# Performing Peptide Bioanalysis Using High Resolution Mass Spectrometry with Target Enhancement MRM Acquisition

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# GOAL

To demonstrate Tof MRM acquisition of commercially available peptide mixture MSQC1 using ionKey™/Xevo® G2-XS QTof platform for peptide bioanalysis. Quantitation for serial diluted samples shows ionKey/Xevo G2-XS is well suited for peptide characterization and quantitation with excellent sensitivity and linear response.

# BACKGROUND

The rise of biotherapeutics in the drug discovery and development pipeline has resulted in the increasing demand for protein/peptide bioanalysis by LC-MS techniques. This has led to a renewed interest in high resolution mass spectrometry (HRMS) platforms for use in quantitation because they enable both a larger mass range and additional options for selectivity. HRMS platforms retain the ability to quickly switch back to characterization mode to elucidate or resolve qualitative, underlying matrix issues with the assay. In this way, both peptide mapping and quantitation can be performed on the same platform.

In addition to full scan data acquisition, newer targeted HRMS modes enable faster tandemlike throughput, while avoiding some of the complexity of full scan approaches. Currently, quantitation through a surrogate peptide approach remains the most popular practice for large molecule bioanalysis. In this approach, proteins and peptides are prepared through enzyme digestion. The resulting mixture is analyzed first to map out peptides thus formed and to search for signature peptides. Informatics tools such as Skyline are tailored for developing quantitative methods. Once the signature peptides are identified, subsequent peptide quantitation is carried out using either tandem quadrupoles or high resolution mass spectrometers.

In this tech brief, peptide quantitation using HRMS is demonstrated using ionKey/MS<sup>™</sup> and Xevo G2-XS with Tof MRM mode for data acquisition (although the acquisition/data processing is amenable to all inlets available at Waters<sup>®</sup>). Tof MRM, or target enhancement

mode of acquisition, provides a selectivity and sensitivity boost for enhanced HRMS quantitation. Microfluidics such as ionKey also produce enhanced selectivity and sensitivity across small and large molecules, particularly with intact molecule characterization for both surrogate and intact quantitation.



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A commercially available peptide mixture called MSQC1 (Sigma Aldrich) is used in the present study. Peptide composition of the mixture, its sequence, and corresponding light/heavy (L/H) stable isotope ratios are summarized in Table 1. The mixture is frequently used by labs as QC standards before running peptides of interest. Table 2 is a summary of the first four peptides which are used to describe the process of creating Tof MRM methods and building a targeted Tof MRM HRMS method.

					-	
Protein (UniProt	Calc'd MW	Approx. per	. protein vial	Corresponding SIL	SIL peptide content per	Theoretical ratio light : heavy
accession number)	(Da)	pmol	μg	peptide sequences	vial (pmol)	(Protein : SIL peptide)
Carbonia Annudraca I/PO0015)	20720	100	2.0	GGPFSDSY[R]	100	1
	20139	100	2.9	VLDALQAI[K]	50	2
	20115	100	2.0	AVQQPDGLAVLGIFL[K]	10	10
Carbonic Annyarase II (P00918)	29115	100	2.9	SADFTNFDP[R]	2	50
NAD(P)H dehydrogenase	20720	20	0.02	EGHLSPDIVAEQ[K]	20	1
(P15559)	20120	20	0.62	ALIVLAHSE[R]	10	2
	22047	20	0.40	ESDTSYVSL[K]	2	10
C-reactive Protein (POZ741)	23047	20	0.40 -	GYSIFSYAT[K]	0.4	50
				FEDENFIL[K]	8	0.5
Peptidyl-prolyl cis-trans	20176	4	0.08	VSFELFAD[K]	4	1
150111e1 ase A (1 02 3 51 )				TAENF[R]	2	2
				GAGAFGYFEVTHDIT[K]	20	0.2
Catalase (P04040)	59625	4	0.24	FSTVAGESGSADTV[R]	0.4	10
				NLSVEDAA[R]	0.08	50

