STANDARD OPERATING PROCEDURE

Title: Preparation of Standard Peptide Samples for the Generation of Reverse Response Curves-Experiment 1

SOP#: WU-SOP-EXP1-01

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1. PURPOSE

The purpose of this document is to describe the preparation of standard solutions of synthetic natural abundance (L, light) and isotope-labeled (H, heavy) peptides to determine the linear range, and estimates of the LOD and LLOQ from a reverse response curve in a tumor digest matrix. The preparation of sufficient quantities for 30 six-point, four-order-of-magnitude standard curves from one freeze-thaw of stocks is described. The methods for LC-MS using parallel reaction monitoring (PRM) are provided in WU-SOP-LC1-01 and WU-SOP-MS2-01.

2. SCOPE

The preparation of response curve standards for performing Experiment 1 in the CPTAC document, “Assay Development Guidelines” is described.

The detailed bench level procedures for preparing standard admixtures of heavy and light peptides from dried, partially purified preparations that are supplied by the vendor in 96 well arrays, and the diluents containing ‘carrier’ peptides is described. The preparation of standard samples containing matrix from a pooled tryptic digest of seven breast cancer patient derived xenografts (PDX) (WU-SOP-TD1-01) is provided.

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, 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)
Tryptic digests of proteins (Michrom Bioresources Inc.)
Yeast Alcohol Dehydrogenase (PTD/00001/36)
Yeast Enolase (PTD/00001/46)
Equine Cytochrome C (PTD/00001/12)
Equine Apo-Myoglobin (PD/00001/04)
Six Bovine Tryptic Digest Equal Molar Mix (PTD/00001/63)
Synthetic partially purified peptides (FLASHPURE™), New England Peptide.

6. REAGENTS

A. Breast Cancer Tumor Matrix (WU-SOP-TD1-01)

A tryptic digest of a pool of patient-derived breast cancer xenografts was prepared by combining the tissues as equal weights of cryopulverized powder. Tryptic peptides were prepared according to the protocol described, WU-SOP-TD-01, “Preparation of purified peptides from solubilized tumor tissue—100 µg scale”. The pool consisted of equal tumor weights of Basal (WHIM’s 2 and 6), Luminal B (WHIM’s 16 and 20, Her-2 –Enriched (WHIM’s 8 and WHIM 11), and claudin-low (WHIM 12) intrinsic subtypes of breast cancer. The tryptic peptide digests were thawed on ice before spiking into the standard samples immediately prior to placing on the autosampler tray for LC-MS. The total quantity that was added to each standard vial (~1 µg) is based on the total protein content of the pooled lysate and assuming a quantitative conversion of peptide from protein.

7. PROCEDURES

A. Preparation of Diluent Solutions

1) ACETONITRILE/FORMIC ACID (1%/1%) (ACN/FA-1)

a) Add 50 mL of LC-MS grade water to a 100 mL volumetric flask that has been labeled, initialed and dated.
b) Dispense 1.0 mL of FA (98%) and 1.0 mL of AcN (LC-MS grade) into the flask.
c) Fill to volumetric flask mark with LC-MS grade water (Q.S.).
d) Store the diluent solution at room room temperature for less than one month.

2) ACETONITRILE/FORMIC ACID (30%/1%) (AcN/FA-30)

a) Add 50 mL of water to a 100 mL volumetric flask that has been labeled, initialed, and dated.
b) Pipette 1 mL of formic acid (98%) into the flask.
c) Add 30 mL of AcN.
d) Q.S. with water.
e) The solution is stored sealed at room temperature for less than one month.

B. Preparation of "Carrier" Tryptic Peptide Solutions for Transfer of Peptides From Vendor Vials and Dilution of These Primary Solutions for Reverse Curve Standard Preparation

NOTE: The naming of the diluent solutions gives the number of proteins as standard tryptic digests, the percent of acetonitrile and the concentration of total peptide content in fmol/µL. For example, TEN-MIX-1-100 is a mixture of the tryptic digests from ten non-human proteins (Tables I and II) in 1% AcN at with a total peptide content of 100 fmol/µL. All solutions contain FA (1%).

Table I. Tryptic peptide digests of proteins and volumes to prepare primary stock solution (~ 1 pmol/µL).

