# 160/180 Labelling Quantitative Analysis of Tryptically Digested Mouse Serum using LC MALDI MS and MS/MS

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Marten F. Snel<sup>1</sup>; Marc Kipping<sup>2</sup>, Richard Tyldesley-Worster<sup>1</sup>; Greg S. Cavey<sup>3</sup>; P. Davidson<sup>3</sup>; James I. Langridge<sup>1</sup>; Therese McKenna<sup>1</sup>; D. Monsma<sup>3</sup>; C. Webb<sup>3</sup> <sup>1</sup>Waters Corporation, Manchester, UK;<sup>2</sup> Waters GmbH, Helfmann-Park 10, 65760 Eschborn, <sup>3</sup>Van Andel Research Institute, Grand Rapids, US

## **OVERVIEW**

- In this presentation we detail the quantitative analysis of Mouse serum by LC-MALDI MS
- Serum samples were obtained from tumour bearing and control mice, tryptically digested and then labelled with either <sup>16</sup>O or <sup>18</sup>O
- Labelled peptides were separated by off-line SCX fractionation and collected into X fractions
- Further fractionation was achieved via RP LC collecting onto 96 well MALDI target plates
- Each position was analysed first via MALDI MS to identify labelled peptide pairs via an automated computer algorithm
- Peptide pairs exhibiting a differential <sup>16</sup>O:<sup>18</sup>O ratio were interrogated via MS/MS on the MALDI Q-Tof mass spectrometer to provide structural information to identified via databank searching

## INTRODUCTION

Mass spectrometry is now an accepted technique for the identification of proteins. In the qualitative analysis of digest mixtures from a cell lysate or sub-cellular fraction, the challenge is often the dynamic range of proteins present in the sample, as the detection and identification of these components is often biased towards the larger and most abundant species. This has resulted in the development of chromatographic techniques, such as two-dimensional chromatography.

Recent developments in MALDI MS/MS and LC-MALDI spotting devices, allows the uncoupling of the chromatographic separation step from the subsequent MS analysis. This permits the use of targeted data acquisition schemes, resulting in additional information content compared to either an ESI LC-MS/MS, or MALDI experiment on the un-separated mixture. In addition, relative quantification of proteins from the MALDI analysis can be achieved through the incorporation of stable isotope labels, in combination with separation and subsequent analysis by mass spectrometry.

### **LC-MALDI**

Samples were separated by reverse phase chromatography on a Waters® nanoACQUITY UPLC<sup>™</sup> System, under full MassLynx software control.

Column:	Waters Atlantis <sup>®</sup> dC18 100 mm x 75 µm
Mobile phase:	A: 0.1% TFA in water , B: ACN
Gradient:	3–40 % B over 60 mins
Flow rate:	300 nL/min; Injection volume: 12 µL

#### LC-MALDI fraction collection

The eluent from the HPLC was collected directly onto a 96 well MALDI target plate using the CTC-MALDI fraction collector.

Collection time: 40 secs/spot

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α-cyano-4-hydroxycinnamic acid (2 mg/mL) added
Matrix:
                post-column at 1.7 µL/min via nanoACQUITY™ UPLC™
                auxiliary solvent manager
                A typical chromatogram obtained from the
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LC-separation is shown below for fraction 17

#### **Mass Spectrometry**

MS analysis was performed on a Waters MALDI Q-Tof<sup>TM</sup> Premier mass spectrometer in the positive ion mode. Each MALDI target position (40 sec HPLC fraction) was analyzed in MS mode on the over the m/z range 800-3,000. Following acquisition each spectrum was automatically deisotoped and a list of monoisotopic mass and intensity pairs for each spot was imported into the MassLynx<sup>™</sup> (Waters Corp.) MALDI merge program to identify <sup>16</sup>O:<sup>18</sup>O doublets; (see Figures 1 and 4).

## RESULTS

From the first dimension SCX fractionation, five of the collected fractions were selected and analysed by LC-MALDI. For each HPLC separation 96 samples were analyzed by MALDI MS. A representative MALDI mass spectrum from fraction 17 is shown in Figure 3. Within the spectrum pairs with different ratios, presumably originating from different proteins, can clearly be seen. Interrogation of the peptides that exhibited a differential ratio allowed identification of the parent protein. The results are summarised in the corresponding Spotfire (Spotfire Inc.) plots (Figure 5 & 6) and the tabulated summary Figure 7.



Figure 5. Spotfire plot of the <sup>16</sup>O peptide intensity vs the <sup>18</sup>O peptide intensity for all peptide pairs determined from the MS data. Highlighted in colour are those peptides identified as matching to a protein sequence from the MS/MS data.



In this study an isotope labelling <sup>18</sup>O quantification technique was used to analyze serum from a xenograph mouse model, and to compare this against a control mouse, to identify potential early detection markers in cancer.



Figure 1. Flow diagram illustrating the analytical method used in this study.



