### GCB Skyline for small molecules: a flexible tool for cross-platform LC-MS/MS Genome ciences UNIVERSITY OF method creation and data analysis for metabolomics.

J. Will Thompson<sup>1</sup>, Brian Pratt<sup>2</sup>, Max Horowitz-Gelb<sup>2</sup>, Laura G. Dubois<sup>1</sup>, Lisa St.John-Williams<sup>1</sup>, Giuseppe Astarita<sup>3</sup>, M. Arthur Moseley<sup>1</sup>, Michael MacCoss<sup>2</sup>, and Brendan MacLean<sup>2</sup> <sup>1</sup>Duke Proteomics and Metabolomics Core, Center for Genomic and Computational Biology, School of Medicine, Duke University, Durham, NC; <sup>2</sup>Department of Genome Sciences, School of Medicine, University of Washington, Seattle, WA; <sup>3</sup>Waters Corporation, Milford, MA

### Abstract

The Skyline software package is a powerful open-source and vendor-neutral software tool which has built a strong reputation for promoting collaboration and cross-platform validation of targeted (SRM) and high-resolution proteomics analysis. The software allows direct import of raw mass spectrometry data from all major instrument vendors, speeds method development by allowing direct export of native instrument methods or transition lists, and performs peak integration with a flexible data reporting environment.<sup>1,2</sup> While each instrument vendor does provide data analysis tools for quantitative analysis of small molecules by liquid chromatography – tandem mass spectrometry (LC-MS/MS), there is no software platform for small molecule analysis which allows cross-vendor method creation and data analysis. This poster demonstrates the initial implementation of the Skyline software package (v3.1) for the creation of custom LC-MS/MS methods for several classes of metabolites, using a custom targeted LC-MS/MS assay for the methionine pathway as an example. The software includes the ability to define precursor and product ions based on empirical formula or m/z, define collision energy specifically by molecule or based on a linear equation, and to define expected retention time. Additionally, we demonstrate the use of Skyline along with retention time and accurate mass lipid libraries for the quantification of lipid species from unbiased high-resolution lipidomics datasets, as an alternative to standard metabolomics workflows.<sup>3,4</sup> Using Skyline for small molecule method creation and data analysis fills a computational gap by easing the translation and validation of targeted metabolomics methods between instruments and laboratories.

## Methionine Pathway

The goal for this experiment was to generate a targeted MRM assay for several metabolites in the methionine pathway, along with internal standards for quantification based on stable-isotope dilution. The pathway was of interest because depletion of methionine had shown a very unique gene expression signature in BT474 breast cancer cell line.

Tang X et al "Comprehensive profiling of amino acid response uncovers unique methionine-deprived response dependent on intact creatine biosynthesis", PLOS Genetics 2015.





# Verification of High-Resolution Differential Metabolomics Results in Skyline

High Resolution Differential Lipidomics analysis of a cancer cell line under drug treatment was performed using UPLC coupled to Synapt G2 HDMS system. Five biological replicates of each were performed, and the data was analyzed in the software package Progenesis QI

Below shows the general informatics wofkflow for unbiased lipidomics:



1.MacLean B, Tomazela DM, Shulman N, et al. Bioinformatics. 2010 Apr 1; 26(7):966-8. 2.Chambers MC, Maclean B, Burke R, et al. Nat Biotechnol. 2012 Oct; 30(10):918-20. 3.Smith CA, Want EJ, O'Maille G et al. Anal. Chem. 2006 Feb 1; 78(3):779-87. 4. Paglia G, Angel P, Williams J et. Al. Anal. Chem. 2015 Jan 20;87(2):1137-44.

# Workflow for Using Skyline for Targeted Small Molecule Analysis

1. Flat File Containing Molecules of Interest

Skyline Targeted Method G	eneration:									
Molecule List Name	Precursor Name	Product Name	Precursor Formula	Product Formula	Precursor m/z	Product m	Precursor	Product Ch	Precursor RT	Precursor CE
Amino Acid	Methionine		C5H12NO2S			104.07	1	1	2.5	1
Amino Acid	d3-Methionine		C5H9H'3NO2S			107.09	1	1	2.5	1
Amino Acid	Isoleucine		C6H14NO2			86.096	1	1	3.05	1
Amino Acid	Leucine		C6H14NO2			86.096	1	1	3.13	1
Amino Acid	d3-leucine		C6H11H'3NO2			89.1	1	1	3.13	1
Amino Acid	Phenylalanine		C9H12NO2			120.08	1	1	3.27	1
Amino Acid	13C6-Phenylalanine		C3C'6H12NO2			126.11	1	1	3.27	1
Amino Acid	Arginine		C6H15N4O2			116.07	1	1	2.01	1
Amino Acid	13C5-Arginine		C1C'5H15N4O2			121.11	1	1	2.01	1
Amino Acid	Ornithine		C5H13N2O2			70.07	1	1	1.1	1
Amino Acid	Ornithine		C5H13N2O2			116.07	1	1	1.1	1
Amino Acid	d2-ornithine		C5H11H'2N2O2			72.07	1	1	1.1	1
Amino Acid	d2-ornithine		C5H11H'2N2O2			118.07	1	1	1.1	1
Organic Acid	creatine		C4H10N3O2			90.06	1	1	1.1	1
Organic Acid	d3-creatine		C4H7H'3N3O2			93.06	1	1	1.1	1
5'-methylthioadenosine	MTA		C11H16N5O3S			136.1	1	1	3.4	1
5'-methylthioadenosine	d3-MTA		C11H13H'3N5O3S			136.1	1	1	3.4	1
S-adenosyl methionine	SAM		C15H23N6O5S			250.11	1	1	3	1
S-Adenosyl homocysteine	SAH		C14H21N6O5S			136.08	1	1	3	1
Polyamine	Spermidine		C7H20N3			129.15	1	1	3.59	1
Polyamine	Spermine		C10H27N4			112.112	1	1	3.82	1

