STANDARD OPERATING PROCEDURE

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

SOP#: WU-SOP-EXP2-01

Version #: 2

Authors: Petra Erdmann-Gilmore
Matthew R. Meyer PhD
Reviewer: Sherri Davies PhD
WU-PCC Directors: Matthew J. Ellis, MB, PhD
R. Reid Townsend, MD, PhD

Date Approved: 6/22/2014 **Date Modified: 11/13/10/14**

1. PURPOSE

The purpose of this document is to describe the preparation of admixture solutions of synthetic natural abundance (L, light) and isotope-labeled (H, heavy) peptides to determine the repeatability of LC-MS measurements in a tumor digest matrix. The preparation of sufficient quantities for replicate injections of LOW, MEDIUM and HIGH samples for analysis as described for Experiment 2 in the "Assay Development Guidelines" is described. The sample block for LC-MS with parallel reaction monitoring (PRM) (WU-SOP-LC1-01 and WU-SOP-MS2-01) is provided.

2. SCOPE

The preparation of LOW, MEDIUM and HIGH concentrations of synthetic peptide admixtures for performing a LC-MS the repeatability study (CPTAC, "Assay Development Guidelines", Experiment 2) is described. The bench procedures for preparing admixtures of heavy and light peptides from at concentrations of 1.5-3.0 x LLOQ (LOW), 50-100 x LLOQ (MEDIUM) and >100 LLOQ (HIGH) from frozen stock solutions is provided. The preparation of the stock solutions, diluents and tumor digest matrix for this SOP is described in WU-SOP-EXP1-01. The peptide concentrations for the three H/L standard admixtures for this experiment are calculated as the average value from the reverse standard curve isotope dilution analysis (WU-SOP-EXP1-01).

QC Standard	Final Concentration (fmol/µL)
QCL	3
QCM	100
QCH	200

3. 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.

4. EQUIPMENT

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

5. MATERIALS

Axygen® MAXYmum™ recovery tips;
P200 and P20: T-200-C-L-STK,
P1000: T-1000-C-L-R
Clear vials (4 mL, National Scientific, B7990-2)
5 and 100 mL volumetric flasks: Kimble KIMAX; rinse 3x with DI water (Millipore, 18.2 MΩ), 3 x with 70% AcN, 1%FA, 5 x with DI water
Volumetric glassware (5 mL flasks): Kimble KIMAX
Pipettes to transfer the 3.6 mL (Rainin, P1000)
PCR tubes: Axygen®, PCR-05-C (321-05-051)
Autosampler vials (Sun-Sri, 200 046)
Microtubes: Axygen® MCT-175-C (311-04-051)
Water, LC-MS grade (Fluka, 39253-1L-R)
Acetonitrile, LC-MS grade (Fluka, 34967-1L)
Formic Acid, 98%, 50 mL (Fluka, 56302-50ML-F)

Synthetic partially purified peptides (FLASHPURE™), New England Peptide. List of sequences shown in Supplemental Table 1 of the WU-SOP-EXP1-01.

6. REAGENTS

A. 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-EXP--01.

B. 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-EXP1-01 and summarized in Table I.

Table I Diluents and Standard Tryptic Peptide "Carrier" Solutions

Solution	Solvents	Peptide Concentration (fmol/µL)
AcN/FA-1	Acetonitrile/formic acid (1%/1%)	0
AcN/FA-30	Acetonitrile/formic acid (30%/1%)	0
TEN-MIX-1-100	Acetonitrile/formic acid (1%/1%)	100
TEN-MIX-30-50	Acetonitrile/formic acid (30%/1%)	50
TEN-MIX-30-200	Acetonitrile/formic acid (30%/1%)	200

C. Preparation of 10X stock H/L admixtures for LOW, MEDIUM and HIGH standard solutions

1) Remove an aliquot of the HPS, LPS and HSS-200 stock peptide solutions (Table II) from the freezer and thaw on ice.
2) Transfer ~1 mL of the TEN-MIX-30-50 diluent into flasks labeled at shown in Table II.
3) Pipette the indicated volumes from either a H or L secondary stock solution to prepare the six varying concentrations of heavy peptides and a constant quantity of light peptides.
4) Q.S. to 1 mL with TEN-MIX-30-50.
5) Dispense as aliquots (220 µL) into 500 µL PCR tubes and freeze at -80ºC.