#### Sample preparation

V12-Harvey ras-expressing NIH3T3 fibroblast cells were implanted into athymic nude mice to induce fibrosarcomas. 50 µL of serum from tumourburdened mice and control mice was diluted in 30% trifluoroethanol, 200 mM Tris pH 8 and 38 mM NaCl. The sample was subsequently reduced with 10 mM TCEP and alkylated (50 mM lodoacetamide) prior t o digestion with Lys -C and trypsin. The resulting tryptic peptides from the tumour bearing mouse serum was labelled with <sup>18</sup>O whilst the normal mouse serum was digested in the presence of <sup>16</sup>O water. Equal amounts of serum from control and experimental states were then mixed. SCX fractionation of the <sup>16</sup>O:<sup>18</sup>O labelled Mouse serum was performed on a 2.1 mm column flowing at 200 µL/min. Peptides were collected into 40 x 200 µL fractions.



Figure 3. MALDI mass spectrum obtained from one 30 second HPLC fraction from SCX fraction 17, (bottom spectrum). The top spectra show expanded m/z regions around isotope doublets with differing  $^{16}O:^{18}O$  ratios.

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		F 1 1 7 3	l n u va l	1		1.10	D II I O	Lunc			52 II 6		D/D	
#	Mass 1	Int 1	Hel Int 1	#	Mass 2	Int 2	Rel Int 2	Int Hatio	Hel Int Ht	Well 1	Well 2	Mass Diff	DID	^
14314	1552.8145	955099.9		14318	1555.8333	639000		./3		HIZ	HIZ	4.0186	0.0101	
14370	1719.8524	667600		14374	1723.8628	470300		./1		H12	HIZ UII	4.0103	0.0018	
13477	1/19.86/6	410500		5481	1/23.0316	007400		.//		H11	HII CA	4.0233	0.0104	
10710	1710 0005	910000		10710	1403.7323	171200		.07		U4	U4	4.0033	-0.0032	
12/09	1/13.0003	247000		12/13	1023.0726	170/00		.63		H10	H10	2 0044	-0.0041	
7510	1002.0634	230300		7522	1000.0730	52270	-	./0		00	00	4.0076	-0.0164	
1/92	1596 7690	222200		1/96	1600 7755	151500	-	22.		00 F2	00 F2	4.0076	-0.0003	
5192	1/79 7998	180300		5196	1483 8074	100800		.00		63	63	4.0036	.0.0027	
6219	1479 8006	165300		6223	1483 8034	90920		.50		65	65	4.0070	-0.0056	
13478	1720 9731	154700		13482	1725.0112	56810		37		H11	H11	4.0020	0.0000	
5765	1536 8145	147500		5769	1540 8213	90410		61		64	G4	4.0069	-0.0016	
7076	1331 7589	143900		7080	1335 7614	31840	2	22		67	67	4.0025	0.000.0-	
1354	1439 8165	134500		1358	1443 8347	85270.01		63		E2	E2	4 0182	0.0097	
14316	1664 8683	130300		14320	1668 8831	13360		10		H12	H12	4 0149	0.0064	
14315	1663 9470	124400	-	14319	1667 9505	88920		71		H12	H12	4 0035	-0.0050	
7028	1274 7302	121600	-	7032	1278 7400	26770		22		67	67	4 0098	0.0013	
13667	949.5428	114900		13671	953,5536	83900		.73	-	H12	H12	4.0107	0.0022	
1543	1653,7907	110400		1547	1657,8001	76100		.69	-	F2	F2	4.0094	0.0009	
1406	1496.8393	104800		1410	1500.8474	66590		.64	-	F2	F2	4.0081	-0.0004	
5196	1483.8074	100800		5200	1487.8027	475.4		.00		G3	63	3.9953	-0.0132	
4713	1479.8050	100600		4717	1483.8117	54760		.54	-	G2	G2	4.0068	-0.0017	
6545	1274.7174	97060		6549	1278.7266	21060		.22		G6	GG	4.0092	0.0007	
11769	1158.6406	85480		11773	1162.6523	53790		.63		H9	H9	4.0117	0.0032	
12170	1719.8679	82220		12174	1723.8963	57510		.70		H9	H9	4.0284	0.0199	
10308	1850.7636	75050		10312	1854.7962	56420		.75		H4	H4	4.0326	0.0241	
6587	1331.7439	74590		6591	1335.7479	15700		.21		G6	G6	4.0039	-0.0046	
14371	1720.9850	73210		14375	1724.9845	57340		.78	-	H12	H12	3.9995	-0.0090	
6066	1274.7286	68710		6070	1278.7368	15160		.22		G5	G5	4.0082	-0.0003	
6268	1536.8252	63600		6272	1540.8310	38700		.61		G5	G5	4.0058	-0.0027	
4354	1681.8638	63410		4358	1685.8681	44620		.70	-	G1	G1	4.0043	-0.0042	
5241	1536.8228	62920		5245	1540.8359	38180		.61		G3	G3	4.0131	0.0046	
9192	1907.8113	62510		9196	1911.8120	45220		.72		G12	G12	4.0007	-0.0078	
12867	949.5605	60930		12871	953.5684	44370		.73	-	H11	H11	4.0078	-0.0007	
10010	1054 7000	EC400		10015	1050 7710	1105	3	02		114	114	2.0740	0.0007	

Int 16O

Figure 6. Spotfire trellis plots for selected proteins identified from the Mouse serum. Each plot displays the <sup>16</sup>O peptide intensity vs the <sup>18</sup>O peptide intensity from the MALDI MS data. The upper panes clearly show proteins not differentially expressed between the control and tumour bearing mice, whilst the lower two plots show proteins up regulated in the tumour bearing mice.

