STANDARD OPERATING PROCEDURE

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

SOP#: WU-SOP-EX2-02

Version #: 1

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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 randomized order of sample analysis is detailed in WU-SOP-LC2-01.

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 admixtures of heavy and light peptides from concentrations of 1.5-3.0 x LLOQ (LOW), 50-100 x LLOQ (MEDIUM) and >100 LLOQ (HIGH) from frozen stock solutions is 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. The peptide concentrations for the three H/L standard admixtures for this experiment are calculated from the average value of the LOD's for all peptides described in WU-SOP-EXP1-02.

QC Standard	Final Concentration (fmol/µL)
QCL	2
QCM	100
QCH	200

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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)

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 and summarized in Table I.

Table I Diluents and Standard Tryptic Peptide "Carrier" Solutions

Solution	Solvents	Peptide Concentration (fmol/µL)
TFA-0.1	Trifluoroacetic acid (0.1%)	0
AcN/FA-30	Acetonitrile/formic acid (30%/1%)	0
NINE-MIX-30-50	Acetonitrile/formic acid (30%/1%)	50

Solutions

Preparation of Stock H/L Admixtures for LOW, MEDIUM and HIGH Standard Solutions.

a. Remove an aliquot of the HPS, LSS-400 and HSS-400 stock peptide solutions (Table II) from the freezer and thaw on ice.
b. Transfer ~1 mL of the NINE-MIX-30-50 diluent into 2 ml volumetric flasks labeled as shown in Table II.
c. Pipette the indicated volumes from either a H primary or secondary stock solution and a L secondary stock solution to prepare the three varying concentrations of heavy peptides and a constant quantity of light peptides (see Table III).
d. Q.S. to 2 mL with NINE-MIX-30-50.
e. Dispense as aliquots (55 µL) into 500 µL microcentrifuge tubes and freeze at -80ºC.

Table II. Primary and Secondary Peptide Stock Solutions for the Preparation of the LOW, MEDIUM and HIGH Primary and Secondary Stock Solutions[1]

Solution	Component	Diluent	Concentration (fmol/µL)
Peptide Stock Solutions			
HPS	Heavy (H) or Light (L) Primary Stock	NINE-MIX-30-50	20,000
HSS-400 LSS-400	Heavy (H) or Light (L) Secondary Stock	NINE-MIX-30-50	400

[1]The preparation of these solutions is described in WU-SOP-EXP-1-02

Table III. Volumes and Reagents for Preparation of Concentrated LOW, MEDIUM and HIGH Stock Solutions (10X)

QC Stock	HEAVY Stock (fmol/µL)	HEAVY Stock (µL)	LSS - 400(µL)	AcN/FA-30 (µL)	Final Volume (µL)	Heavy Concentration (fmol/(µL)
HIGH	HPS	20	125	1275*	2000	200
MEDIUM	HPS	10	125	1575*	2000	100
LOW	HSS-400	10	125	1865	2000	2

*there are 18 primary stock solutions that comprise the peptide panel. All primary stock solutions are combined in the secondary stock solutions.

Procedure

1. **Preparation of Standard Samples for LC-MS Repeatability Study**
   a. Remove an aliquot for each of the three H/L admixtures and a vial of the diluted tumor matrix from the freezer and thaw on ice.
   b. Pipette 3.3 µL of tumor digest into all autosampler vials.

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c. Pipette 20 µL of the respective concentrated stock solution into the designated autosampler vial as shown in Table IV.
d. Speedvac to dryness.
e. Add 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-LC2-01 and WU-SOP-MS4-01.

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

Standard	Diluent (TFA-0.1) (µL)	Stock (µL)	Tumor matrix spike solution (µL)	Final volume	Final (fmol/(µL)
HIGH	50	20	3.3	50	80
MEDIUM	50	20	3.3	50	40
LOW	50	20	3.3	50	0.8

