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| STANDARD OPERATING PROCEDURE |
| |  |  | | --- | --- | | **Title: CPTAC Verification SOP:** **Study 9-1 SOP for NCI CPTAC Consortium-Wide Multiple Reaction Monitoring (MRM) Experiment**  **9-Point Calibration Curve of 123 target peptides** | | |  |  | | **Version #: 1** | **Author: CTPAC Experimental Design and Statistics Verification Studies Working Group** | | **Date: August 1, 2016** |  | |

# Overview

Samples from CPTAC Study 9-1 were used for the CPTAC Assay Portal of direct MRM assays in immunodepleted human plasma. Below are excerpts from that protocol for reference on their designed preparation and use.

Study 9-1, the 9 point calibration curve will entail monitoring 123 signature peptides and corresponding stable-isotope labeled internal standard (SIS) peptides (~750 total transitions) that will be quantitatively assayed by time-scheduled LC-MRM-MS against a background of MARS-14 depleted human K2EDTA plasma (0.5 µg/µL).

**Aims:** The study described in this SOP is designed to accomplish the following:

1. To generate a 9 point standard curve for 123 potentially cancer relevant peptide targets spanning 1 amol/µL to 100 fmol/µL in a depleted digested plasma background (500 ng/µL) that will be analyzed by LC-MRM-MS on four different triple quadrupole platforms at volunteer CPTAC sites.
2. To enable calculations of LOD, LOQ, accuracy and precision for all peptides from the calibration curve.
3. A set of 6 blinded samples is provided that mimic real biomarker samples.
4. To evaluate the level of endogenous and interfering signals for all peptides by replicate measurements of blank plasma samples.

The number of transitions that will be targeted in this method (~750) is a critical challenge and will require the use of retention time scheduled LC-MRM-MS. To achieve success, the following protocol is designed to assess the system suitability and retention time stability of the LC-MRM-MS instrument, as well as design a single MRM-MS method empirically. The protocol outlined herein should be followed as strictly as possible and all deviations from this protocol must be outlined in detail on the last sheet. The experimental design is outlined below.

**Experimental Outline**

1. Run System Suitability Sample 5 times to condition column and assess LC-MRM-MS performance
2. Using Skyline, create 6 MRM-MS transition lists for the heavy target peptides only (maximum of 60 transitions in each unscheduled method)
3. Analyze the heavy-only peptide sample using the 6 different MRM-MS methods
   * 1. Import the 6 raw files into Skyline
     2. Obtain retention times for each heavy target peptide
     3. Export a scheduled MRM-MS transition list (or MRM-MS method) with a 4 min RT window
     4. Run the scheduled LC-MRM-MS method twice using the sample containing heavy peptides spiked into depleted, digested plasma.
     5. Import the 2 raw files into Skyline
     6. Add in the light (unlabeled) peptides into the Skyline document
     7. Export a new scheduled LC-MRM-MS method with all ~750 transitions and a 2 min RT window
4. Execute calibration curve and blinded samples according to specified run order.
5. Import data into Skyline and integrate.
   * 1. Import System Suitability Sample files into System Suitability Skyline file and integrate. Ensure peak area CVs and retention times are within specified error limits.
     2. Import Calibration Curve files into Study 9-1 Skyline file and integrate.
6. Export reports from each Skyline file and upload to the NIST ftp server (see separate document).

# Materials and Reagents

1. **System Suitability** **Sample (**Study 9-1-SSS)
   1. Digest of 6 equimolar proteins (Michrom Bioresources, #PTD/00001/63)
      1. Four 10 µL aliquots, 1 pmol/µL, supplied in 30% acetonitrile/0.1 % formic acid in water
2. **Heavy-Only IS Peptide Mixture (in 25 fmol/uL 6 protein mix)**
   1. 123 heavy IS peptides at 800 fmol/L each
      1. Two 10 L aliquots, supplied in 30% acetonitrile/0.1% formic acid
3. **QC Sample** (**Sample 9-1-QC**)
   1. Equimolar mixture of the 123 unlabeled and 123 labeled synthetic peptides in 25 fmol/uL 6 Protein mix matrix
      1. Four 25 µL aliquot supplied at 10 fmol/µL each peptide in 0.1% formic acid in water. Background of 25 fmol/uL of 6 protein mix added to minimize adsorption/loss of hydrophobic peptides.
4. **Digested human plasma spiked with labeled IS peptides only**, (Sample9-1-A)
   1. Depleted plasma diluted to a final concentration after digestion of approximately 0.5 μg/µL
      1. 123 labeled IS peptides spiked at a concentration of 10 fmol/µL (Sample9-1-A)
      2. Ten 25 L aliquots supplied in 0.1 % formic acid in water (after desalting by SPE, note: desalting was already performed at Vanderbilt University)
5. **Digested human depleted plasma spiked with 123 unlabeled synthetic peptides and 123 labeled IS peptides** (Samples9-1-Bto 9-1-J)
   1. Depleted plasma diluted to a final concentration after digestion of ~ 0.5 μg/µL
      1. 123 labeled IS peptides spiked at a concentration of 10 fmol/µL
      2. Four 25 µL aliquot of each spike level supplied for 4 singlicate curves
      3. 123 unlabeled synthetic peptides are spiked in at the following approximate concentrations:

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| Sample  (Study 9-1) | Spiked [12C/14N] peptide Concentration  (fmol/µL) |
| 9-1-J | 100.00 |
| 9-1-I | 23.71 |
| 9-1-H | 5.62 |
| 9-1-G | 1.33 |
| 9-1-F | 0.316 |
| 9-1-E | 0.075 |
| 9-1-D | 0.018 |
| 9-1-C | 0.004 |
| 9-1-B | 0.001 |

Important note: each of the 4 different singlicate calibration curves will be completed before the next replicate curve will be started: Specific naming including the first blank (A sample) is required, for details also see run order below:

9-1-A1 to 9-1-J1; 9-1-A2 to 9-1-J2; 9-1-A3 to 9-1-J3; 9-1-A4 to 9-1-J4.

