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| STANDARD OPERATING PROCEDURE |
| |  |  | | --- | --- | | **Title: Response curve for PRM assays** | | | **Version #: 1.1** | **Author: Hui Zhang Laboratory – Johns Hopkins University** | | **Date: 02/18/2016** |  | |

# 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

* 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: LC-MS Ultra (Sigma-Aldrich; cat. # 14265)
* Methanol: Optima LC/MS-grade (Fisher Scientific; cat. # A456-4)
* Ammonium formate (Sigma-Aldrich; cat. # 70221)
* Ammonium hydroxide (Sigma-Aldrich; cat. # 320145)
* Polysulfoethyl A TopTips 100 – 200 µL (Glygen; cat. # TT2SSA.96)

# Reagents

* 10 mM Ammonium formate in 25% ACN, pH 3.0
* 500 mM Ammonium formate in 25% ACN, pH 6.8
* 80:15:5 (vol:vol:vol) Methanol: Water: Ammonium hydroxide
* Crude unlabeled peptides (~60% purity)
* Stable isotope-labeled standards (SIS)
  + Crude unlabeled peptides and SIS (both ~60% purity) from Thermo Fisher Scientific (PEPotec SRM peptide library): SIS peptides incorporate 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 should be provided in 0.1% TFA/50% ACN and stored at -80 °C until use.
  + Following de-salting via strong cation exchange (SCX) as detailed in Procedure #1 below, prepare stock solutions of the unlabeled and SIS peptides at a concentration of 1 nmol/µL and store at -20 °C. The peptide recovery following SCX clean-up is estimated to be 40%.
* Matrix
  + Prepare a background matrix consisting of peptides from the trypsin digestion of ovarian tumor tissue homogenate according to the SOP entitled “SOP\_Tissue background matrix preparation v1\_1-HuiZhang lab.” This background matrix will be used for the preparation of the response curves and for the preparation of the mini-validation of repeatability experiments.

# Procedure

1. SCX de-salting of crude peptides and SIS
   1. All centrifugation steps are performed at 2,000 rpm for 1.5 min, unless otherwise specified. De-salt 1 mg of each peptide via SCX.
      1. Condition Polysulfoethyl A TopTip 2x with 330 µL of Methanol
      2. Wash 2x with 330 µL of 10 mM Ammonium Formate in 25% ACN, pH 3.0
      3. Wash 2x with 330 µL of 500 mM Ammonium Formate in 25% ACN, pH 6.8
      4. Wash 2x with 330 µL of 10 mM Ammonium Formate in 25% ACN, pH 3.0
      5. Wash 2x with 330 µL of Water
      6. Wash 4x with 330 µL of 10 mM Ammonium Formate in 25% ACN, pH 3.0
      7. Slowly load acidified sample (pH < 3.0) 2x; centrifuge at 1,100 rpm for 5 min
      8. Wash 6x with 330 µL of 10 mM Ammonium Formate in 25% ACN, pH 3.0
      9. Allow TopTip to dry out. Elute sample 2x with 300 µL of 80:15:5 (vol:vol:vol) Methanol: Water: Ammonium hydroxide; centrifuge at 1,100 rpm for 5 min
      10. Dry eluted sample in a vacuum centrifuge
2. Preparation of samples
   1. Samples are prepared to create 7 points of varying concentrations (0.2, 1, 2, 10, 20, 100, and 200 fmol/µL; or 1, 2, 10, 20, 100, 200 and 1000 fmol/µL) of unlabeled peptide. Blank matrix containing SIS is also prepared. An adequate volume of each sample is prepared for at least 6 runs (response curve concentration points) or 12 runs (blanks). The final preparation of each sample contains background matrix (0.1 µg/µL), unlabeled peptides and SIS peptides (200 fmol/µL).
   2. Dilute matrix to 0.1 µg/µL with 0.2% formic acid.
   3. Prepare sample in diluted matrix for the highest point on the curve.
   4. Beginning with the highest point on the curve (200 fmol/µL or 1000 fmol/µL), prepare 1:5 or 1:2 serial dilutions using the diluted matrix.
   5. Store samples at -20 °C until LC-PRM MS analysis.
3. Execution of LC-PRM MS analysis
   1. Vortex samples, centrifuge briefly and transfer to autosampler vials. Add sufficient volume to each vial for all of the replicate injections.
   2. Perform LC-PRM MS analysis according to the SOPs entitled “SOP\_Liquid Chromatography CPTAC Assays v1\_1-HuiZhang lab” and “SOP\_PRM mass spectrometry QExactive CPTAC Assays v1\_1-HuiZhang lab.”
4. 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.

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| Run  order | Sample |
| 1 | Blank |
| 2 | Blank |
| 3 | Blank |
| 4 | 0.2 fmol/µL |
| 5 | 1 fmol/µL |
| 6 | 2 fmol/µL |
| 7 | 10 fmol/µL |
| 8 | 20 fmol/µL |
| 9 | 100 fmol/µL |
| 10 | 200 fmol/µL |
| 11 | Blank |
| 12 | Blank |
| 13 | Wash |
| 14 | Wash |

# Referenced Documents

* SOP\_Liquid Chromatography CPTAC Assays v1\_1-HuiZhang lab
* SOP\_PRM mass spectrometry QExactive CPTAC Assays v1\_1-HuiZhang lab
* SOP\_Tissue background matrix preparation v1\_1-HuiZhang lab

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2. Thomas SN, Harlan R, Chen J, Aiyetan P, Liu Y, Sokoll LJ, Aebersold R, Chan DW, Zhang H. Multiplexed Targeted Mass Spectrometry-Based Assays for the Quantification of N-Linked Glycosite-Containing Peptides in Serum. Anal Chem. 2015 Nov 3;87(21):10830-8. doi: 10.1021/acs.analchem.5b02063. Epub 2015 Oct 21. PubMed PMID: 26451657; PubMed Central PMCID: PMC4708883.