#### Table 1. Protein digest and stable isotope labeled peptide composition of MSQC1 mix

#### Table 2. A summary of peptide sequence, charge, and transition for the first four peptides (Sigma Aldrich)

Protein (UniProt accession number)	Peptide	MH+ (mono)	z(Q1)	Q1 <i>m/z</i>	Q3 m/z	Fragment ion type	CE	DP	СХР	EP
			2	493.2	871.4	y7	21	80	12	10
	GGPFSDSYR	985.44	2	493.2	627.3	y5	27	80	12	10
			2	493.2	774.3	уб	33	80	12	10
			2	498.2	881.4	y7	21	80	12	10
	GGPFSDSY[R]	995.44	2	498.2	637.3	y5	27	80	12	10
Carbonic Anhydrase I			2	498.2	784.3	уб	33	80	12	10
(P00915)			2	485.8	643.4	уб	26	80	12	10
	VLDALQAIK	970.59	2	485.8	758.4	y7	26	80	12	10
			2	485.8	871.5	y8	20	80	12	10
			2	489.8	651.4	yб	26	80	12	10
	VLDALQAI[K]	978.60	2	489.8	766.4	y7	26	80	12	10
			2	489.8	879.5	y8	20	80	12	10
			2	835.0	1242.8	y12	36	80	12	10
	AVQQPDGLAVLGIFLK	1668.97	2	835.0	1030.7	y10	42	80	12	10
			2	835.0	973.6	y9	42	80	12	10
			2	839.0	1250.8	y12	36	80	12	10
	AVQQPDGLAVLGIFL[K]	1676.97	2	839.0	1038.7	y10	42	80	12	10
Carbonic Anhydrase II			2	839.0	981.6	y9	42	80	12	10
(P00918)			2	585.3	896.4	y7	25	80	12	10
	SADFTNFDPR	1169.52	2	585.3	749.4	у6	25	80	12	10
			2	585.3	1011.5	y8	25	80	12	10
			2	590.3	906.4	y7	25	80	12	10
	SADFTNFDP[R]	1179.52	2	590.3	759.4	уб	25	80	12	10
			2	590.3	1021.5	y8	25	80	12	10

# RESULTS

Results are described in three sections:

I – Setting up a Tof MRM experiment in MassLynx®

II – Developing a Tof MRM scouting experiment to identify the best peptide transitions and also the best conditions (CE) to monitor the peptide

III – Quantification using TargetLynx™

# I. Setting up TofMRM data acquisition in MassLynx

Step 1. Open MS method editor and choose "Tof-MRM" function. A function line appears in the function table as shown in Figure 1.

Step 2. Click on the function to open a new window/ dialog. In the first tab, "Acquisition," enter acquisition time, polarity, etc. as shown in Figure 2a. Next, click on the "TOF MS" tab and enter mass range and scan time as shown in Figure 2b. Mass ranges from 50 to 1200 or 50 to 2000 *m/z* and scan time of 0.1 s are typical. Scan time should be adjusted based on run time and peak width to ensure a minimum of 10 data points base-to-base are collected across the peaks for the number of MRMs scanning concurrently.

Compound information is entered in the third tab called "MRM" as shown in Figure 3. In this tab, choosing the "Add" button opens the MRM editor. Enter the time window and set mass, CE, CV, and "Target Enhancement m/z" to the appropriate cells shown. Additional fragments may be entered for monitoring, but typically only one is chosen for maximum sensitivity and entered in fragment box 1. "Set Mass" and "Target enhancement *m/z*" are critical for data acquisition. Repeat this process for all the transitions of interest. In this example four peptides along with their internal standards are being monitored. A time window is entered for each peptide providing maximum signal for each pair. For the time window, ending at 1.0 min and starting next at 1.0 min is considered overlapping, which results in two traces of data. Try to start the second window at 1.01 min to produce one data trace.