D. Preparation of the diluted tumor digest for direct addition to the matrix blank and standard peptide samples for reverse curve generation

Table II. Primary and secondary peptide stock solutions for the preparation of the LOW, MEDIUM and HIGH concentrated (10X) Solutions[1]

Solution	Component	Diluent	Concentration (fmol/µL)
Peptide Stock Solutions			
HPS-5000 LPS-5000	Heavy (H) or Light (L) Primary Stock	ACN/FA-30	5000
HSS-200	Heavy (H) Secondary Stock	TENMIX-30-50	200

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

Table III. Volumes and reagents for preparation of concentrated (10X) LOW, MEDIUM and HIGH stocks

QC Stock	HEAVY Stock (fmol/µL)	HEAVY Stock (µL)	LPS (µL)	TEN-MIX-30-50 (µL)	Final Volume (µL)	Heavy Concentration (fmol/(µL)
10X-HIGH	HPS-5000	80	20	1900	2000	200
10X-MEDIUM	HPS-5000	40	20	1940	2000	100
10X-LOW	HSS-200	30	20	1950	2000	3

7. PROCEDURE

A. Preparation of Tumor Digest Dilutions for Addition to the Standard Peptide Samples for the Mini-validation Study

1) Remove an aliquot of the purified tumor digest peptides prepared according to WU-SOP-TD1-01 and dilute, if necessary, with the AcN/FA-1 diluent to an appropriate concentration to spike into the final standard sample (see below for example).

B. Preparation of Standards and Matrix Blanks for LC-MS

NOTE: The preparation of the samples for the repeatability study is performed on the day of LC-MS analysis. The samples are not frozen and are discarded after acquisition of the MS data.

1) Remove an aliquot for each of the three 10X H/L admixtures and a vial of the diluted tumor matrix from the freezer and thaw on ice.
2) Pipette 36 µL of AcN/FA-1 into all autosampler vials.
3) Pipette 4.5 µL of the respective concentrated stock soluion into the designated autosampler vial as shown in Table IV.
4) Add 4.7 µL of the tumor digest to all autosampler vials.
5) Vortex each autosampler vial (~ 15 s) and centrifuge in Sorval at 8,000 rpm for 20 min.
6) Place on autosampler tray at 4ºC for LC-MS analysis using WU-SOP-LC1-01 and WU-SOP-MS2-01.

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

Standard	Diluent (AcN /FA-1) (µL)	10X Stock (µL)	Tumor matrix spike solution (µL)	Final volume	Final (fmol/(µL)
HIGH	36	4.5	4.7	46	20
MEDIUM	36	4.5	4.7	46	10
LOW	36	4.5	4.7	46	0.3

Table V. Sample queue for LC-MS analysis of LOW (QCL), MEDIUM (QCM) and HIGH (QCH) for the 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)
Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7
QCL	4	QCH	4	QCH	4	QCL	4	QCL	4
Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7
QCH	4	QCM	4	QCL	4	QCH	4	QCH	4
Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7
QCL	4	QCL	4	QCH	4	QCM	4	QCM	4
Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7
QCL	4	QCM	4	QCM	4	QCM	4	QCH	4
Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7
QCM	4	QCL	4	QCM	4	QCL	4	QCM	4
Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7
QCM	4	QCH	4	QCM	4	QCL	4	QCH	4
Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7
QCH	4	QCL	4	QCL	4	QCH	4	QCL	4
Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7
QCH	4	QCH	4	QCH	4	QCM	4	QCM	4
Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7	Cal	1.7
QCM	4	QCM	4	QCL	4	QCH	4	QCL	4

Runtime (days): 10.7

8. REFERENCED DOCUMENTS

A. WU-SOP-EXP1-01- Preparation of Standard Peptide Samples for the Generation Reverse Response Curves-Experiment 1

B. WU-SOP-MS2-01- Mass Spectrometry Using Parallel Reaction Monitoring for Experiments 1 and 2

C. WU-SOP-TD1-01- Preparation of Peptides from Tumor Lysates

D. WU-SOP-LC1-01- nano-Liquid Chromatography for Experiment 1 and 2

9. LIST OF ABBREVIATIONS

AcN, acetonitrile
FA, formic acid
LC-MS, *nano*-LC interfaced to a high-resolution quadrupole-time-of-flight mass spectrometer as described in WU-SOP-LC-1 and WU-SOP-MS-1
H or heavy, stable isotopically labeled synthetic peptide
L or light, natural abundance synthetic peptide
Q.S., *quantum satis*
PS, primary stock solution; 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 W-SOP-EXP1
PRM, parallel reaction monitoring
PDX, patient derived xenograft