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| STANDARD OPERATING PROCEDURE |
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| **Title: Mini-Validation of Repeatability for MRM assays run on TSQ Vantage triple quadrupole mass spectrometer**  |
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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
* 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 2 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 human ovarian tissue according to the SOP entitled “SOP\_Tissue background matrix preparation v1\_2-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 stock SIS mix
	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. Determination of spike levels and preparation of samples
	1. Determine peptide concentrations to use 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 are as follows:
		1. Low: 0.004, 0.041, 0.417 or 4.17 pmol/µL
		2. Medium: 0.167, 1.67, 16.7, or 167 pmol/µL
		3. High: 0.417, 4.17, 41.7, or 417 pmol/µL
	2. These concentrations were selected to approximate 1.5-3.0x LLOQ (Low), 50-100x LLOQ (Medium), and >100x LLOQ (High). The final preparation of each sample contains background matrix (0.1 µg/µL), unlabeled peptides and SIS peptides (91 or 909 fmol/µL).
	3. Dilute matrix to 0.1 µg/µL with 0.2% formic acid.
	4. Prepare the High sample in the diluted matrix.
	5. Prepare 1:2.5 and 1:40 serial dilutions using the diluted matrix to generate the Medium and Low samples, respectively.
	6. Store samples at 4 °C (no longer than 48 hours) until LC-MRM MS analysis.
3. Execution of LC-MRM MS analysis
	1. Prepare 80 µL of each sample and spike with 8 µL of the SIS peptide mixture.
	2. Vortex samples, centrifuge briefly and transfer to autosampler vials. Add sufficient volume to each vial for all of the replicate injections.
	3. Perform LC-MRM MS analysis according to the SOPs entitled “SOP\_Liquid Chromatography CPTAC MRM TSQ Vantage Assays v1\_1-HuiZhang lab” and “SOP\_MRM mass spectrometry TSQ Vantage CPTAC Assays v1\_1-HuiZhang lab.”
4. Run order
	1. To avoid artificially minimizing variability, the run order of the samples should be randomized. To minimize carryover, 1 wash is inserted after each “High” sample.

**Referenced Documents**

* SOP\_Liquid Chromatography CPTAC MRM TSQ Vantage Assays v1\_1-HuiZhang lab
* SOP\_MRM mass spectrometry TSQ Vantage CPTAC Assays v1\_1-HuiZhang lab
* SOP\_Tissue background matrix preparation v1\_2-HuiZhang lab

1. Hoofnagle AN, Whiteaker JR, Carr SA, Kuhn E, Liu T, Massoni SA, Thomas SN, Townsend RR, Zimmerman LJ, Boja E, Chen J, Crimmins DL, Davies SR, Gao Y, Hiltke TR, Ketchum KA, Kinsinger CR, Mesri M, Meyer MR, Qian WJ, Schoenherr RM, Scott MG, Shi T, Whiteley GR, Wrobel JA, Wu C, Ackermann BL, Aebersold R, Barnidge DR, Bunk DM, Clarke N, Fishman JB, Grant RP, Kusebauch U, Kushnir MM, Lowenthal MS, Moritz RL, Neubert H, Patterson SD, Rockwood AL, Rogers J, Singh RJ, Van Eyk JE, Wong SH, Zhang S, Chan DW, Chen X, Ellis MJ, Liebler DC, Rodland KD, Rodriguez H, Smith RD, Zhang Z, Zhang H, Paulovich AG. Recommendations for the Generation, Quantification, Storage, and Handling of Peptides Used for Mass Spectrometry-Based Assays. Clin Chem. 2016 Jan;62(1):48-69. doi: 10.1373/clinchem.2015.250563. PubMed PMID: 26719571.