File E	dit Op	tions	Toolbars	Fu	nctions Hel	р							
3 🚅	8		X 🔺	V	<ul> <li>LockSpray</li> </ul>		Method	Events					
1	MS		MSMS		PID Product		PID Neutral		45 Continuum	MS <sup>E</sup> Centroid		Tof-MRM	Fast DDA
otal Ru	un Time: 1	0.00	↔					0		5	1	1 2	10mins
No.			In	form	ation			1.0		Time	-		

Figure 1. A snapshot of the user interface after opening the method editor and choosing "Tof MRM."

function:1 Tof-MRM		-1/ Function/1 Tof-MRM	-0- <b></b>
Acquisition TOF MS M	(FM	Acquisition TOF MS MEM	
Acquisition Times		Da sange	
Total time for this acq	winition	Acquire TOF MS over the range	
Start Time	E min	Low Mass 🔣 Da	
End Time	10 min	High-Mass 1200 Da	
Source			
Source	ES *	Scanning Conditions	
		Scan Time 0.1 • sec	
		Data Format Continuum *	
Acquisition Mode			
Polarity	Positive O Negative		
Analyzer Mode	C Resolution @ Sensitivity		

Figure 2. (a) Acquisition, (b) "TOF MS" tab of the MS method.

when singu DARAAR PADAR Interval 5 = Ure MRM coan padding MRM Windows			
Use MRM scan padding MRM windows			
MRM Windows			
Nove     Single Isotope     Isotope Cluster			
C'MassLyw/MSQC1_Frag.mm	MRM Editor		
No Name Mass Fragm., Start End RT EDC	That Editor		
00PF505YR_ 4032200 8272700 2.4 4.1 82727			
00FF505V78 4982200 6372700 3.4 4.1 83727( 140/F70F0F8	GGPESDSYR F		
LACFTNFDP[	admocart		
VLDALDAIK 485.000 871.520 47 5.5	Datantian Time Cta		24
VLDALDAVC. 409.000 879.500 47 5.5 879.501 879.501	netention Time Sta	IC.	3.4
#JOOPDOLAZ 838.9900 838.9900 6.8 7.7	Retention Time End	t	4.1
			400.00
	Set Mass:		493.22
	-> Fragments:		
	627.27	0	0
	0	0	0
n	CE Ramp:	27	27
New Add Delete Sot Save Save As	Cone Voltage:		100
	Target Enhanceme	nt m/z:	627.27

Figure 3. MRM table and MRM editor shows an example of Precursor  $([M^+2H2^+] = 493.22 \text{ m/z})$ >Fragment  $([M^+H^+] = 627.27 \text{ m/z})$  transition (middle), and a tuneless Precursor>Precursor transition (right).

Once all information is entered, click "Save" to store the MRM table as a ".mrm" file. A .mrm file is a collection of compounds, time window, and other information shown in the MRM editor page. Once it is created, it can be imported to easily create the MRM function. An Excel Macro file to help generate .mrm files is freely available from Waters upon request.

Because data acquisition reads from the .mrm file, if the "Save" button is not clicked, any modified information is not used. If one also wants to collect full scan spectrum simultaneously, the "RADAR" box should be checked (note: this reduces the duty cycle and is recommended for method development, but not final assay method). MRM window choices describe the tolerance of data shown with the target enhancement region of about 50 Da around the target mass. "None" indicates the entire 50 Da range centered around ion of interest; "single isotope" only retains the single isotope (1 Da), the "isotope cluster" retains a 5 Da window. In all cases, the data can be further extracted during the XIC creation to an HRMS mDa tolerance. thus further increasing specificity of the extracted ion chromatogram (XIC).

### II. Performing scouting experiments for MSQC1

The first set of MRM experiments is carried out to select the best transitions for eventual quantitation for each of the peptides. The transitions tested are the three precursor>fragment transitions provided in the catalog (Table 2), and a fourth transition based on precursor>precursor without fragmentation (tuneless transition). A spreadsheet containing the transitions is shown (Figure 4) and when translated to a .mrm file it can be imported or transcribed into the Tof MRM method editor.

A resulting XIC for each of the peptides is then overlaid. Peptide 1 and peptide 2 are shown in Figure 5 and Figure 6 as an example. As expected, the precursor>precursor transition produces the highest signal for all four peptides. In this example the precursor>precursor transition and one of the precursor>fragment transitions with the highest response for subsequent quantitation and instrument performance check are chosen.