PROTEIN	Species	pmol/each vial	Volume (µL) to make primary solution (1 pmol/µL)
Cytochrom C	Equine	500	500
Apomyoglobin	Equine	500	500
Alcohol Dehydrogenase	Yeast	500	500
Enolase	Yeast	500	500

Table II. Tryptic peptide digests of six bovine proteins and volume to prepare primary stock solution (1.2 pmol/µL).

<table>
<tr><th>Proteins</th><th>Species</th><th>pmol/one vendor vial</th><th>volume to make primary stock solution
(1.2 pmol /µL)</th></tr>
<tr><td>Beta Lactoglobulin</td><td>bovine</td><td>100</td><td rowspan="6">500</td></tr>
<tr><td>Lactoperoxidase</td><td>bovine</td><td>100</td></tr>
<tr><td>Carbonic Anhydrase</td><td>bovine</td><td>100</td></tr>
<tr><td>Glutamate Dehydrogenase</td><td>bovine</td><td>100</td></tr>
<tr><td>Alpha Casein</td><td>bovine</td><td>100</td></tr>
<tr><td>Serum Albumin</td><td>bovine</td><td>100</td></tr>
</table>

1) Preparation of TEN-MIX-1-100.

a) Pipette 500 µL of AcN/FA-1 diluent solution to each of four vendor vials containing 500 pmol of dried tryptic digests of cytochrome C, apomyoglobin, alcohol dehydrogenase and enolase (Table I).
b) Vortex vials for 30 s and spin in the microcentrifuge for 10 s at the maximum setting (~ 14,000 rcf x g). Place the centrifuged vials on ice.
c) Combine an aliquot (200 µL) from each of the four primary solutions into a 1.5 mL vial, add 200 µL of AcN/FA-1, vortex for 30 s and place this secondary, four- protein peptide solution on ice.
d) Add 500 µL of AcN/FA-1 to the vendor vial that contains the tryptic digests of six bovine proteins (Table II), vortex for 30 s, centrifuge as above for 30 s and place on ice.
e) Add 3.6 mL of AcN/FA-1 to the 4 mL glass vial.
f) Prepare the TEN-MIX-100-1 solution by adding 200 µL of the secondary solution of the four protein mix (Step 3 above) and 200 µL of the primary solution of bovine tryptic digests (Step 4 above) to the 4 mL vial. Mix by vortexing.
g) TEN-MIX-1-100 peptide solutions are stored at -20ºC.

2) Preparation of TEN-MIX-30-100.

This solution is prepared exactly as described for the TEN-MIX-1-100 solution except that the the **AcN/FA-30** instead of **AcN/FA-1** is used as the diluent for preparing the primary, secondary and final solutions (TEN-MIX-30-100 aliquots).

Calculations for total peptide content of the ten protein digest equimolar mix (TEN-MIX-1-100 and TEN-MIX-30-100): Four protein secondary solution = 800 pmols/mL = 0.8 pmols/µL and primary six bovine protein mix = 600 pmol/500 µL=1.2 pmol/µL. Two hundred µL of each of the above solutions (160 + 240 pmol) are added to 3.6 mL of diluent to give a final concentration

of total peptide of 100 fmol/µL or 150 pg/µL of total peptide (assuming average molecular weight of peptide to be 1500). The "1-100" and "30-100"designation indicates that these peptide diluents contain 1% and 30% acetonitrile, respectively, and a total peptide content of 100 fmol/µL.

3) Preparation of TEN-MIX-30-50.

a) Pipette 4750 µL of diluent solution **AcN-FA-30** into the 4 mL glass vial.
b) Add 125 µL of the secondary stock solution of the yeast-equine four protein tryptic digest mix.
c) Add 125 µL of the primary bovine six protein tryptic digest stock solution.
d) The TEN-MIX-30-50 peptide solutions are stored at -20ºC.

Calculation the total peptide content of TEN-MIX-30-50: Four protein mix = 800 pmols/mL = 0.8 pmol/µL and six bovine protein mix = 600 pmol/500 µL=1.2 pmol/µL. One hundred twenty five µL of the yeast-equine (100 pmol) and bovine (150 pmol) tryptic digests added to final volume of 5 mL (100 +150 pmol/5000 µL). The "30-50" designation of this solution indicates AcN(30%)/FA (1%) and a total peptide content of 50 fmol/µL or 75 pg/µL of total peptide content (assuming average molecular weight of peptide to be 1500).