Example of High versus Low-Methionine Sample, Chromatograms in Skyline lethod: 2.1 mm x 10 cm BEH C18 column with MPA 0.1% Formic acid/0.02% HFBA in water, MPB is 90/10 MeCN/IPA. F = 0.4 mL/min @ 35C. Acquity TQ-S in ESI+ MRM mode; full method and Skyline file available in Tang et al, *PLOS Genetics* **2015**)





A compound list including saturated, mono- and polyunsaturated Fatty Acids was generated in Skyline, and set to perform high-resolution MS1 extraction at 10,000 Rs.

Skyline Targeted Methe	od Generation:											
Molecule List Name	Precursor Name	Product Name	Precursor Formula	Product Formula	Precursor m/z	Product m/z	Precursor	Product C	Explicit Re	e Explicit Co	Precursor	Explicit Dri
Fatty Acid	FA 16:0 (palmitic)		C16H31O2			255.2	1	1	1			
Fatty Acid	FA 16:1 (palmitoleic)		C16H29O2			253.2	1		1			
Fatty Acid	FA 17:1		C17H31O2			267.2	1	1	1			
Fatty Acid	FA 18:0 (stearic)		C18H35O2			283.3	1	1	1			
Fatty Acid	FA 18:1 (oleic)		C18H33O2			281.2	1	1	1			
Fatty Acid	FA 18:2 omega-6 (LA)		C18H31O2			279.2	1	1	1			
Fatty Acid	FA 18:3 omega-3 (ALA)		C18H29O2			277.2	1	1	1			
Fatty Acid	FA 20:0 (eicosanoic)		C20H39O2			311.3	1	1	1			
Fatty Acid	FA 20:3 omega-6		C20H33O2			305.2	1	1	1			
Fatty Acid	FA 20:3 omega-9 (mead acid)		C20H33O2			305.2	1	1	1			
Fatty Acid	FA 20:4 omega-6 (AA)		C20H31O2			303.2	1	1	1			
Fatty Acid	d8 FA 20:4 (d8-AA)		C20H23H'8O2			311.2	1	1	1			
Fatty Acid	FA 20:4 omega-3 (ETA)		C20H31O2			303.2	1	-	1			
Fatty Acid	FA 20:5 omega-6 (EPA)		C20H29O2			301.2	1	1	1			
Fatty Acid	FA 22:4 omega-3		C22H35O2			331.3	1	1	1			
Fatty Acid	FA 22:4 omega-6 (adrenic)		C22H35O2			331.3	1	1	1			
Fatty Acid	FA 22:5 omega-3 (DPA)		C22H33O2			329.3	1	1	1			
Fatty Acid	FA 22:5 omega-6 (Osbond)		C22H33O2			329.3	1	1	1			
Fatty Acid	FA 22:6 omega-3 (DHA)		C22H31O2			327.2	1	-	1			

#### 2. Import Into Skyline, building an analysis template

argets	ųх								_							_
Amino Acid Amino	Î	No	Insert   No errors   Transition List												x	
d3-leucine Phenylalanine 13C6-Phenylalanine Arginine	- 18			Molecule List Name	Precursor Name	Product Name	Precursor Formula	Product Formula	Precursor m/z	Product m/z	Precursor Charge	Product Charge	Explicit Retention Time	Explicit Collision Energy	Explicit Drift Time (msec)	*
		ШÞ		Amino Acid	Methionine		C5H12N		150.058	104.07	1	1	2.5	15		Ξ
				Amino Acid	d3-Methi		C5H9H'3		153.077	107.09	1	1	2.5	15		
Organic Acid	E			Amino Acid	Isoleucine		C6H14N		132.101	86.096	1	1	3.05	15		
				Amino Acid	Leucine		C6H14N		132.101	86.096	1	1	3.13	15		
. S'-methylthioadenosine				Amino Acid	d3-leucine		C6H11H'		135.120	89.1	1	1	3.13	15		
🕀 🙀 MTA				Amino Acid	Phenylal		C9H12N		166.086	120.08	1	1	3.27	15		
⊞… 🙀 d3-MTA				Amino Acid	13C6-Ph		C3C'6H1		172.106	126.11	1	1	3.27	15		
🖻 🙀 SAM				Amino Acid	Arginine		C6H15N		175.118	116.07	1	1	2.01	15		
S-Adenosyl homocysteine				Amino Acid	- 13C5-Ar		C1C'5H1		180.135	121.11	1	1	2.01	15		
				Amino Acid	Omithine		C5H13N		133.097	70.07	1	1	1.1	15		Ŧ

#### 3. Export an instrument acquisition method or MRM list



Metabolite overlays on the mechanism: Met depletion drives changes in central methyl donors (SAM), which then causes epigenetic changes in gene expression



Targeted molecule extraction verified the statistical changes observed in Progenesis QI, and suggests a general trend towards no change in saturated FA, but an accumulation of essential omega-3 and omega-6 FA with drug Tx.



Skyline provides a seamless way to share MRM metabolomics data and methods, improving transparency in metabolomics experiments. The software eases cross-laboratory verification experiments by using a common data analysis pipeline and method development tool.

Skyline can currently be used to perform novel MRM

metabolomics experiments or to perform targeted interrogation of high-resolution metabolomics data