Name	RT start	RT end	Precursor m/z	fragment m/z	TE on Fragment?	ConeVoltage	CE start	CE end	MRM File Name	
GGPPSDSYR_P	0	30	493.2	493.2	Y	100	6	6	MSQC1_P1	
GGPFSOSYR_F1	0	30	493.2	871.4	Y	100	21	21	MSQC1_P1	C
GGPFSOSVR_F2	0	30	493.2	627.8	Y	100	27	27	MSQC1_P1	Generate .MRM hies
GGPTSOSYR_F3	0	30	493.2	774.5	¥	100	33	33	MSQC1_P1	for Xevo Tofs
VLDALQAIK_P	0	10	485.8	485.8	Y	100	6	6	MSQC1_P2	
VLDALQAIK_F1	0	10	485.8	643.4	Y	100	26	26	MSQC1_P2	Constate NDM files
VLDALQAIK_F2	0	10	485.8	758.4	Y	100	26	26	MSQC1_F2	Generate .meda mes
VLDALQAIK_FS	0	10	485.8	871.5	¥	100	20	20	MSQC1_P2	for synapt
AVQQPOGLAVLGIFLK_P	0	10	835.0	835.0	¥	100	6	6	MSQC1_P3	
AVQQPDGLAVLGIPLK_F1	0	10	835.0	1242.8	Y	100	36	56	MSQC1_F3	
AVQQPDGLAVLGIFLK_F2	0	10	835.0	1050.7	Y	100	42	42	MSQC1_P3	
AVQQPDGLAVLGIFLK_F3	0	30	835.0	973.6	¥	100	42	42	MSQC1_F3	
SADFTNFDPR_P	0	30	585.3	585.3	Y	100	6	6	MSQC1_F4	
SADFTNFDFR_F1	0	10	585.3	895.4	Y	100	25	25	MSQC1_P4	
SADFTNFDFR_F2	0	30	585.3	749.4	¥	100	25	25	MSQC1_P4	
SADFTNFDPR F3	0	10	585.3	1011.5	Y	100	25	25	MSOC1 P4	

Figure 4. Excel macro display for creation of .mrm files.



Figure 5. Overlaid extracted ion chromatograms for peptide 1 scouting results from the four transitions.



*Figure 6. Overlaid extracted ion chromatograms for peptide 2 scouting results from the four transitions.* 

### III. Quantitation

A standard curve is prepared for MSQC1 by successive 1:2 dilutions based on instruction from Sigma Aldrich. The range of the dilution starts with 1:16 down to 1:4096 fold. Four peptides are monitored simultaneously according to the time window shown in Figure 3. A sample chromatogram is shown in Figure 7. Gradient conditions for ionKey are shown in Figure 8.

Figure 9 shows TargetLynx results of Peptide 4 based on precursor>precursor transition. A good linear range from 1:64 to 1:4096 fold dilution is obtained with R<sup>2</sup> = 0.99. Above 1:64 fold, the signal suggests saturation of the mass detector. At the lowest prepared concentration of 1:4096 fold, the average signal-to-noise ratio is 310, indicating low detection limit has not been reached and the mass spec is capable of detecting sample at lower concentration.

Figure 10 shows TargetLynx results of Peptide 4 based on isotope labeled precursor>precursor transition. As expected, the lower abundance of the labeled peptide produces a lower response. The linear range now extends to all concentrations with no signal saturation at higher concentration. An R<sup>2</sup> of 0.99 is also obtained. The average signal-to-noise ratio at 1:4096 fold dilution is 25 which is expected based on lower response observed.



Figure 7. Overlaid extracted mass chromatogram of the four peptides monitored. The iKey<sup>™</sup> Separation Device used is the iKey HSS T3, 150  $\mu$ m x 50 mm (P/N <u>186007260</u>). Injection volume is 1  $\mu$ L and iKey temperature is 45 °C. Scan time is 0.1 s.

	Time (min)	Flow (µL/min)	%A	%В	Curve
1	Initial	3.000	99.0	1.0	Initial
2	7.00	3.000	55.0	45.0	6
3	7.50	3.000	5.0	95.0	6
4	8.50	3.000	5.0	95.0	6
5	9.00	3.000	98.0	2.0	6
6	20.00	3.000	98.0	2.0	6
7	21.00	1.000	5.0	95.0	6

Figure 8. Gradient conditions used in the present analysis.

