STANDARD OPERATING PROCEDURE

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

SOP#: WU-SOP-EXP1-02

Version #: 3.0
(Metabolic Panel)

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Purpose

The purpose of this document is to describe the preparation of standard solutions of high purity, synthetic natural abundance (L, light) and stable isotope-labeled (H, heavy) peptides to determine the linear range, and the limits of the blank (LOB), limit of detection (LOD), and the lower limit of quantification (LLOQ) from a reverse response curve in a tumor digest matrix (https://assays.cancer.gov/guidance-document/). The preparation of sufficient quantities of H/L peptide standard admixtures to generate replicate measurements (n=3) at six concentrations (over four orders of magnitude) from a single freeze-thaw cycle of stock solutions is described.

Scope

The detailed bench procedures for preparing admixtures of standard heavy and light peptides from high purity, quantified peptide solutions that are supplied by the vendor is described. The preparation of primary and secondary stock solutions using diluents containing matrix peptides or carrier peptides is described. The preparation of standard samples containing matrix from a pooled tryptic digest of breast cancer patient derived xenografts (PDX) (WU-SOP-TD1-01) is provided. The preparation of standard samples for LC-MS to generate the reverse response curves that is described in Experiment 1 in the CPTAC document, "Assay Development Guidelines" is described.

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 (1, 2, 5, 20, 50, 100 mL flasks): Kimble KIMAX ;rinse 3x with DI water, 3 x with 70% AcN, 1%FA, 5 x with DI water
- Autosampler vials (Sun-Sri, 200 046)
- Microcentrifuge tubes: Fisher, 02-681-333
- Microcentrifuge tube caps: 02-681-368

Reagents

- Water, LC-MS grade (Fluka, 39253-1L-R)
- Acetonitrile, LC-MS grade (Fluka, 34967-1L)
- Formic Acid, 98%, 50 mL (Fluka, 56302-50ML-F)
- Triflouroacetic Acid, 99.5%, 10 x 1 mL (Thermo Scientific, 28904)
- Tryptic digests of proteins for TEN-MIX solutions (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)
- Tryptic digests of proteins for NINE-MIX solutions (Protea Biosciences, Inc.)
- Yeast Enolase (PS-217-1)
- Equine Cytochrome C (PS-200)
- Equine Myoglobin (PS-209)
- Six Bovine Tryptic Digest Equal Molar Mix (PS-215-1)
- Synthetic high purity peptides, New England Peptide.

Solutions

- **Breast Cancer Tumor Matrix (WU-SOP-TD1-01)**
  The matrix for the preparation of standard samples is a tryptic digest of a pool of patient-derived breast cancer xenografts as detailed in WU-SOP-TD-01, "Preparation of purified peptides from solubilized tumor tissue—100 µg scale". The aliquoted tryptic peptide digests were thawed on ice before. The total quantity that

was added to each standard vial (~1 µg per injection) is based on the starting protein concentration of the pooled lysate and assumes complete conversion and recovery of peptides from proteins.

Procedure

1. **Preparation Of Diluent Solutions**

   a. **Breast Cancer Tumor Matrix (0.5µg/µL) (TD)**
      1. Remove an aliquot of the purified tumor digest peptides prepared according to WU-SOP-TD1-01 and dilute with TFA-1, to a concentration of 0.5 mg/mL (see below for example used for Experiment 1).

   2. **Trifluoroacetic acid/Water (1%) (TFA-1)**
      1. Add 50 mL of LC-MS grade water to a 100 mL volumetric flask that has been labeled, initialed, and dated.
      2. Pipette 1 mL of TFA (98%) into the flask.
      3. Q.S. with LC-MS grade water.
      4. The solution is stored sealed at room temperature for < one month.