# Procedure

**Overview**

In Main Study 9-1 (9-point calibration curve), synthetic [12C/14N] and [13C/15N] signature peptides will be spiked into MARS-14 depleted, digested human K2EDTA plasma and analyzed by scheduled LC-MRM-MS at selected volunteer sites. All sample preparation will be performed at Vanderbilt University prior to distribution of the sample kits. Results from Study 9-1 from all CPTAC sites, will provide LOD/LOQ values for each peptide (this design contains minimal sample handling, and no target protein digestion and eliminates key factors of experimental variability). The Study 9-1 scheduled LC-MRM-MS samples will consist of a 9-point standard curve ranging in concentration from 100 fmol/L to 1 amol/L, with all concentrations in the presence of 0.5 µg/µL depleted digested plasma. Synthetic signature internal standard (IS) peptides uniformly labeled with a 13C/15N amino acid will be spiked into all plasma samples at a constant concentration of 10 fmol/µL.

The following paragraphs provide a description of the experimental procedure, with each major step separated into its own paragraph and heading. Paragraphs are followed by outlined “checklists” that have matching headings for reference.

* + - 1. **System Suitability Sample, Column Conditioning and Instrument Performance**

System Suitability Sample (SSS) runs using the Michrom 6 protein mix (based on Study 9S) will be interspersed into Study 9-1 (to guarantee system suitability and performance and, in particular, monitor peak area stability and potential RT drift). The SSS will first be analyzed in an unscheduled LC-MRM-MS method (Study 9S) to condition the column and assess the performance of the LC-MS instrument platform. Upon obtaining the specified results for retention time and peak area, sites may proceed to the generation of the scheduled LC-MRM-MS runs for Study 9.

The SSS will also be run every 6-8 runs in the sample queue, in order to track instrument performance. Data will be analyzed in Skyline and will be submitted in a separate “Michrom Study 9S” file.

* + - 1. **Generation of Scheduled LC-MRM-MS Method for Study 9 Peptides**

In preparation for the scheduled calibration curve LC-MRM-MS runs, in which 738 transitions will be monitored in one run, participating sites will monitor all heavy synthetic labeled peptides with about 369 transitions (123 peptides with 3 transitions each) in six unscheduled LC-MRM-MS runs (~60 transitions per run), with each run represented by a separate LC-MRM-MS method. Retention times for scheduling will be determined empirically using these six runs and will be verified by analysis with a single scheduled LC-MRM-MS run prior to analyzing the samples from which the calibration curve will be generated. All method building and data analysis will be performed using Skyline.

* + - 1. **Data Analysis**

The experimentally determined molar concentration of the spiked peptide or protein will be calculated and compared to its theoretical value for accuracy. Operators will import all acquired data files into Skyline and check and if necessary adjust peak integration. Check "Integrate All" (Menu, Settings, Integrate all), so that heavy and light transitions will be "integrated together". While checking peak integration open RT replicate views (Menu, View, Retention Time, Replicate Comparison), and Peak Area replicate views (Menu, View, Peak Areas, Replicate Comparison) to visually help confirm proper peak integration. Specifically, check for interferences and RT drift problems during scheduling. Use Skyline Custom Annotation features to annotate any observations or notes for Study Statisticians to consider (Menu, View, Results Grid; and annotate observations on the precursor or transition level). Finally use "AuDIT" to analyze data for interferences before submitting data to statisticians. Linear plots of response versus known concentration from each of the 9-point standard curves will be used to evaluate the linearity of the MRM measurements across the range of spiked peptide concentrations, thus providing evidence of a quantitative measurement process. Replicate analyses of the spiked plasma samples will provide estimates of assay precision (standard deviation and % CV), plus LOQ and LOD will be determined at defined signal-to-noise ratio (S/N) values. Blank runs of digested plasma with labeled peptides will provide estimates of chemical background levels in the absence of unlabeled signature peptide peaks as well as the presence of endogenous peptides in the sample (*i.e.*, CRP). Furthermore, an estimate of carryover will be determined by running a series of gradient HPLC washout runs. Finally, variation across CPTAC sites will be assessed for each of these characteristic analytical metrics.

* + - 1. **Troubleshooting**

This study contains a large number of samples that need to be run in a defined order and requires very reproducible peptide RTs. Problems may arise that will affect RT stability, including, but not limited to, increased column pressure, tip blockage, significant change in ambient temperature or injection of air into the system. The troubleshooting section provides some suggested routes of diagnosis and an outline of how to resolve the problem and continue with data acquisition. Importantly, operators are instructed to use the SSS and Skyline to observe any problems with retention time shifting, asymmetric peaks, low signal, and asymmetric peak shape. These problems can be caught and rectified without much down time and with minimal re-running of sample.

# Referenced Documents

CPTAC Study 9 -1 SOP\_0831\_2011\_v14