STANDARD OPERATING PROCEDURE

Title: Preparation of Standard Peptide Samples for a Mini-validation of Repeatability Study-Experiment 2

SOP#: WU-SOP-EXP2-02

Version #: 2
(Metabolic Panel)

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Purpose

The purpose of this document is to describe the preparation of admixture solutions of high purity synthetic natural abundance (L, light) and stable isotope-labeled (H, heavy) peptides to determine intra- and inter-assay variability using the protocol details in Experiment 2 of the CPTAC "Assay Development Guidelines" (https://assays.cancer.gov/guidance-document/). The preparation of sufficient quantities for replicate injections (n = 3) of LOW, MEDIUM and HIGH samples for LC-MS analysis over five experimental blocks is described . The order of sample analysis is detailed in WU-SOP-LC2-02.

Scope

The preparation of LOW, MEDIUM and HIGH concentrations of synthetic peptide admixtures for performing the mini-repeatability study (CPTAC, "Assay Development Guidelines", Experiment 2) is described. The bench procedures for preparing three admixtures of light (13 fmol) and heavy peptides, LOW (2.6 fmol), MEDIUM (13 fmol) and HIGH (130 fmol) from frozen stock solutions are detailed. The preparation of the samples from the primary stock solutions, the diluents and preparation of tumor digest matrix from frozen aliquots is described in WU-SOP-EXP1-02_V3.

Table I. Standards for Determining Method Repeatability (EXP2)

QC Standard	Label	Heavy Final Concentration (fmol on column)	Light Final Concentration Internal Standard (fmol on column)
LOW	QCL	2.6	13
MEDIUM	QCM	13	13
HIGH	QCH	130	13

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, Eppendorf 5415D
- Sorval centrifuge RC6Plus; rotor: HB-6
- Rainin™ Pipet-lite XLS, P20, P200, P1000

Materials

- Axygen® MAXYmum™ recovery tips; P200 and P20: T-200-C-L-STK; P1000: T-1000-C-L-R
- Volumetric glassware (2, mL flasks): Kimble KIMAX; rinse 3x with DI water (Millipore, 18.2 MΩ), 3 x with 70% AcN, 1%FA, 5 x with DI water
- Microcentrifuge tubes: Fisher, 02-681-333
- Microcentrifuge tubes: Fisher, 02-681-368
- Autosampler vials (Sun-Sri, 200 046)
- Water, LC-MS grade (Fluka, 39253-1L-R)
- Acetonitrile, LC-MS grade (Fluka, 34967-1L)
- Formic Acid, 98%, 50 mL (Fluka, 56302-50ML-F)
- Trifluoroacetic Acid, 99.5%, 10 x 1 mL (Thermo Scientific, 28904)

Reagents

- **Synthetic high purity peptides; New England Peptide.**
- **Breast Cancer Tumor Matrix**

  Preparation of stock tumor peptide digest described in WU-SOP-TD1-01 and preparation of matrix solution described in WU-SOP-TD1-01.
- **Diluents and Standard Tryptic Peptide "Carrier" Solutions**

  The solutions for preparing the blanks and LOW, MEDIUM and HIGH standards are described in the WU-SOP-EXP1-02 (V3) and summarized in Table II.

Table II Diluents and Standard Tryptic Peptide "Carrier" Solutions

Solution	Solvents	Peptide Concentration (fmol/µL)
TFA-1	Trifluoroacetic acid (1%)	0
AcN/FA-30	Acetonitrile/formic acid (30%/1%)	0
NINE-MIX-30-50	Acetonitrile/formic acid (30%/1%)	50

Procedure

1. **Preparation of Standard Samples for LC-MS Repeatability Study**
    a. Remove an aliquot for each of the H/L primay stock and a vial of the diluted tumor matrix from the freezer and thaw on ice.
    b. Pipette 26.32, 10.53, and 10.53 µL of tumor digest into 3 autosampler vials.
    c. Pipette 1, 2, and 20 µL of the respective concentrated Heavy stock and 2 µL Light stock solution into the designated autosampler vial as shown in Table III and Figure 1.
    d. Speedvac to dryness.
    e. Add 125, 50, and 50 µL of TFA-0.1. Vortex each autosampler vial (~ 45 s) and centrifuge in Sorval at 8,000 rpm for 20 min.
    f. Place on autosampler tray at 4ºC for LC-MS analysis using WU-SOP-LC2-02 and WU-SOP-MS4-01.

Table III. Volumes and Reagents used for Preparation of Standards for LC-MS

Standard	Diluent (TFA-1) (µL)	Stock (µL)	Tumor matrix spike solution (µL)	Final volume	Final (fmol/(µL)
LOW	125	1	26.32	125	1.04
MEDIUM	50	2	10.53	50	5.2
HIGH	50	20	10.53	50	52