2. **Preparation of "Carrier" Tryptic Peptide Solutions for Primary Transfer of Peptides from Vendor Vials and Dilution of the Primary Solutions.**
   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, Nine-MIX-1-100 is a mixture of the tryptic digests from nine non-human proteins (Tables I and II) in 1% AcN 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
Enolase	Yeast	500	500

Table II. Tryptic peptide digests of six bovine proteins and volume to prepare primary stock solution (1.2 pmol/µL).			
Protein	**Species**	**pmol/one vendor vial**	**volume to make primary stock solution (1.2 pmol /µL)**
Beta Lactoglobulin	bovine	100	500
Lactoperoxidase	bovine	100	
Carbonic Anhydrase	bovine	100	
Glutamate Dehydrogenase	bovine	100	
Alpha Casein	bovine	100	
Serum Albumin	bovine	100	

Table III. Diluents and Standard Tryptic Peptide "Carrier" Solutions		
Solution	**Solvents**	**Peptide Concentration (fmol/µL)**
TFA-1	Trifluoroacetic acid/Water (1%/99%)	0
TD matrix	breast cancer tumor matrix in TFA-1	0.5 mg/mL
AcN/FA-30	Acetonitrile/formic acid (30%/1%)	0
NINE-MIX-30-5000	Acetonitrile/formic acid (30%/1%)	5000
NINE-MIX-30-200	Acetonitrile/formic acid (30%/1%)	200
NINE-MIX-30-50	Acetonitrile/formic acid (30%/1%)	50

3. Preparation of NINE-MIX-30-5000 Solution.

a. Pipette 500 µL of AcN/FA-30 diluent solution to each of three vendor vials containing 500 pmol of dried tryptic digests of cytochrome C, apomyoglobin, 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).
c. Add 500 µL of AcN/FA-30 to a vendor vial that contains the tryptic digests of six bovine proteins (Table II), vortex for 30 s, and centrifuge as above for 30 s.
d. Combine 10 µL each of the cytochrome C, apomyoglobin, and enolase solutions and 50 µL of the six bovine solution and store at -20 °C.

4. **Preparation of NINE-MIX-30-200 Solution.**
    a. Pipette 500 µL of AcN/FA-30 diluent solution to each of three vendor vials containing 500 pmol of dried tryptic digests of cytochrome C, apomyoglobin, 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). Place the centrifuged vials on ice.
    c. transfer entire contents of each vial to a 50mL volumetric flask. Rinse vial with another 500 µL of AcN/FA-30, vortex, spin and transfer entire volume to 50 mL volumetric flask.
    d. Add 500 µL of AcN/FA-30 to each of 5 vendor vials that contain the tryptic digests of six bovine proteins (Table II), vortex for 30 s, centrifuge as above for 30 s and transfer entire contents of each vial to the 50 mL volumetric flask .
    e. QS the volumetric flask to 50 mL mark with AcN/FA-30.
    f. Aliquot into 4mL into each of 12 glass vials.
    g. Store aliquots of NINE-MIX-30-200 peptide solutions at -20ºC.

5. **Preparation of NINE-MIX-30-50.**
    a. Pipette 2500µL of NINE-MIX-30-200 solution into the 10 mL volumetric flask.
    b. QS the volumetric flask to the 10 mL mark with AcN-FA-30. Mix by inversion.
    c. Aliquot into 4 mL into each of 2 glass vials
    d. Store aliquots of NINE-MIX-30-50 peptide solutions at -20ºC.

6. **Preparation of the Primary Heavy and Light Peptide Stock Solutions in tumor digest matrix (HPS-TD and LPS-TD).**

    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 generate secondary solutions and then discarded. The synthetic peptides for this SOP are vendor quantified and qualified high purity peptides. A portion of the contents of each vendor vial is used to prepare the primary heavy and primary light solutions, HPS and LPS.

    a. Remove the vendor vials for all selected peptides for the designed multiplex assay from the -80 ºC freezer and allow warming to room temperature.
    b. Group into heavy and light sets.
    c. Label two 1 mL volumetric flasks as either “HEAVY” or “LIGHT” and add ~ 0.5 mg of tumor digest matrix in 40µL 1%TFA diluent.
    d. Add 13 µL of each peptide from each vendor vial to the 1mL volumetric flask.

e. Once all selected peptides have been transferred, Q.S. the volumetric flask with 1%TFA for a final concentration of ~ 130 fmol/µL for each peptide in 0.5 mg/mL TD matrix.
f. The panel consists of 1 primary stock solution composed of **Primary Heavy (HPS) and Primary Light (LPS)** peptide solutions diluted in TD matrix for the reverse calibration curve (Figure 1).