4) Preparation of TEN-MIX-30-200.

a) Pipette 1.6 mL of diluent solution (AcN-FA-30) into the 4 mL glass vial.
b) Add 200 µL of the secondary yeast-equine four protein tryptic digest mix.
c) Add 200 µL of the primary bovine six protein tryptic digest mix.
d) The TEN-MIX-30-200 peptide solutions are stored at -20ºC.

Calculations for total peptide content of TEN-MIX-30-200: Equine-yeast tryptic digest (200 µL x 0.8 pmols/µL = 160 pmols) and six bovine protein mix (200 µL x 1.2 pmols/µL = 240 pmols) in 4 mL. The "30-200" designation of this solution indicates AcN(30%)/FA (1%) and a total peptide content of 200 fmol/µL or 300 pg/µL of total peptide content (assuming an average molecular weight of peptide to be 1500).

Table III. 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 the primary peptide stocks as combined mixture of either heavy or light peptides

NOTE: A primary peptide stock solution is defined as the solution that is prepared from the vendor vial. A stock solution is prepared, frozen and thawed once to prepare secondary solutions and then discarded. The synthetic peptides for this SOP are are vendor estimated quantities of 400-700 nmols/vial that have been transferred from a 96 well plate, dried and shipped. The estimated concentrations are based on the lesser quantity quoted (e.g. 400 nmol). The peptides from eight 96 well plates (384 H/L) pairs were used. The list of gene names and sequences are provided as a Supplementary Table at the end of this document.

1) Remove all selected peptides for a planned multiplex assay from the -80 $^{\circ}$C freezer and allow to warm to room temperature.
2) Group into heavy and light sets.
3) Label two 5 mL volumetric flasks as either “HEAVY” or “LIGHT” and add ~ 1 mL of the TEN-MIX-30-50 diluent.
4) Add 100 µL of the AcN/FA-30 diluent to each vendor vial.
5) Vortex for 30 sec and shake liquid to the bottom of the vendor vial.
6) Pipette ~25 nmol from each vendor vial into either the “HEAVY” or “LIGHT” labeled volumetric flask. Pipette up and down 3 times with the contents of the 5 mL volumetric flask.
7) Once all selected peptides have been transferred, Q.S. the volumetric flask with TEN-MIX-30-50 for a final concentration of ~ 5 pmol/µL for each peptide.
8) Dispense as aliquots of the primary H or L combined peptide stock solutions (220 µL) into 500 µL PCR tubes and freeze at -80°C.

D. Preparation of Secondary Peptide Stock Solutions as Admixtures of the H and L Primary Peptide Stock Solutions.

1) Remove an aliquot of each H and L primary stock from the freezer and thaw on ice.
2) Label six 2 mL volumetric flasks as HSS-200, LSS-200, HSS-20, LSS-20 and LSS-2.
3) Transfer ~1 mL of the TEN-MIX-30-50 diluent to each flask
4) Pipette into each flask the volumes from the H and L primary stocks as indicated in Table III and Q.S. to 2 mL with TEN-MIX-30-50.
5) Dispense as aliquots (220 µL) into 500 µL PCR tubes and freeze at -80ºC.

Table IV. Primary and Secondary Peptide Stock Solutions			
Solution	Component	Diluent	Concentration (fmol/µL)
Peptide Stock Solutions			
HPS-5000 LPS-5000	Heavy (H) or Light (L) Primary Stock	30%/1% AcN/FA	5000
HSS-200 LSS-200	Heavy (H) or Light (L) Secondary Stock	TENMIX- 30-50	200
HSS-20 LSS-20	Heavy (H) or Light (L) Secondary Stock	TENMIX- 30-50	20
HSS-2 LSS-2	Heavy (H) or Light (L) Secondary Stock	TENMIX- 30-50	2

E. Preparation of 10X Stock H/L Admixtures for Standard Reverse Curve Generation.

1) Remove an aliquot of each of the six H and L secondary stock solutions from the freezer and thaw on ice.
2) Transfer ~1 mL of the AcN/FA-1 diluent into flasks labeled as shown in Table V.
3) Pipette the indicated volumes (**Table V**) 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.