								-			
-	#/ Name	RT	Area	Туре	Std. Conc	IS Area	Response	Primer	Conc.	%Dev	SA
	1 0702_2015_MSQC1_029	4.39	13.794	Blank			13.794	bb	0.1	-	19.78
	2 0702_2015_MSQC1_030	4.40	24.095	Blank			24.095	bb	0.1	-	10.105
	3 0702_2015_MSQC1_031	4.30	11.231	Blank			11.231	60	0,1		5.39
	4 0702_2015_M5QC1_032	4.39	805.489	Standard	0.244		805.489	MM.	0.2	-7.3	342.140
	5 0702_2015_MSQC1_033	4.40	811.728	Standard	0.244		811.728	00	0.2	-6.8	276.52
	6 0702_2015_MSQC1_034	4,40	2097.095	Standard	0.488		2097.095	60	0.5	-3.1	458.420
	7[0702_2015_MSQC1_035	4.40	2116.442	Standard	0.488		2116.442	bù	0.5	-2.3	653.518
	e10/02_2015_MSGC1_036	4.39	4557.114	Drahdard	0.977		4557.114	DO	0.9	-3.4	444.264
	9[0/02_2015_MSQC1_037	4.40	4561.970	brahdard	0.977		4561.970	00	0.9	-3.3	663.37
0	10 0/02_2015_MSQC1_038	4.39	9/40.426	Standard	1.953		9/40.425	00	1.9	-0.9	1649.15
	11[0/02_2015_MSQC1_039	4.39	9910.728	Standard	1.953		9910.728	60	2.0	0.7	1363.22
2	12 0702_2015_MSQC1_040	4.40	22011.468	Standard	3.906		22011.468	00	4.3	9.6	650.50
3	13/0/02_2015_MSQC1_041	4.39	21/69.156	Scandard	3.906		21/69.156	00	4.2	0.4	1904.96
•	14 0702_2015_MSQC1_042	4.39	45194.700	Standard	7.813		45194,700	00	0.7	11.5	5529.77
	15 0702_2015_MSQC1_043	4.40	40030.882	Standard	7.013		40030.992	00	0.0	12.4	8/1.32/
	10 0702_2015_MSGC1_044	4.39	75056.977	Scandard	15.043		75050.977	00	14,4	-1.1	041.42
	17 0702_2015_MSQC1_045	4.39	14023-172	Standard	15.645		14029.172	DO NAV	14.4	-0.0	4041.404
2	10 0702 2015 85001 045	4.37	112001.000	Standard	31,250		112001.000	DDA NOO	21.0	-31.2	3060.40
	19 0702_2015_MSGC1_047	4.30	111200.713	Standard	31,230		111200.719	DEA	21.3	-21.7	2626.40
-	24 6702 2015 85001 649	4.37	163630 438	Clandard	602.500		153630 438	bby	20.4	62.0	4000.070
	22 0702 2015 MSGC1_049	4.57	221 452	Black	02.500		221 452	hh	22.4	-04.8	4000.071
	23 0702 2015 MSOC1 051	4.40	88 975	Black			88 976	hh	0.1		20.055
Chro	matogram					Calibrat	ion: 07 Jul 201	5 13:34:0	8		
4096	P4_parent_ 4.39 805.49 19800 342.15			F2TOF D	ugnter,ES+ 585,2809 2,015e+004	Correlation Calibration Response I Curve type: 300000	coefficient r courve: 5229.7 hpe: External Linear, Origin	= 0.9951 5 * x + -3 Std, Area Exclud	91, r*2 = 0.99040 378,256 a e, Weighting: 1/x,/	5 Axis trans: f	lone

Figure 9. TargetLynx results of P4 peptide showing summary data, chromatogram of 1:4096 fold of dilution, and plot of response versus concentration.