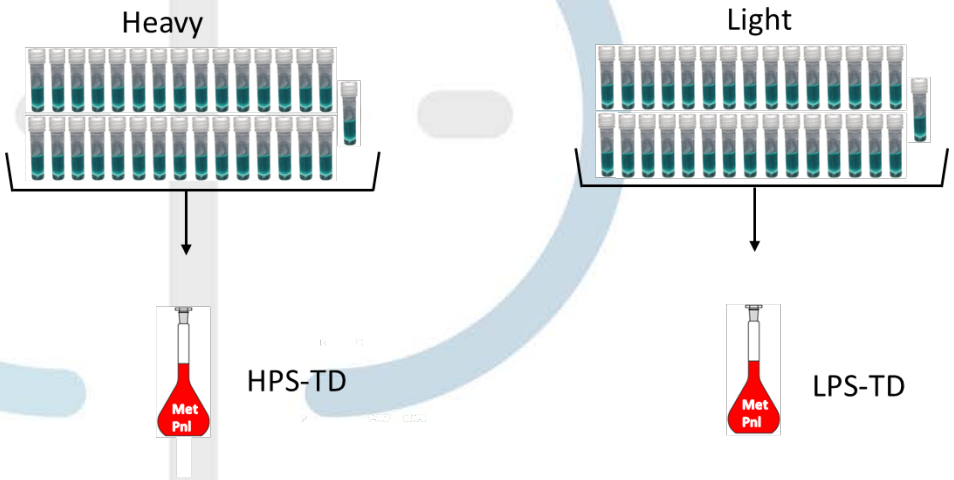

Figure 1.

7.

a.
b.
c.
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e.
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8. **Preparation of Secondary Stock H/L Solutions for Standard Reverse Curve Generation (Figure 1).**
   a. Remove an aliquot of each H and L primary stock solution from the freezer and thaw on ice.
   b. Pipette the indicated volumes (**Table V**) of diluent (TD matrix) into a 1.7 mL Eppendorf tube.

c. Pipette the indicated volumes (**Table V**) from either a H or L primary stock solution to prepare the six varying concentrations of heavy peptides and a constant quantity of light peptides.
d. Freeze at -80ºC.

Table V. Preparation of Stock Solutions (H/L) for Standard Reverse Curve Generation						
STANDARD	**HEAVY fmol/µL**	**Stock Concentration fmol/µL**	**Dilution Factor**	**Volume Stock**	**Volume Diluent**	**Total Volume**
STD6/HPS	130	10000	76.92	13.00*	51	1000
STD5	13	130	10	5	45	50
STD4	2.6	13	5	10	40	50
STD3	1.3	2.6	2	25	25	50
STD2	0.13	1.3	10	5	45	50
STD1	0.013	0.13	10	5	45	50
LPS	130	10000	76.92	13.00*	51	1000
ISTD (L)	13	130	10	20	180	200

*stock volume for each of the 73 peptides in the solution

9. **Preparation of Standards and Matrix Blanks for Six-Point Reverse Curve Data Generation.**
   a. Remove an aliquot for each of the six H standards (**Table V**) and an aliquot of the ISTD L from the freezer and thaw on ice.
   b. Add equal volumes of H and L peptide solution to the AS vial according to the table below (**Table VI**).
   c. Vortex for ~ 30 sec; dry in speedvac
   d. Solubilize in appropriate volume of TFA-1 and vortex for ~30 sec.
   e. Centrifuge in the Sorvall centrifuge for 20 min at 8000 rpm.
   f. Inject 2.5 µL on column.

Table VI. Preparation of Standards and Matrix Blank				
STANDARD	**HEAVY fmol/µL**	**volume in AS vial (µL)**	**ISTD (L) 13 fmol/µL**	**Final total volume (µL)**
STD6	130	8	8	20
STD5	13	8	8	20
STD4	2.6	8	8	20
STD3	1.3	8	8	20
STD2	0.13	8	8	20
STD1	0.013	8	8	20
MB	0	16*	16	40

*TD matrix without heavy peptides

10. Generating the Assay Response Curve

Standards, blanks and the equimolar solution are analyzed by *nano*-LC-MS according to WU-SOP-LC2-02 and WU-SOP-MS4-01. **Table VII** shows the injection queue. A total of 3 replicates are acquired for each of the six standard admixture concentrations. The injection volume of STD samples is 2.5 µL in 1 µg tumor digest matrix.

