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| STANDARD OPERATING PROCEDURE |
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| **Title: Mini-Validation of Repeatability** |
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# Purpose

The purpose of this document is to describe the protocol used to characterize a set of assays based on its repeatability of measurement over 5 days. Estimation as to the performance of the assay measured in a complex sample across several days is thus facilitated.

# Scope

This procedure addresses the preparation and running of samples for generating validation samples in accordance with 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
* Vacuum centrifuge

# Materials

* Water: Optima LC/MS-grade (Fisher Scientific; cat. # W6-4)
* Acetonitrile: Optima LC/MS-grade (Fisher Scientific; cat. # A955-4)
* Formic Acid: Pierce Formic Acid, LC-MS grade (Pierce; cat. # 28905)
* Autosampler 96-well plate: PCR plate, 150 µL max. well volume (Eppendorf; cat. # 951020401)
* Autosampler 96-well plate cover: Adhesive free film (Excel Scientific; cat. # ZAF-PE-50)

# Reagents

* Stable isotope-labeled standards (SIS)
	+ Crude SIS phosphorylated peptides were synthesized (~60% purity) by Thermo Fisher Scientific (PEPotec SRM peptide library). SIS 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 provided in 0.1% TFA/50% ACN and stored at -80 °C until use.
	+ A stock SIS mix was prepared at a concentration of 4 pmol/µL for each peptide.
* Stable non-labeled standards (IS)
	+ Crude IS phosphorylated peptides were synthesized (~60% purity) by Thermo Fisher Scientific (PEPotec SRM peptide library). Peptides were provided in 0.1% TFA/50% ACN and stored at -80 °C until use.
	+ A stock IS mix was prepared at a concentration of 200 fmol/µL for each peptide
* Matrix
	+ A background matrix consisting of HEK293 cell lysate was prepared as described in the SOP entitled “Lysis of cell lines.” Digestion of an aliquot of the lysate was performed as described in the SOP entitled “Trypsin digestion of cell lysate.” Following digestion, aliquots were constructed, lyophilized, and stored at -80**°**C until subsequent processing. Separate aliquots of this background matrix were used to make each validation sample. Validation samples were generated on the day of analysis following the protocol described below.

# Procedure

1. 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 the CPTAC Assay Characterization Guidance Experiment #1 in order to prepare Validation samples at an appropriate concentration. The three SIS spike levels were as follows:
		1. Low: 3 fmol/µL (30 fmol on column)
		2. Medium: 30 fmol/µL (300 fmol on column)
		3. High: 150 fmol/µL (1.5 pmol on column)

These concentrations were selected to approximate 1.5-3.0x LLOQ (Low), 50-100x LLOQ (Medium), and >100x LLOQ (High).

* 1. Dilute matrix to 0.2 µg/µL with 0.1% formic acid.
	2. Prepare dilution mix containing 100 µL matrix (0.2 µg/µL), 10 µL unlabeled IS peptide stock (200fmol/µL) and 80 µL 0.1 % formic acid (final concentration of matrix in dilution mix is 0.1 µg/µL). A total volume of dilution mix should be prepared to permit at least 20 injections of each sample.
	3. Prepare SIS stock by taking resuspending 500 pmol of SIS in 125 µL of HPLC water for a final SIS stock concentration of 4 pmol/µL.
	4. In separate microcentrifuge tubes, prepare daily “low,” “medium,” and “high” concentrations of SIS by diluting the SIS stock.
		1. Prepare “High” concentration sample by adding 7.5 µL of SIS to 190 ul of dilution mix and add 2.5 ul of 0.1% formic acid.
		2. Prepare “Medium” concentration sample by adding 1.5 µL of SIS to 190 µL of dilution mix and add 8.5 ul of 0.1% formic acid.
		3. Prepare “Low” concentration sample by adding 1.5 ul of SIS (from intermediate stock of 400fmol/ul) to 190 ul of dilution mix and add 8.5 ul of 0.1% formic acid.
	5. Vortex samples for 30 seconds to thoroughly mix, centrifuge briefly and transfer to auto sampler 96-well plate (Eppendorf; cat. # 951020401), placing 96-well cover seal onto the plate. Insert plate into EASY-nLC auto sampler for immediate LC-PRM MS analysis.
1. Execution of LC-PRM MS analysis
	1. Perform LC-PRM MS analysis according to the SOPs entitled “Liquid Chromatography, Thermo Scientific EASY-nLC 1200 LC System” and “Parallel Reaction Monitoring (PRM) Mass Spectrometry, Orbitrap Lumos Fusion.”
2. Run order
	1. To avoid artificially minimizing variability, the run order of the samples should be randomized using a random number generator. To minimize carryover, 2 washes are inserted after triplicate analysis of each “Low” “Medium” and “High” sample.

# Referenced Documents

* SOP Liquid Chromatography, Thermo Scientific EASY-nLC 1200 LC System
* SOP Parallel Reaction Monitoring (PRM) Mass Spectrometry, Orbitrap Lumos Fusion
* SOP Cell lysate preparation
* SOP Trypsin digestion of cell lysate