STANDARD OPERATING PROCEDURE

Title: Mini-Validation of Repeatability

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Purpose

The purpose of this document is to describe the characterization of a set of assays based on its repeatability of measurement over 5 days. This is to estimate the performance of the assay measured in a complex sample across several days.

Scope

This procedure addresses the preparation and running of samples for generating the 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: 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 repeatability study.

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. Determination of spike levels and preparation of samples
    a. 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:
        i. Low: 0.288 pmol/µL (1.728 pmol on column)
        ii. Medium: 7.5 pmol/µL (45 pmol on column)
        iii. High: 12 pmol/µL (72 pmol on column)

        These concentrations were selected to approximate 1.5-3.0x LLOQ (Low), 50-100x LLOQ (Medium), and >100x LLOQ (High).
    b. Dilute matrix to 0.25 µg/µL with 2% ACN/0.2% formic acid.
    c. Prepare dilution mix containing 366.67 µL matrix (0.25 µg/µL), 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 µg/µL and final dilution of iRT is 1:100). A total volume of dilution mix should be prepared to permit at least 15 injections of each sample.
    d. Prepare SIS stock 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. Prepare “High” concentration sample by adding 9.6 µL of SIS to 110.4 µL of dilution mix.
    f. Prepare “Medium” concentration sample by adding 6.0 µL of SIS to 114 µL of dilution mix.
    g. Prepare “Low” concentration sample by adding 2.3 µL of 1:10 dilution of SIS to 117.7 µL of dilution mix.
    h. 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. To avoid artificially minimizing variability, the run order of the samples should be randomized. To minimize carryover, 1 wash is inserted after the “Low” and “Medium” samples, and 2 washes are inserted after the “High” samples.

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