Table V. Diluents for Preparation of Stock Solutions (H/L) for Standard Reverse Curve Generation						
STD Stock Solution	Reagents Needed	Carrier	fmol H/L	HSS (µL)	HPS (µL)	LPS (µL)
10XSTD1	HSS-2 & LPS	TENMIX-30-50	0.025/50	2	25	20
10XSTD02	HSS-20 & LPS	TENMIX-30-50	0.25/50	20	25	20
10XSTD03	HSS-20 & LPS	TENMIX-30-50	1.15/50	20	115	20
10XSTD04	HSS-200 & LPS	TENMIX-30-50	7.0/50	200	70	20
10XSTD05	HPS & LPS	TENMIX-30-50	70/50	5000	28	20
10XSTD06	HMAP-5000 & LMAP-5000	TENMIX-30-50	250/50	5000	100	20

E. Preparation of Tumor Digest Dilutions for Addition to the Standard Peptide Samples for Reverse Curve Generation.

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

F. Preparation of Standards and Matrix Blanks for Six-Point Reverse Curve Generation.

NOTE: The preparation of the samples for the standard curve 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 six 10X H/L admixtures (**Table V**) and a vial of the tumor matrix from the freezer and thaw on ice.
2) Pipette 46 µL of AcN/FA-1 into all autosampler vials.
3) Add 3.5 µL of the tumor matrix peptide solution
4) Add 5. 5 µL of the standard peptide admixtures.
5) Vortex for ~ 10 s and centrifuge in the Sorvall centrifuge for 20 min at 8000 rpm.

G. Generating the Assay Response Curve

Samples and standards are analyzed by *nano*-LC-MS according to WU-SOP-LC1-01 and WU-SOP-MS1-01. **Table VI** shows the injection queue. A total of 3 replicates are acquired for each concentration of the curve. Injection volume of STD samples is 10 µL in TEN-MIX-1-100 diluent.

Table IV. Reverse-Curve Run Block[1]

Sample Injection	Run Time (h)
Matrix Blank-1	6
Matrix Blank-2	6

Matrix Blank-3	6
STD 01	6
STD 02	6
QCM 01	6
STD 03	6
STD 04	6
STD 05	6
STD 06	6
Matrix Blank-4	6
Matrix Blank-5	6
Matrix Blank-6	6
STD 01	6
STD 02	6
STD 03	6
STD 04	6
STD 05	6
QCM 02	6
STD 06	6
Matrix Blank-9	6
Matrix Blank-10	6
Matrix Blank-11	6
STD 01	6
STD 02	6
STD 03	6
QCM 03	6
STD 04	6
STD 05	6
STD 06	6
Matrix Blank-13	6
Matrix Blank-14	6
Matrix Blank-15	6
Total Run Time (Days)	8.25

[1]The “Run time” includes the calibration runs on the calibration column (see WU-SOP-LC1-01).

8. REFERENCED DOCUMENTS

A. WU-SOP-TD-01, “Preparation of purified peptides from solubilized tumor tissue—100 µg scale”.

B. WU-SOP-LC1-01-“nano-Liquid Chromatography for Experiment 1 and 2”

C. WU-SOP-MS1-01-“Optimizing Mass Spectrometer Performance for Experiments 1 and 2”

D. WU-SOP-MS2-01-“Mass Spectrometry Using Parallel Reaction Monitoring for Experiments 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-01 and WU-SOP-MS1-01

H or heavy, stable isotopically labeled synthetic peptide

L or light, natural abundance synthetic peptide

Q.S., *quantum satis*

PDX, patient-derived xenografts

PRM, parallel reaction monitoring

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.

Supplemental Table. Sequences and estimated quantities of peptides for reverse curve generation.