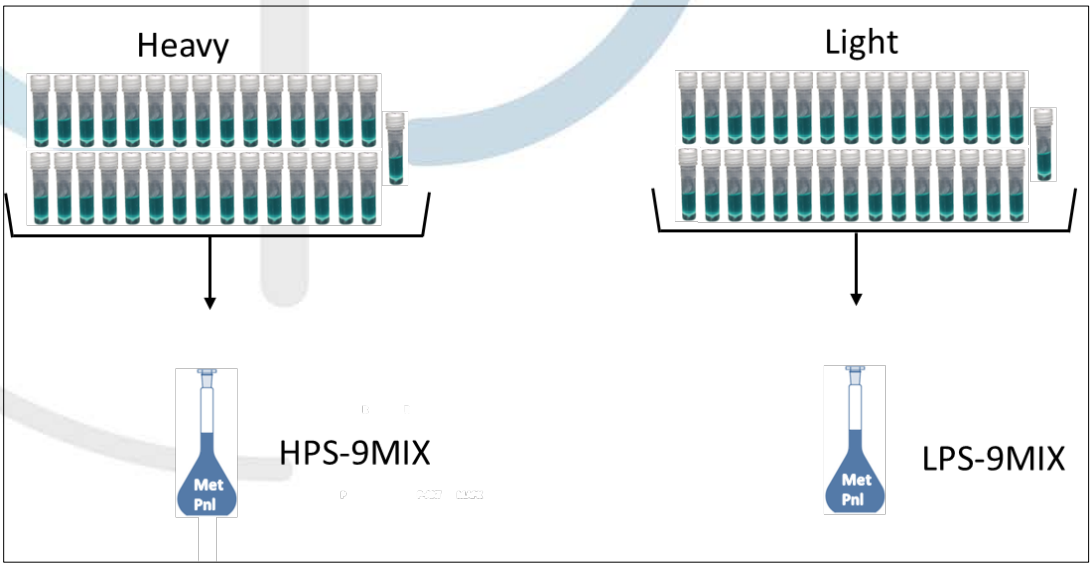
Table VII. Reverse-Curve Run Block[1]

Sample Injection	Run Time (h)
Matrix Blank-1	3.25
Matrix Blank-2	3.25
Matrix Blank-3	3.25
STD 01	3.25
STD 02	3.25
STD 03	3.25
STD 04	3.25
STD 05	3.25
STD 06	3.25
Matrix Blank-4	3.25
Matrix Blank-5	3.25
Matrix Blank-6	3.25
STD 01	3.25
STD 02	3.25
STD 03	3.25
STD 04	3.25
STD 05	3.25
STD 06	3.25
Matrix Blank-9	3.25
Matrix Blank-10	3.25
Matrix Blank-11	3.25
STD 01	3.25
STD 02	3.25
STD 03	3.25
STD 04	3.25
STD 05	3.25
STD 06	3.25
Matrix Blank-13	3.25
Matrix Blank-14	3.25
Matrix Blank-15	3.25
Total Run Time (Days)	4.06

11. Preparation of Primary Heavy Peptide Stock (HPS) and Primary Light Peptide Stock (LPS) QC Sample Solutions (Figure 2).

g. Remove an aliquot of each H and L vendor vial from the freezer and thaw on ice.
h. Label two 2 mL volumetric flasks as HPS-9MIX, LPS-9MIX.
i. Transfer ~80 µL of NINE-MIX-30-5000 diluent to each flask
j. Add 26 µL of each peptide from each vendor vial to the 2mL volumetric flask.
k. Once all selected peptides have been transferred, Q.S. the volumetric flask with AcN/FA-30 for a final concentration of ~ 130 fmol/µL for each peptide in 50 fmol/µL NINE-MIX-30-50.
l. Dispense as aliquots (110 µL) into 500 µL screw cap tubes and freeze at -80ºC.

Figure2



Referenced Documents

- WU-SOP-TD-01, "Preparation of purified peptides from solubilized tumor tissue—100 µg scale".
- WU-SOP-LC2-02-"nano-Liquid Chromatography for Experiment 1 and 2 using EASY-nLC1000"
- WU-SOP-MS3-01-"Optimizing Mass Spectrometer Performance for Experiments 1 and 2 on the Q-Exactive™ system"
- WU-SOP-MS4-01-"Mass Spectrometry Using Parallel Reaction Monitoring for Experiments 1 and 2 on the Q-Exactive™ system"

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-MS4-01
- 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
- 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.

