peptides and sequence matched pure light versions were synthesized. Pure light peptides were purified to >95% purity by HPLC from the vendor and spiked in as internal standards (IS). Light peptides were quantified by amino acid analysis and aliquots were stored in 5% acetonitrile/0.1% formic acid at -80°C until use. Different light peptides were spiked in at different concentration level depending on the response of peptides and served as light stable isotope standard (SIS). The stock of light internal standard was stored in -80 ºC freezer. Crude 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. Crude heavy peptides were mixed together at three different concentrations, including low, median, and high and further stored in -80 ºC until use. And the concentration of crude heavy peptides was estimated from the ratio of heavy/light ratio times the known concentration of pure light peptides.

**Matrix:**

A background matrix consisting of ovarian cancer tumor tissue digest was used for experiment 2. Tissue sample was processed as described in SOP TP-1 (Tissue sample Preparation). Digestion was performed according to SOP TD-1 (Trypsin Digestion of tissue sample). The tissue digest was aliquoted and independent digestions were performed for each replicate of repeatability validation to make complete process replicates for each sample. Following digestion, tissue digest was first diluted to a concentration of 0.25 ug/ul 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. Crude heavy peptides were spiked into tissue digest matrix with the following spike levels:

Low: 2-3 LOQ

Med: 50-60 LOQ

High: half of the highest concentration from experiment 1

1. The stock heavy SIS mix is serially diluted with tissue digest matrix (0.25 ug/ul) to create the required concentration above.
2. 2 ul of each concentration point of heavy SIS mix is added to different aliquots of tissue digest matrix (36 ul) to create low, med and high concentrations of heavy peptide for each day.
3. 2 ul of light peptide mix is added to each sample, and the final volume of each sample is 40 ul, while both heavy and light peptide mix account for 5% of final volume.
4. Store sample in the autosampler (4 ºC) and get ready for MRM analysis (See SOP LC-1 for Liquid Chromatography and SOP PM-1 for Peptide MRM on TSQ Vantage).
5. Analyze different samples in triplicates from low concentration to high concentration on 5 consecutive days.

# Referenced Documents

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

SOP TP-1 for Tissue Sample Preparation.pdf

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