Gene Name	Peptide Sequence	Concentration (nmoles/mL)[a]		Peptide Sequence[b]	Concentration (nmoles/mL)[a]
ACACA	SS[+80]MSGLHLVK	196		SS[+80]MSGLHLVK^	258
AKT1	FFAGIVWQHVYEK	434		FFAGIVWQHVYEK^	152
AKT1	QEEEEMDFR	857		QEEEEMDFR^	248
AKT1S1	C[+57]LHDIALAHR	417		C[+57]LHDIALAHR^	83
AKT1S1	LNT[+80]SDFQK	284		LNT[+80]SDFQK^	182
AKT1S1	SLPVSVPVWGFK	875		SLPVSVPVWGFK^	229
AKT2	APGEDPMDYK	669		APGEDPMDYK^	232
AKT2	YDSLGLLELDQR	309		YDSLGLLELDQR^	131
BIRC2	AVEDISSSR	244		AVEDISSSR^	85
BIRC2	LGDSPIQK	464		LGDSPIQK^	213
CASP10	HEDILSILTAVNDDVSR	251		HEDILSILTAVNDDVSR^	131
CASP10	LLIIDSNLGVQDVENLK	191		LLIIDSNLGVQDVENLK^	166
CASP8	FLLQEEISK	397		FLLQEEISK^	261
CASP8	FLSLDYIPQR	393		FLSLDYIPQR^	170
CDK1	IGEGT[+80]YGVVYK	530		IGEGT[+80]YGVVYK^	385
CDK1	IGEGTY[+80]GVVYK	440		IGEGTY[+80]GVVYK^	286
CDK1	IGEGTYGVVYK	461		IGEGTYGVVYK^	348
CDK1	VY[+80]THEVVTLWYR	432		VY[+80]THEVVTLWYR^	293
CDK1	VYT[+80]HEVVTLWYR	456		VYT[+80]HEVVTLWYR^	306
CDK1	VYTHEVVTLWYR	376		VYTHEVVTLWYR^	288
EGFR	GSHQISLDNPDY[+80]QQDFFPK	355		GSHQISLDNPDY[+80]QQDFFPK^	169
EGFR	MHLPSPTDSNFY[+80]R	486		MHLPSPTDSNFY[+80]R^	169
GSK3B	TTS[+80]FAESC[+57]KPVQQPSAFGSMK	321		TTS[+80]FAESC[+57]KPVQQPSAFGSMK^	330
IKBKG	EC[+57]QALEGR	246		EC[+57]QALEGR^	165
IKBKG	ELLQEQLEQLQR	412		ELLQEQLEQLQR^	178
JUN	LAS[+80]PELER	343		LAS[+80]PELER^	196
MAP2K1	QLMVHAFIK	503		QLMVHAFIK^	386
MAP2K1	VSHKPSGLVMAR	245		VSHKPSGLVMAR^	138
MAP2K2	LNQPGTPT[+80]R	176		LNQPGTPT[+80]R^	176
MAP2K2	LNQPGTPTR	728		LNQPGTPTR^	206
MAP2K2	LPNGVFTPDFQEFVNK	610		LPNGVFTPDFQEFVNK^	191
MAP2K2	SYMAPER	597		SYMAPER^	156
MAP2K3	FSPEFVDFTAQC[+57]LR	399		FSPEFVDFTAQC[+57]LR^	128
MAP2K3	QVVEEPSPQLPADR	528		QVVEEPSPQLPADR^	184
MAP2K4	AVEVAC[+57]YVC[+57]K	545		AVEVAC[+57]YVC[+57]K^	212
MAP2K4	FTLNPNPTGVQNPHIER	514		FTLNPNPTGVQNPHIER^	141
MAP2K5	ILANGQMNEQDIR	241		ILANGQMNEQDIR^	214