Protein ID	Accession	Description	Mass	Score	Peptides matched	<sup>18</sup> O/ <sup>16</sup> O ratio	Std dev.
ALBU_MOUSE	(P07724)	Serum albumin precursor	70700	767	35	0.74	0.29
HEMO_MOUSE	(Q91X72)	Hemopexin precursor	52049	260	16	0.60	0.36
TRFE_MOUSE	(Q92111)	Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal binding globulin)	78841	232	10	0.87	0.45
A1AT1_MOUSE	(P07758)	Alpha-1-antitrypsin 38718 precursor (Serine protease inhibitor 1-1) (Alpha-1 protease inhibi	46145	224	13	0.84	0.18
A2MG_MOUSE	(Q61838)	Alpha-2-macroglobulin precursor (Alpha- 2-M) [Contains: Alpha-2-macroglobulin 165 kDa subun	167091	202	9	0.91	0.17
FIBB_MOUSE	(Q8K0E8)	Fibrinogen beta chain precursor [Contains: Fibrinopeptide B]	55402	160	11	<sup>16</sup> O only	-
APOA1_MOUSE	(Q00623)	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I)	30569	155	8	0.85	0.12
FIBG_MOUSE	(Q8VCM7)	Fibrinogen gamma chain precursor	50044	112	8	0.70	0.15
HBB1_MOUSE	(P02088)	Hemoglobin beta-1 subunit (Hemoglobin beta-1 chain) (Beta-1-globin) (Hemoglobin beta-major	15813	94	9	0.24	0.06
HPT_MOUSE	(Q61646)	Haptoglobin precursor [Contains: Haptoglobin alpha chain; Haptoglobin beta chain]	39241	94	4	<sup>16</sup> O only	-
KNG1_MOUSE	(O08677)	Kininogen-1 precursor [Contains: Kininogen-1 heavy chain; Bradykinin; Kininogen-1 light ch	74140	89	2	1.30	0.05
CO3_MOUSE	(PO1027)	Complement C3 precursor (HSE-MSF) [Contains: Complement C3 beta chain; Complement C3	187904	69	6	0.75	0.29
TTHY MOUSE	(P07309)	Transthyretin precursor (Prealbumin)	15880	47	2	0.64	0.07
HBB2_MOUSE	(P02089)	Hemoglobin beta-2 subunit (Hemoglobin beta-2 chain) (Beta-2-globin) (Hemoglobin beta-minor	15851	45	6	1.07	1.41
FIBA_RAT	(P06399)	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A]	87373	34	3	<sup>16</sup> O only	
NOL6_MOUSE	(Q8R5K4)	Nucleolar protein 6 (Nucleolar RNA- associated protein) (Nrap)	128878	34	2	0.85	0.29
MUG1_MOUSE	(P28665)	Murinoglobulin-1 precursor (MuG1)	166460	33	3	0.64	0.27
PLMN_RAT	(Q01177)	Plasminogen precursor (EC 3.4.21.7) [Contains: Plasmin heavy chain A; Activation peptide;	93214	32	3	0.38	-
MUCM_MOUSE	(PO1873)	lg mu chain C region membrane-bound form	53121	30	2	0.70	0.24

Figure 7. Identified proteins quantified from their  $^{16}O/^{18}O$  isotope labels, showing score, number of matched peptides, expression ratio and standard deviation (where appropriate).

## **CONCLUSION**

• Here we present the use of an <sup>18</sup>O isotope labeling strategy in combination with LC-MALDI analysis for the quantitative analysis of Mouse serum, from control and tumor bearing mice.



Figure 2. Base Peak Intensity plot from the reverse phase LC-MS analysis of SCX Fraction 17.

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	9871	1907.8406	54680	-	9875	1911.8377	37930	 .69		H1	H1	3.9971	-0.0114

Figure 4. Output from the MassLynx<sup>™</sup> MALDI merge module. Listed are the <sup>16</sup>O:<sup>18</sup>O isotope labelled peptide pairs and the ratios for all peptides identified from the MALDI MS data for fraction 17. A total of 4377 peptide pairs were identified from this HPLC fraction.

- A new processing algorithm allows the interrogation of the LC-MALDI MS data to identify isotope labeled doublets and rank them by their intensity ratio, in this case  $^{16}O/^{18}O$ . This information can be used to supply precursor ion information for the MALDI Q-Tof mass spectrometer and in addition this can be exported for further statistical treatment or display.
- The analysis of serum from tumor-bearing mice showed changes in regulation of numerous previously wellcharacterized serum proteins.
- Further work will focus on the analysis of all fractions obtained from the SCX fractionation and comparison to data obtained from electrospray LC-MS experiments.

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