

STANDARD OPERATING PROCEDURE

Title: Response curve

Version #: 1

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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

- 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
- Stable isotope-labeled standards (SIS)
 - Crude SIS formerly N-glycosylated peptides were synthesized (~60% purity) by Thermo Fisher Scientific (PEPotec SRM peptide library). SIS peptides incorporated a fully atom-labeled ^{13}C and ^{15}N 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. Deamidated asparagine residues corresponding to N-glycosylation sites were synthesized as aspartic acid residues. 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 1320 pmol/ μL for each peptide, followed by de-salting via strong cation exchange (SCX) as indicated in Procedure 1, below. The peptide recovery following SCX clean-up was assumed to be 50%.
- Matrix
 - A background matrix consisting of trypsin digested serum-derived N-glycopeptides was prepared according to the SOP entitled “Trypsin digestion of serum and enrichment of N-glycopeptides using an automated liquid handler”. This background matrix was used for the preparation of the response curves and for the preparation of the mini-validation of repeatability experiments. Given the limitation of a maximum yield of 20 μg of glycopeptides per preparation of 40 μL serum, N-glycopeptides prepared using multiple DART pipette tips packed with hydrazide resin were combined to achieve the required amount of N-glycopeptides that were needed for the response curves.

Procedure

1. SCX de-salting of stock SIS mix
 - a. All centrifugation steps are performed at 2,000 rpm for 1.5 min, unless otherwise specified. The maximum binding capacity of each TopTip is 1 mg.
 - i. Condition Polysulfoethyl A TopTip 2x with 330 μL of Methanol
 - ii. Wash 2x with 330 μL of 10 mM Ammonium Formate in 25% ACN, pH 3.0
 - iii. Wash 2x with 330 μL of 500 mM Ammonium Formate in 25% ACN, pH 6.8
 - iv. Wash 2x with 330 μL of 10 mM Ammonium Formate in 25% ACN, pH 3.0
 - v. Wash 2x with 330 μL of Water
 - vi. Wash 4x with 330 μL of 10 mM Ammonium Formate in 25% ACN, pH 3.0
 - vii. Slowly load acidified sample (pH < 3.0) 2x; centrifuge at 1,100 rpm for 5 min
 - viii. Wash 6x with 330 μL of 10 mM Ammonium Formate in 25% ACN, pH 3.0

- ix. 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
- x. Dry eluted sample in a vacuum centrifuge

2. Preparation of samples

- a. Samples are prepared to create 7 points of varying concentrations (0.0576, 0.288, 1.44, 7.2, 36, 180 and 900 pmol on column) of SIS. Blanks not containing SIS are also prepared. Adequate volume of each sample is prepared for at least 3 runs (SIS peptides) or 9 runs (blanks). The final preparation of each sample contains background matrix, SIS and iRT peptides.
- b. Dilute matrix to 0.25 $\mu\text{g}/\mu\text{L}$ with 2% ACN/0.2% formic acid.
- c. Prepare dilution mix containing 366.67 μL matrix (0.25 $\mu\text{g}/\mu\text{L}$), 110 μL iRT standard diluted 1:10 and 623.33 μL 0.1 % formic acid (final concentration of matrix in dilution mix is 0.083 $\mu\text{g}/\mu\text{L}$ and final dilution of iRT is 1:100). This dilution mix will also be used as the blank (no SIS added).
- d. Prepare sample for the highest point on the curve by taking 84 μL of SIS (660 pmol/ μL) and drying in a vacuum centrifuge. Resuspend in 369.6 μL dilution mix (prepared in Step 1c, above) for a final SIS stock concentration of 150 pmol/ μL .
- e. Beginning with the highest point on the curve (150 pmol/ μL), prepare a 1:5 dilution by adding 14 μL of the 150 pmol/ μL solution to 56 μL of the dilution matrix. Prepare six 1:5 serial dilutions by adding 14 μL of the previously diluted sample to 56 μL of dilution matrix.
- f. Store samples at -80 °C until LC-PRM MS analysis.

3. Execution of LC-PRM MS analysis

- a. Vortex samples, centrifuge briefly and transfer to autosampler vials. Add sufficient volume to each vial for all of the replicate injections.
- b. Perform LC-PRM MS analysis according to the SOPs entitled "Liquid Chromatography, Dionex UltiMate 3000 RSLCnano LC System" and "Parallel Reaction Monitoring (PRM) Mass Spectrometry, Q-Exactive".

4. Run order

- a. 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.

<i>Run order</i>	<i>Sample</i>
1	Blank
2	Blank
3	Blank
4	0.0576 pmol
5	0.288 pmol
6	1.44 pmol
7	7.2 pmol
8	36 pmol
9	180 pmol
10	900 pmol
11	Blank
12	Blank
13	Wash
14	Wash

Referenced Documents

- SOP Liquid Chromatography, Dionex UltiMate 3000 RSLCnano LC System
- SOP Parallel Reaction Monitoring (PRM) Mass Spectrometry, Q-Exactive
- SOP Trypsin digestion of serum and enrichment of N-glycopeptides using an automated liquid handler