MAP2K5	LC[+57]DFGVSTQLVNSIAK	615		LC[+57]DFGVSTQLVNSIAK^	202
MAP2K6	DVKPSNVLINALGQVK	285		DVKPSNVLINALGQVK^	95
MAP2K6	GTDVASFVK	599		GTDVASFVK^	230
MAP2K7	TGHVIAVK	361		TGHVIAVK^	169
MAP2K7	TSGVLSQPHLPFFR	567		TSGVLSQPHLPFFR^	148
MAP3K1	ASSAPAAAAGLLR	591		ASSAPAAAAGLLR^	181
MAP3K1	LLQPVVDTILVK	440		LLQPVVDTILVK^	231
MAP3K1	LSAS[+80]SEDISER	194		LSAS[+80]SEDISER^	230
MAP3K1	LSASS[+80]EDISER	177		LSASS[+80]EDISER^	243
MAP3K1	LSASSEDISER	317		LSASSEDISER^	179
MAP3K1	NS[+80]MTLDLNSSSK	394		NS[+80]MTLDLNSSSK^	364
MAP3K1	NSMTLDLNSSSK	647		NSMTLDLNSSSK^	337
MAP3K1	RAPS[+80]PDGFSPYSPEETNR	351		RAPS[+80]PDGFSPYSPEETNR^	206
MAP3K1	RAPSPDGFSPYSPEETNR	339		RAPSPDGFSPYSPEETNR^	235
MAP3K11	AIQLEPAEPGQAWGR	672		AIQLEPAEPGQAWGR^	194
MAP3K11	GGTVS[+80]PPPGTSR	156		GGTVS[+80]PPPGTSR^	231
MAP3K11	GGTVSPPPGTSR	871		GGTVSPPPGTSR^	210
MAP3K11	ITVQAS[+80]PGLDR	334		ITVQAS[+80]PGLDR^	417
MAP3K11	ITVQASPGLDR	290		ITVQASPGLDR^	218
MAP3K11	LEEVIGIGGFGK	1277		LEEVIGIGGFGK^	411
MAP3K11	NVFEVGPGDS[+80]PTFPR	430		NVFEVGPGDS[+80]PTFPR^	348
MAP3K11	NVFEVGPGDSPTFPR	1208		NVFEVGPGDSPTFPR^	272
MAP3K15	ETLLNDIR	36		ETLLNDIR^	184
MAP3K15	IGVRLNSLLGR	173		IGVRLNSLLGR^	227
MAP3K2	AQSYPDNHQEFSDYDNPIFEK	344		AQSYPDNHQEFSDYDNPIFEK^	166
MAP3K2	LSIIGPTSR	553		LSIIGPTSR^	228
MAP3K3	AQSFPDNR	620		AQSFPDNR^	136
MAP3K3	SADSENALSVQER	472		SADSENALSVQER^	176
MAP3K4	SIILQLLNAAAGK	119		SIILQLLNAAAGK^	163
MAP3K4	VDYGSFAFVR	859		VDYGSFAFVR^	290
MAP3K5	AC[+57]ANDLLVDEFLK	577		AC[+57]ANDLLVDEFLK^	277
MAP3K5	NLYTGKELAAELAR	254		NLYTGKELAAELAR^	219
MAP3K6	C[+57]LSYGGTSQLR	608		C[+57]LSYGGTSQLR^	160
MAP3K6	FSGPQLR	797		FSGPQLR^	264
MAP3K7	DLKPPNLLLVAGGTVLK	600		DLKPPNLLLVAGGTVLK^	355
MAP3K7	IAATTGNGQPR	289		IAATTGNGQPR^	106
MAP3K7	MS[+80]ADMSEIEAR	245		MS[+80]ADMSEIEAR^	293
MAP3K7	MSADMSEIEAR	228		MSADMSEIEAR^	217
MAP3K7	S[+80]IQDLTVTGTEPGQVSSR	280		S[+80]IQDLTVTGTEPGQVSSR^	133
MAP3K8	IEASLER	581		IEASLER^	143
MAP3K8	NIGSDFIPR	793		NIGSDFIPR^	265