Table VIII. Primary Stocks for Assay

Primary Stock_018

Light

HUGO Gene Symbol	Sequence	Vendor Vial amount
ACLY	TIAIIAEGIPEALTR	1 nmol
ACO1	AVLAESYER	1 nmol
ACO2	LTGSLSGWSSPK	1 nmol
ALDOA	AAQEEYVK	1 nmol
ALDOA	GILAADESTGSIAK	1 nmol
ALDOA	LQSIGTENTEENR	1 nmol
ALDOB	LSFSYGR	1 nmol
ALDOB	VENTEENR	1 nmol
CS	GLVYETSVLDPDEGIR	1 nmol
CS	IVPNVLLEQGK	1 nmol
CS	VVPGYGHAVLR	1 nmol
DERA	IGASTLLSDIER	1 nmol
DLAT	GVETIANDVVSLATK	1 nmol
DLD	ADGGTQVIDTK	1 nmol
DLST	VEGGTPLFTLR	1 nmol
ENO1	IGAEVYHNLK	1 nmol
ENO1	LNVTEQEK	1 nmol
FBP2	TSEDEPSEK	1 nmol
FBP2	YFDAATTEYVQK	1 nmol
FH	IYELAAGGTAVGTGLNTR	1 nmol
G6PD	GGYFDEFGIIR	1 nmol
GAPDH	LVINGNPITIFQER	1 nmol
GLUD1	HGGTIPIVPTAEFQDR	1 nmol
GLUD2	TAAYVNAIEK	1 nmol
GPI	VWYVSNIDGTHIAK	1 nmol
IDH1	VEITYTPSDGTQK	1 nmol
IDH2	ATDFVADR	1 nmol
IDH2	TIEAEAAHGTVTR	1 nmol
IDH2	YFDLGLPNR	1 nmol
IDH3A	APIQWEER	1 nmol
IDH3B	GELASYDMR	1 nmol
LDHA	LVIITAGAR	1 nmol
LDHA	VTLTSEEEAR	1 nmol
LDHB	FIIPQIVK	1 nmol
MDH1	LGVTANDVK	1 nmol
MDH2	IQEAGTEVVK	1 nmol

MDH2	GYLGPEQLPD(Cam)LK	1 nmol
MDH2	MISDAIPELK	1 nmol
PDHA1	LEEGPPVTTVLTR	1 nmol
PDHB 1	ILEDNSIPQVK	1 nmol
PFKL	TFVLEVMGR	1 nmol
PFKM	IGLIQGNR	1 nmol
PGAM1	HGESAWNLENR	1 nmol
PGAM1	YADLTEDQLPS(Cam)ESLK	1 nmol
PGD	VDDFLANEAK	1 nmol
PGK1	YSLEPVAVELK	1 nmol
PGLS	VTLTLPVLNAAR	1 nmol
PHGDH	ILQDGGLQVVEK	1 nmol
PHGDH	VTADVINAAEK	1 nmol
PHGDH	AGTGVDNVDLEAATR	1 nmol
PHGDH	GGIVDEGALLR	1 nmol
PHGDH	GTIQVITQGTSLK	1 nmol
PKM2	EAEAAIYHLQLFEELR	1 nmol
PKM2	IYVDDGLISLQVK	1 nmol
PKM3	(Cam)DENILWLDYK	1 nmol
PSAT1	IINNTENLVR	1 nmol
PSAT1	FGVIFAGAQK	1 nmol
PSAT1	ASLYNAVTIEDVQK	1 nmol
PSPH	LNIPATNVFANR	1 nmol
PSPH	LALIQPSR	1 nmol
R/L PKLR	GDLGIEIPAEK	1 nmol
RPIA	FIVIADFR	1 nmol
SLC1A5	LSAFVFPGELLLR	1 nmol
SLC1A5	EVLDSFLDLAR	1 nmol
SLC2A1	VTILELFR	1 nmol
SLC2A1	TFDEIASGFR	1 nmol
SLC2A12	ALSDTTEELTVIK	1 nmol
SLC2A12	GQEGAASK	1 nmol
SUCLG2	INFDDNAEFR	1 nmol
TALDO1	SYEPLEDPGVK	1 nmol
TKT	IIALDGDTK	1 nmol
TPI1	HVFGESDELIGQK	1 nmol
TPI2	VVLAYEPVWAIGTGK	1 nmol