# [TECHNOLOGY BRIEF]

						<i>u</i>						Danein_
	10	Name	RT	Area	Type	Std. Con	IS Area	Response	Primar	Conc.	%Dev	
	1	0702_2015_MSQC1_029	4.30	1.676	Blank			1.676	bb	0.1		3.
	2	0702_2015_MSQC1_030	4.59	0.469	Blank			0.469	bb	0.1		2
	3	0702_2015_MSQC1_031	4.36	2,451	Blank			2.451	MM	0.1	2	1
	4	0702_2015_MSQC1_032	4.39	14.187	Standard	0.24	4	14.187	60	0.2	-3.5	19
	5	0702_2015_MSQC1_033	4.40	17.920	Standard	0.24	4	17.920	bb	0.3	11.4	31.
	6	0702_2015_MSQC1_034	4.40	41.967	Standard	0.48	В	41.967	MM	0.5	3.5	30
	7	0702_2015_MSQC1_035	4.40	38.305	Standard	0.48	3	38.305	MM	0.5	-3.8	22
	8	0702_2015_M5QC1_036	4.39	80.850	Standard	0.97	7	80.850	MM	0.9	-9.6	51
Т	9	0702_2015_MSQC1_037	4.40	81,696	Standard	0.97	7	81.696	MM	0.9	-8.8	45
	10	0702_2015_MSQC1_038	4.39	170.662	Standard	1.95	3	170.662	MM	1.8	-10.2	81
	11	0702_2015_M5QC1_039	4.39	166.971	Standard	1.953	3	166.971	MM	1.7	-12.0	75
	12	0702 2015 MSQC1_040	4.40	353.014	Standard	3.90	5	353.014	MM	3.5	-9.8	153
	13	0702_2015_MSQC1_041	4.39	370.075	Standard	3.90	5	370.075	MM	3.7	-5.5	82
	14	0702_2015_MSQC1_042	4.39	835.405	Standard	7.81	3	835.405	MM	8.2	5.0	131
	15	0702 2015 MSQC1 043	4.40	825.639	Standard	7.81	3	825.639	MM	8.1	3.8	126
	16	0702_2015_MSQC1_044	4.39	1729.708	Standard	15.625	5	1729.708	MM	16.9	8.1	236
	17	0702_2015_MSQC1_045	4.39	1674.404	Standard	15.625	5	1674.404	MM	16.4	4.6	141
	18	0702 2015 MSQC1 046	4.38	3317,980	Standard	31,25		3317,980	MM	32.3	3.4	472
	19	0702 2015 MSQC1 047	4.38	3305.051	Standard	31.25		3305.051	MM	32.2	3.0	250
	20	0702_2015_MSQC1_048	4.37	6932,166	Standard	62.50		6932.166	MM	67.4	7.8	544
	21	0702 2015 MSQC1 049	4.37	7235.652	Standard	62.50		7235.652	MM	70.3	12.5	1005
	22	0702_2015_MSQC1_050			Blank							
	23	0702 2015 MSOC1 051	4.40	2 710	Blank			2 710	Nh.	0.1		5
hro	matog	gram					Calibration:	08 Jul 2015 1	3:39:58			00
96	P4_	parent_H_ 4.40 17.92 407 31.90 P4_parent_H 4.40			5/ 4.39	90.2809 4e+002	Correlation coe Calibration cun Response type Curve type: Line 6000-	fficient r = 0 ve: 103.023 ' : External Str ear, Origin: E	995555, 'x + -10.0 d, Area xclude, W	r*2 = 0.991130 889 /eighting: 1/k*2, A	xis trans: I	None
	P4_	parent_H17.92 4.40 407 17.92 31.90 407 31.90 4.60 6.00 5.60	600	6.60	700 70	m min	4000 2000- -0-					Con

Figure 10. TargetLynx results of isotope labeled P4 peptide showing summary data, chromatogram of 1:4096 fold of dilution, and plot of response versus concentration.

### SUMMARY

Tof MRM mode of data acquisition provides an effective approach for analyte quantitation using HRMS. Setting up the Tof MRM MS method is straightforward. In the present example, Tof MRM mode of data acquisition is used for the identification of the monitoring mass of a representative peptide in MSQC1. Subsequent quantitation for serial diluted samples shows that the ionKey/Xevo G2-XS is well suited for the peptide characterization and quantitation with excellent sensitivity and linear response. In this way, one can go from characterization to high performance guantitative method using a single MS platform. The use of a simple turnkey microfluidics technology such as ionKey offers high sensitivity. In summary, the coupling of targeted HRMS (Tof MRM with target enhancement) on the Xevo G2-XS HRMS QTof with ionKey microfluidics, offers a powerful, simple workflow for HRMS peptide bioanalysis.



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