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| STANDARD OPERATING PROCEDURE |
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| **Title: Validation samples preparation (Assessment of repeatability)** |
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| **Version #: 1** | **Author: Paulovich Lab with input from CPTAC Assay Development Working Group** |
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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

* Waters positive pressure displacement

# Materials

* HPLC water: Fisher Cat# W6-4
* Formic Acid: EDM Cat# 11670-1
* Acetonitrile: Fisher Cat# A955-4

# Reagents

**Standards:**

* Heavystable isotope-labeled standards (SIS) were synthesized and purified to >95% purity by HPLC. 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. Peptides were quantified by amino acid analysis and aliquots were stored in 30% acetonitrile/0.1% formic acid at -80°C until use.
* Synthetic peptide standards were synthesized and purified to >95% purity by HPLC. Peptides were quantified by amino acid analysis and aliquots were stored in 5% acetonitrile/0.1% formic acid at -80°C until use.
* The stock heavy SIS mix is at 100 nM
* The stock light synthetic peptide mix is at 100nM

**Matrix:**

* An equal mix (by total protein mass) of 10 breast cancer-related cell lines (BT474, HCC1187, HCC1419, HCC1954, HCC70, MCF12A, MDAMB175VII, MDAMB415, ZR751, ZR7530) was used as a background matrix. Lysates were prepared as described in SOP P-1 (Cell line lysis). The pooled lysate was aliquotted and independent digestions were performed for each replicate of repeatability validation to make complete process replicates for each sample. Following digestion, heavy synthetic peptide was added to the samples at three concentrations (low, med, high) and the samples were desalted. Measurements of heavy:light (endogenous) ratio were used as validation of repeatability at the concentrations described below. Digests for different days are performed >= 24 hours apart.

# Procedure

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

1. Peptides were multiplexed according to the LLOQ and linear range determined from the response curves in order to prepare Validation Samples at an appropriate concentration. Therefore peptides were grouped into 4 groups with the following heavy SIS spike levels in three levels:
	1. Group 1: Low – 0.5 nM, Med – 10 nM, High – 100 nM
	2. Group 2: Low – 2 nM, Med – 10 nM, High – 100 nM
	3. Group 3: Low – 2 nM, Med – 10 nM, High – 50 nM
	4. Group 4: Low – 10 nM, Med – 50 nM, High – 100 nM
2. The stock heavy SIS mix at 100nM is serially diluted with 0.1% formic acid / 3% acetonitrile to create the five concentrations above.
3. 100 uL of each concentration point of heavy SIS mix is added to independently digested aliquots (see SOP-D-02 for digestion protocol) of the background matrix to create three replicates of low, med, and high concentrations of heavy peptide for each day. Each aliquot of background matrix contains 100ug of total protein.
4. For peptides with endogenous signal above the LLOQ, no light peptide was added. For peptides with endogenous signal below the LLOQ, 10uL of light peptide mix is added to each sample, for a final concentration of 10nM.
5. The digested matrix plus heavy peptides (and light if added) are desalted (see step B).

**B. Desalting Samples Offline by Positive Pressure**

1. Set the system pressure to 80psi.
2. Condition desalting plate with 3 x 400μL of 0.1% formic acid in 80% ACN at 12psi.
3. Equilibrate desalting plate with 4 x 400μL of 0.1% formic acid in 100% water at 12psi.
4. Add sample to desalting plate at 12psi.
5. Wash with 4 x 400μL of 0.1% formic acid in 100% water at 6psi.
6. Elute peptides with3 x 400μL 0.1% formic acid in 80% acetonitrile at 3psi into a deep well plate.
7. Freeze eluates on dry ice or at -80°C for approximately 1 hour.
8. Lyophilize samples overnight to dryness.
9. Samples can be stored lyophilized at -80°C until ready for SRM analysis.

**C. Reconstituting Samples (To be performed just prior to executing LC-SRM)**

1. Reconstitute dried and desalted digests with 100μL of 3% acetonitrile, 0.1% formic acid to each sample to achieve 1μg/μL digest solution for the cell lysates.
2. Vortex sample, spin down, and transfer ~95μL to an autosampler vial.
3. LCMS analysis is performed according to SOP LC-01 and SOP MS-01.

**D. Run Order**

1. Samples are run in increasing order of concentration, one pass per replicate. System suitability standards (SSS) are acquired in between replicates to check instrument performance.

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| **Sample** |
| **Initial conditions solvent blank (2x for dual column systems)** |
| **SSS replicate (2x for dual column systems)** |
| **Yeast blank** |
| **Low QC, replicate 1** |
| **Low QC, replicate 2** |
| **Low QC, replicate 3** |
| **Med QC, replicate 1** |
| **Med QC, replicate 2** |
| **Med QC, replicate 3** |
| **High QC, replicate 1** |
| **High QC, replicate 2** |
| **High QC, replicate 3** |
| **Wash (2x for dual column systems)** |

# Referenced Documents

SOP D-02 Trypsin digestion in cell lysate.pdf

SOP LC-01 Liquid Chromatography Ultra nanoflex dual col.pdf

SOP MS-01 peptide MRM on 5500 QTRAP.pdf

SOP P-01 Cell Line Lysis.pdf