Heavy

HUGO Gene Symbol	**Sequence**	Vendor Vial amount
ACLY	TIAIIAEGIPEALTR^	1 nmol
ACO1	AVLAESYER^	1 nmol
ACO2	LTGSLSGWSSPK^	1 nmol

ALDOA	AAQEEYVK^	1 nmol
ALDOA	GILAADESTGSIAK^	1 nmol
ALDOA	LQSIGTENTEENR^	1 nmol
ALDOB	LSFSYGR^	1 nmol
ALDOB	VENTEENR^	1 nmol
CS	GLVYETSVLDPDEGIR^	1 nmol
CS	IVPNVLLEQGK^	1 nmol
CS	VVPGYGHAVLR^	1 nmol
DERA	IGASTLLSDIER^	1 nmol
DLAT	GVETIANDVVSLATK^	1 nmol
DLD	ADGGTQVIDTK^	1 nmol
DLST	VEGGTPLFTLR^	1 nmol
ENO1	IGAEVYHNLK^	1 nmol
ENO1	LNVTEQEK^	1 nmol
FBP2	TSEDEPSEK^	1 nmol
FBP2	YFDAATTEYVQK^	1 nmol
FH	IYELAAGGTAVGTGLNTR^	1 nmol
G6PD	GGYFDEFGIIR^	1 nmol
GAPDH	LVINGNPITIFQER^	1 nmol
GLUD1	HGGTIPIVPTAEFQDR^	1 nmol
GLUD2	TAAYVNAIEK^	1 nmol
GPI	VWYVSNIDGTHIAK^	1 nmol
IDH1	VEITYTPSDGTQK^	1 nmol
IDH2	ATDFVADR^	1 nmol
IDH2	TIEAEAAHGTVTR^	1 nmol
IDH2	YFDLGLPNR^	1 nmol
IDH3A	APIQWEER^	1 nmol
IDH3B	GELASYDMR^	1 nmol
LDHA	LVIITAGAR^	1 nmol
LDHA	VTLTSEEEAR^	1 nmol
LDHB	FIIPQIVK^	1 nmol
MDH1	LGVTANDVK^	1 nmol
MDH2	IQEAGTEVVK^	1 nmol
MDH2	GYLGPEQLPD(Cam)LK^	1 nmol
MDH2	MISDAIPELK^	1 nmol
PDHA1	LEEGPPVTTVLTR^	1 nmol
PDHB 1	ILEDNSIPQVK^	1 nmol
PFKL	TFVLEVMGR^	1 nmol
PFKM	IGLIQGNR^	1 nmol
PGAM1	HGESAWNLENR^	1 nmol
PGAM1	YADLTEDQLPS(Cam)ESLK^	1 nmol
PGD	VDDFLANEAK^	1 nmol
PGK1	YSLEPVAVELK^	1 nmol

PGLS	VTLTLPVLNAAR^	1 nmol
PHGDH	ILQDGGLQVVEK^	1 nmol
PHGDH	VTADVINAAEK^	1 nmol
PHGDH	AGTGVDNVDLEAATR^	1 nmol
PHGDH	GGIVDEGALLR^	1 nmol
PHGDH	GTIQVITQGTSLK^	1 nmol
PKM2	EAEAAIYHLQLFEELR^	1 nmol
PKM2	IYVDDGLISLQVK^	1 nmol
PKM3	(Cam)DENILWLDYK^	1 nmol
PSAT1	IINNTENLVR^	1 nmol
PSAT1	FGVIFAGAQK^	1 nmol
PSAT1	ASLYNAVTIEDVQK^	1 nmol
PSPH	LNIPATNVFANR^	1 nmol
PSPH	LALIQPSR^	1 nmol
R/L PKLR	GDLGIEIPAEK^	1 nmol
RPIA	FIVIADFR^	1 nmol
SLC1A5	LSAFVFPGELLLR^	1 nmol
SLC1A5	EVLDSFLDLAR^	1 nmol
SLC2A1	VTILELFR^	1 nmol
SLC2A1	TFDEIASGFR^	1 nmol
SLC2A12	ALSDTTEELTVIK^	1 nmol
SLC2A12	GQEGAASK^	1 nmol
SUCLG2	INFDDNAEFR^	1 nmol
TALDO1	SYEPLEDPGVK^	1 nmol
TKT	IIALDGDTK^	1 nmol
TPI1	HVFGESDELIGQK^	1 nmol
TPI2	VVLAYEPVWAIGTGK^	1 nmol