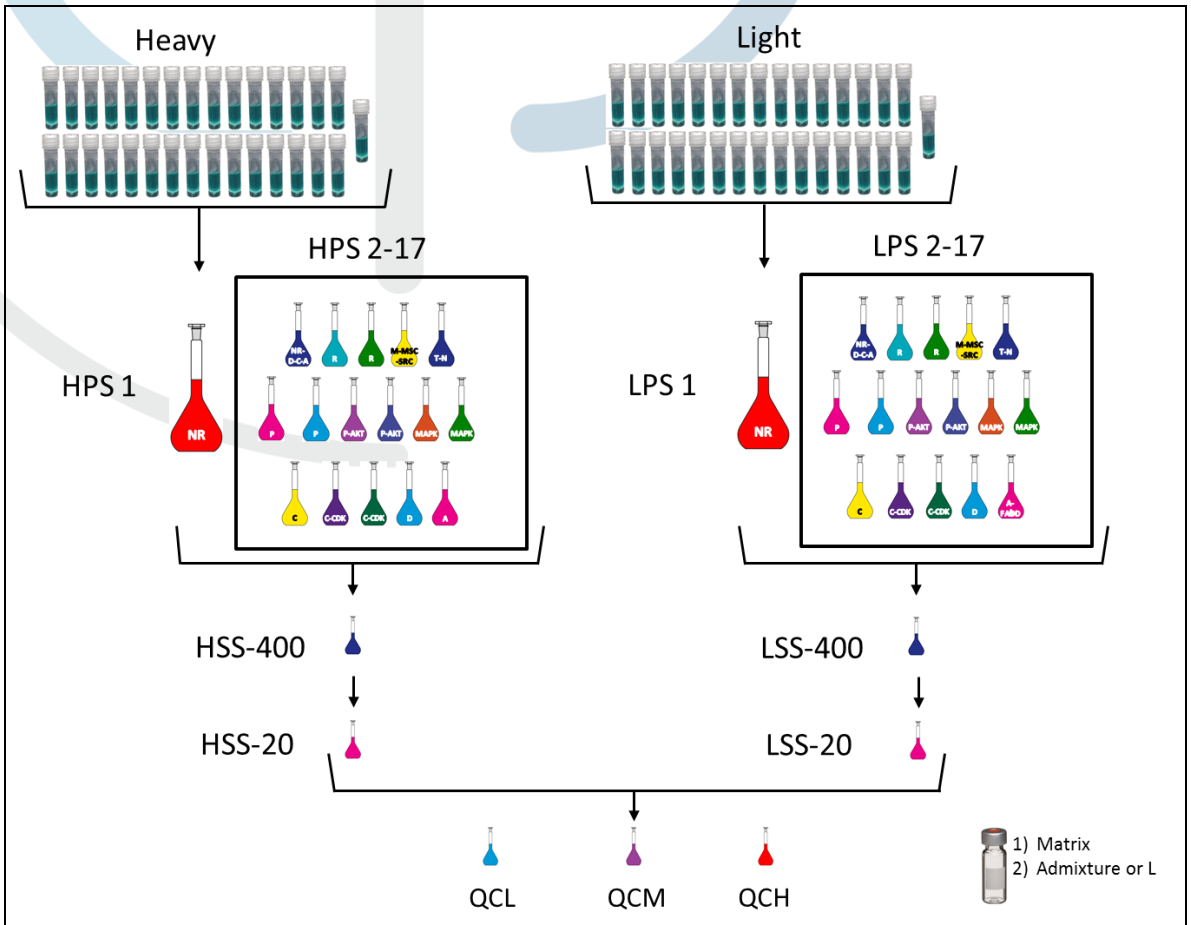

Figure 1. Preparation of standard admixtures of H and L peptides for the mini-repeatability study (Eperiment 2). The preparation of the primary and secondary stocks is described in WU-SOP-EXP2-02. Aliquots of the secondary stocks (HSS and LSS) are used to prepare the QCL, QCM and QCH samples for transfer to autosampler vials with tumor digest matrix (Table x).

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Table V. Example Sample Queue for LC-MS Analysis of LOW (QCL), MEDIUM (QCM) and HIGH (QCH) 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)
PRTC	1	PRTC	1	PRTC	1	PRTC	1	PRTC	1
QCL	2	QCH	2	QCH	2	QCL	2	QCL	2
QCH	2	QCM	2	QCL	2	QCH	2	QCH	2
QCL	2	QCL	2	QCH	2	QCM	2	QCM	2
QCL	2	QCM	2	QCM	2	QCM	2	QCH	2
QCM	2	QCL	2	QCM	2	QCL	2	QCM	2
QCM	2	QCH	2	QCM	2	QCL	2	QCH	2
QCH	2	QCL	2	QCL	2	QCH	2	QCL	2
QCH	2	QCH	2	QCH	2	QCM	2	QCM	2
QCM	2	QCM	2	QCL	2	QCH	2	QCL	2

Runtime (days): 10.7

Referenced Documents

- WU-SOP-EXP1-02- 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-LC2-01- 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-LC2-01 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
- PRM, parallel reaction monitoring
- PDX, patient derived xenograft