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| STANDARD OPERATING PROCEDURE |
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| **Title: Response curve** |
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# Purpose

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

# Scope

This procedure addresses the preparation and running of samples for generating a response curve in accordance with CPTAC Assay Characterization Guidance Experiment #1.

# 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

* Benchtop 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 HEK293 cell lysate was prepared as described in the SOP entitled “Lysis of cell lines.” Digestion of lysate was performed as described in the SOP entitled “Trypsin digestion of cell lysate.” This background matrix was used for the preparation of the response curves and for the preparation of the mini-validation of repeatability experiments.

# Procedure

1. Preparation of samples
	1. Samples are prepared to create 7 points of varying concentrations (0.025. 0.1, 0.5, 1, 10, 100, 1000, and 2000 fmol on column) of SIS. Blanks not containing SIS are also prepared. Adequate volume of each sample is prepared for at least 10 runs. The final preparation of each sample contains background matrix, SIS and IS peptides.
	2. Dilute matrix to 0.2 µg/µL with HPLC water.
	3. Prepare dilution mix containing 50 µL matrix (0.2 µg/µL), 5 µL unlabeled stock IS peptide mix (200 fmol peptide/µL) and 40 µL 0.1 % formic acid (final concentration of matrix in dilution mix is 0.105 µg/µL).
	4. Prepare blank sample containing 50 µL matrix (0.2 µg/µL) and 50 µL 0.1 % formic acid (final concentration of matrix in dilution mix is 0.1 µg/µL). Adequate volume of blank sample is prepared for at least 10 runs.
	5. In separate microcentrifuge tubes, prepare serial dilutions for calibration curve points by serially diluting SIS stock (4 pmol/µL) with HPLC water to generate the following concentrations of SIS peptide: 2 pmol/µL. 200 fmol/µL, 20 fmol/µL, 2 fmol/µL, 1 fmol/µL, 0.2 fmol/µL, 0.05 fmol/µL.
	6. Prepare sample for the highest point on the curve by taking 5 µL of SIS stock (4 pmol/µL) and diluting in 95 µL dilution mix (prepared in Step 1c, above) for a final SIS peptide concentration of 0.2 pmol/µL and a final concentration of matrix of 0.1 µg/µL. Adequate volume of sample is prepared for at least 10 runs.
	7. Prepare sample for the remaining points on the curve by taking 5 µL of diluted SIS (prepared individually in Step 1e) and diluting in 95 µL dilution mix (prepared in Step 1c, above) for a final concentration of matrix of 0.1 µg/µL. and a final SIS concentration of 0.1 pmol/µL. 10 fmol/µL, 1 fmol/µL, 0.1 fmol/µL, 50 amol/µL, 10 amol/µL, 2.5 amol/µL respectively. Adequate volume of each sample is prepared for at least 10 runs.
	8. Vortex samples for 30 seconds to thoroughly mix, centrifuge briefly and transfer to autosampler 96-well plate (Eppendorf; cat. # 951020401), placing 96-well cover seal onto the plate. Insert plate into EASY-nLC autosampler for immediate LC-PRM MS analysis.
2. Execution of LC-PRM MS analysis
	1. Perform LC-PRM MS analysis according to the SOPs entitled “Liquid Chromatography, Thermo Scientific EASY-nLC LC 1200 System” and “Parallel Reaction Monitoring (PRM) Mass Spectrometry, Orbitrap Lumos Fusion.”
3. Run order
	1. Samples are run in order of increasing concentration as indicated below. Three replicates are acquired for each concentration. Three blanks are run prior to the first replicate run of the curve and two blanks are run following each curve. To minimize carryover, 2 washes are inserted after triplicate analysis of each sample.

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| Runorder | Sample |
| 1 | Blank |
| 2 | Blank |
| 3 | Blank |
| 4 | 0.025 fmol |
| 5 | 0.1 fmol |
| 6 | 0.5 fmol |
| 7 | 1 fmol |
| 8 | 10 fmol |
| 9 | 100 fmol |
| 10 | 1 pmol |
| 11 | 2 pmol |
| 12 | Blank |
| 13 | Blank |

# 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