MAP3K9	LQHSPSQSYLC[+57]IPFPR	511		LQHSPSQSYLC[+57]IPFPR^	183
MAP3K9	TPSDGALKPETLLASR	521		TPSDGALKPETLLASR^	190
MAP4K2	IHAAVTWIHPVTR	635		IHAAVTWIHPVTR^	174
MAP4K2	VFNGC[+57]PLR	673		VFNGC[+57]PLR^	251
MAP4K3	HIDFPIPC[+57]PLR	777		HIDFPIPC[+57]PLR^	150
MAP4K3	LLQHPFVTQHLTR	666		LLQHPFVTQHLTR^	173
MAP4K4	VYPLINR	793		VYPLINR^	260
MAP4K4	DSPLQGSGQQNSQAGQR	289		DSPLQGSGQQNSQAGQR^	120
MAP4K5	ISSYPEDNFPDEEK	552		ISSYPEDNFPDEEK^	252
MAP4K5	LLTHTFVAQPGLSR	459		LLTHTFVAQPGLSR^	165
MAPK1	AAAAAAGAGPEMVR	498		AAAAAAGAGPEMVR^	205
MAPK1	ELIFEETAR	289		ELIFEETAR^	258
MAPK11	DLSSIFR	321		DLSSIFR^	296
MAPK11	GANPLAIDLLGR	256		GANPLAIDLLGR^	323
MAPK12	DLQPVGSGAYGAVC[+57]SAVDGR	420		DLQPVGSGAYGAVC[+57]SAVDGR^	245
MAPK12	QADSEMTGYVVTR	301		QADSEMTGYVVTR^	181
MAPK13	EIVNFSPIAR	524		EIVNFSPIAR^	242
MAPK13	SYIQSLPQTPR	677		SYIQSLPQTPR^	116
MAPK14	IVTAKQGAVNSFYTVSK	426		IVTAKQGAVNSFYTVSK^	156
MAPK14	NYIQSLTQMPK	479		NYIQSLTQMPK^	324
MAPK3	IADPEHDHTGFLTEY[+80]VATR	347		IADPEHDHTGFLTEY[+80]VATR^	182
MAPK3	IADPEHDHTGFLT[+80]EYVATR	265		IADPEHDHTGFLT[+80]EYVATR^	132
MAPK3	IADPEHDHTGFLTEYVATR	360		IADPEHDHTGFLTEYVATR^	224
MAPK4	GYLSEGLVTK	548		GYLSEGLVTK^	338
MAPK4	VGSPSYLDK	463		VGSPSYLDK^	301
MAPK6	SSPQIPHQTYSSILK	454		SSPQIPHQTYSSILK^	231
MAPK6	SSSYLDNLVWR	604		SSSYLDNLVWR^	195
MAPK7	DLKPSNLLVNENC[+57]ELK	268		DLKPSNLLVNENC[+57]ELK^	142
MAPK7	GLC[+57]TSPAEHQYFMTEYVATR	279		GLC[+57]TSPAEHQYFMTEYVATR^	151
MAPK8	EVMDLEER	837		EVMDLEER^	241
MAPK8	NIIGLLNVFTPQK	417		NIIGLLNVFTPQK^	91
MAPK8	TAGTSFMMTPY[+80]VVTR	378		TAGTSFMMTPY[+80]VVTR^	154
MAPK8	TAGTSFMMTPYVVTR	298		TAGTSFMMTPYVVTR^	229
MAPK9	FEELFPDWIFPSESER	335		FEELFPDWIFPSESER^	176
MAPK9	VIEQLGTPSAEFMK	454		VIEQLGTPSAEFMK^	221
MLKL	LAGFELR	420		LAGFELR^	292
MLKL	LQAGSIAIVR	386		LQAGSIAIVR^	228
PDPK1	ANS[+80]FVGTAQYVSPELLTEK	291		ANS[+80]FVGTAQYVSPELLTEK^	215
RB1	IPGGNIYIS[+80]PLK	182		IPGGNIYIS[+80]PLK^	182
RIPK1	IADLGLASFK	482		IADLGLASFK^	429
RIPK1	MQS[+80]LQLDC[+57]VAVPSSR	227		MQS[+80]LQLDC[+57]VAVPSSR^	343

RIPK1	MQSLQLDC[+57]VAVPSSR	154		MQSLQLDC[+57]VAVPSSR^	235
RIPK1	YQAIFDNTTSLTDK	343		YQAIFDNTTSLTDK^	392
RIPK3	DLKPSNVLLDPELHVK	190		DLKPSNVLLDPELHVK^	124
RIPK3	VNWDQDPKPALVTK	511		VNWDQDPKPALVTK^	251
SRC	LIEDNEY[+80]TAR	150		LIEDNEY[+80]TAR^	188
TAB1	GTESHPPEDSWLK	395		GTESHPPEDSWLK^	229
TAB1	QVGIIC[+57]GGQESTR	307		QVGIIC[+57]GGQESTR^	232
TAB2	AIGNNSATSPR	111		AIGNNSATSPR^	150
TAB2	TSSTSSSVNSQTLNR	272		TSSTSSSVNSQTLNR^	105
TBK1	EPLNTIGLIYEK	488		EPLNTIGLIYEK^	210
TBK1	YQEYTNELQETLPQK	348		YQEYTNELQETLPQK^	183

[a]Concentrations were calculated based on average nmoles of fiducial residues (A, L, F, R).

[b] "^" denotes isotopically labeled residue.