Figure 1

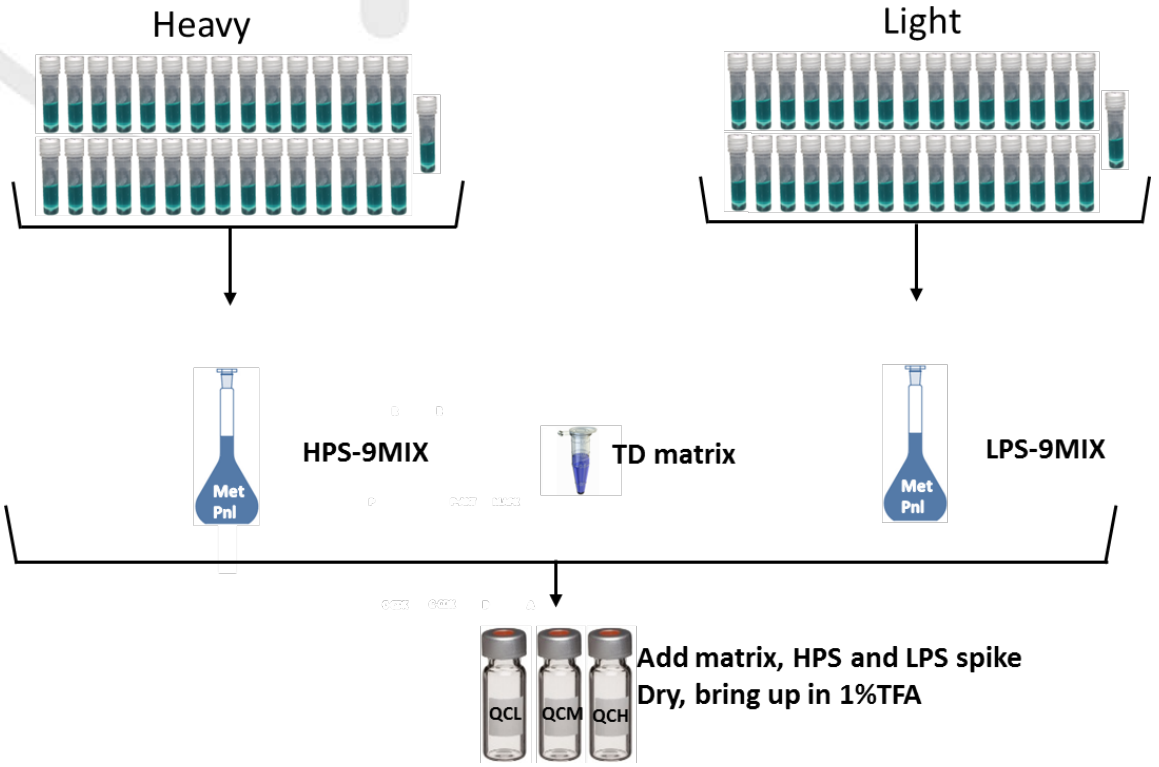

Figure 1. Preparation of standard admixtures of H and L peptides for the mini-repeatability study (Experiment 2). The preparation of the primary stocks is described in WU-SOP-EXP1-03. Aliquots of the primary stocks (HPS and LPS) are used to prepare the QCL, QCM and QCH samples in autosampler vials with tumor digest matrix (Table III).

Runtime (days): 10.7

Table IV. Example Sample Queue for LC-MS Analysis of LOW (QCL), MEDIUM (QCM) and HIGH (QCH) Standard Samples for Repeatability Study									
BLOCK 1		**BLOCK 2**		**BLOCK 3**		**BLOCK4**		**BLOCK5**	
Sample	**Runtime (hr)**	**Sample**	**Runtime (hr)**	**Sample**	**Runtime (hr)**	**Sample**	**Runtime (hr)**	**Sample**	**Runtime (hr)**
Hela	3.25	Hela	3.25	Hela	3.25	Hela	3.25	Hela	3.25
PRTC	3.25	PRTC	3.25	PRTC	3.25	PRTC	3.25	PRTC	3.25
PRTC	3.25	PRTC	3.25	PRTC	3.25	PRTC	3.25	PRTC	3.25
QCL	3.25	QCL	3.25	QCL	3.25	QCL	3.25	QCL	3.25
QCL	3.25	QCL	3.25	QCL	3.25	QCL	3.25	QCL	3.25
QCL	3.25	QCL	3.25	QCL	3.25	QCL	3.25	QCL	3.25
QCM	3.25	QCM	3.25	QCM	3.25	QCM	3.25	QCM	3.25
QCM	3.25	QCM	3.25	QCM	3.25	QCM	3.25	QCM	3.25
QCM	3.25	QCM	3.25	QCM	3.25	QCM	3.25	QCM	3.25
QCH	3.25	QCH	3.25	QCH	3.25	QCH	3.25	QCH	3.25
QCH	3.25	QCH	3.25	QCH	3.25	QCH	3.25	QCH	3.25
QCH	3.25	QCH	3.25	QCH	3.25	QCH	3.25	QCH	3.25

Runtime (days): 8.13

Referenced Documents

- WU-SOP-EXP1-02 (V3)- Preparation of Standard Peptide Samples for the Generation Reverse Response Curves-Experiment 1
- WU-SOP-MS4-01- Mass Spectrometry Using Parallel Reaction Monitoring for Experiments 1 and 2
- WU-SOP-TD1-01- Preparation of Peptides from Tumor Lysates
- WU-SOP-LC2-02- nano-Liquid Chromatography for Experiment 1 and 2

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List of Abbreviations

- AcN, acetonitrile
- FA, formic acid
- LC-MS, nano-LC interfaced to a high-resolution Quadrupole-Orbitrap Mass Spectrometer as described in WU-SOP-LC2-02 and WU-SOP-M3-01
- H or heavy, stable isotopically labeled synthetic peptide
- L or light, natural abundance synthetic peptide
- Q.S., quantum satis
- HPS , primary stock solution of the heavy peptide; prepared by direct dilution and transfer from the vendor
- LPS, primary stock solution of the light peptide; prepared by direct dilution and transfer from the vendor vials.

- HSS, secondary stocks of the heavy primary peptide stock solution.
- LSS, secondary stocks of the light primary peptide stock solution.
- QCL, sample with 1.5-3.0 x the LLOQ of the set of peptides described in WU-SOP-EXP1-02 (V3)
- PRM, parallel reaction monitoring
- PDX, patient derived xenograft