Developing Reliable MRM Assays for Protein Quantification Based on Parallel Multiplexing LC-MS/MS Analysis

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

Catalin E. Doneanu, Weibin Chen, Asish B. Chakraborty, Scott J. Geromanos, Gordon Fujimoto, and John C. Gebler

Biopharmaceutical Sciences, Waters Corporation, 34 Maple Street, Milford, MA 01757

INTRODUCTION

Successful and accurate MRM measurements require the selection/inclusion of at least one unique *proteotypic* peptide for each protein isoform and at least one of the most intense fragment ions generated during the CID process of this peptide, although two fragment ions are preferred in many cases. Although this information could be obtained from the protein identification process, the procedure to obtain proper MRM transitions for proteins of interest is lengthy and time consuming. Furthermore, if the fragmentation data is acquired using a data-dependent acquisition (DDA) method, the data may not contain the best MRM transition information for all the identified proteins because the selection of precursors in the DDA method at the MS/MS fragmentation step is rather random, especially in the case of analysis of very complex samples (e.g. whole cell or serum digests).

Here we describe a method using parallel multiplexing data acquisition (LC/MS^E method) to simultaneously collect the information of all the *proteotypic* peptides as well as the corresponding fragments. As a result, the probability of missing some of the *proteotypic* peptides (which could occur in a DDA experiment) and the corresponding fragment ions is eliminated. In addition, a software (**MRM Extractor)** was developed to automatically extract the best MRM transitions for a large number of proteins based on the MS^E data. The unique approach allows us to quickly develop reliable MRM methods without the need of theoretical predictions. The utility and effectiveness of the approach are demonstrated for the analysis of 40 serum proteins from a human serum digest, using two peptides/protein and two transitions/peptide for a total of 160 MRM transitions.

METHODS

nanoLC Conditions:

nanoLC System:	Waters nanoACQUITY™ UPLC
Trap Column:	180 µm x 20 mm, Symmetry C_{18}, 5 µm dp
Analytical Column:	75 µm x 100 mm, BEH 130 C_{18}, 1.7 µm dp
Mobile Phases:	
Eluent A:	Water with 0.1% formic acid (FA)
Eluent B:	ACN with 0.1% formic acid (FA)
Flow Rate:	300 nL/min
Column Temp:	45 °C
Gradient:	1% - 50% B in 30 minutes
Sample Volume:	5 µL

Mass spectrometry

Instrument:	Waters Quattro Premier™ XE
Capillary Voltage	2.9 KV
Source Temperature:	90 °C
Cone Voltage:	37 V
MS1/MS2 mass window:	1 Da (FWHH)
Dwell Time:	5/25 ms
Collision Energy Formula:	CE=0.034x*m/z* + 3.3

Samples

Top20 depleted serum digests were prepared using the ProteoPrep20 plasma immunodepletion kit purchased from Sigma. The amount of sample loaded onto the 75 µm column was equivalent to 360 ng of digested protein in each run. MRM experiments were replicated 6 times.

Data Processing Workflow

Protein ID Data from IDENTITY^E

Remove non-proteotypic peptides

Remove peptides containing labile AA (Cys,Met, etc.)

Remove peptides containing >18 AA

Select fragments with *m/z* values greater than precursors

Remove interfering transitions within ± 2 m/z window originating from peptides ± 0.5 min apart

Rank top 5 best responding peptides per protein

Rank fragments for the top 5 peptides

List of Transitions for each protein

MRM chromatograms were batched integrated using the QuanLynx™ application manager and the reports containing MRM transitions monitored, chromatographic retention times and peak areas were exported to Excel for subsequent data analysis.

RESULTS

No	Protein name	Peptide I	RT (min)	m/z	CE	Frag I	Frag II
1	1328 HRG_HUMAN (P04196) Histidine-rich glycoprotein precursor	IADAHLDR	17.8	455.74	18.8	540.30	611.32
2	370 APOH_HUMAN (P02749) Beta-2-glycoprotein I precursor	ATVVYQGER	18.6	511.77	20.7	652.30	751.37
3	6596 A2AP_HUMAN (P08697) Alpha-2-antiplasmin precursor	LGNQEPGGQTALK	19.0	656.85	25.6	771.44	900.48
4	5828 FA12_HUMAN (P00748) Coagulation factor XII precursor	EQPPSLTR	19.1	464.25	19.1	573.33	670.38
5	5853 KAL_HUMAN (P03952) Plasma kallikrein precursor	LSMDGSPTR	19.2	482.23	19.7	517.27	763.34
6	6574 CBG_HUMAN (P08185) Corticosteroid-binding globulin precursor	HLVALSPK	19.6	432.77	18.0	614.38	727.48
7	4869 PRTS_HUMAN (P07225) Vitamin K-dependent protein S precursor	ETISHEDLQR	19.7	670.84	26.1	614.29	884.42
8	6595 PEDF_HUMAN (P36955) Pigment epithelium-derived factor precursor	LSYEGEVTK	19.7	513.26	20.8	662.34	912.43
9	7385 VTNC_HUMAN (P04004) Vitronectin precursor	GSQYWR	19.8	398.69	16.9	524.26	652.32
10	363 APA4_HUMAN (P06727) Apolipoprotein A-IV precursor	SLAPYAQDTQEK	19.9	675.83	26.3	819.38	982.44
11	363 APA4_HUMAN (P06727) Apolipoprotein A-IV precursor	IDQNVEELK	20.2	544.29	21.8	518.29	974.47
12	3625 LEM1_HUMAN (P14151) L-selectin precursor	AEIEYLEK	20.6	497.76	20.2	681.36	794.43
13	7367 GELS_HUMAN (P06396) Gelsolin precursor	TGAQELLR	20.9	444.25	18.4	729.44	786.44
14	5169 CRP_HUMAN (P02741) C-reactive protein precursor	ESDTSYVSLK	21.1	564.78	22.5	609.36	696.36
15	5853 KAL_HUMAN (P03952) Plasma kallikrein precursor	IAYGTQGSSGYSLR	21.4	730.37	28.1	954.49	1112.54
16	1194 CO5_HUMAN (P01031) Complement C5 precursor	LQGTLPVEAR	21.5	542.31	21.8	684.40	1083.61
17	6592 HEP2_HUMAN (P05546) Heparin cofactor II precursor	TLEAQLTPR	21.5	514.79	20.8	486.30	814.44
18	1135 CLUS_HUMAN (P10909) Clusterin precursor	ELDESLQVAER	22.0	644.82	25.2	602.32	1046.50
19	5861 THRB_HUMAN (P00734) Prothrombin precursor	VTGWGNLK	22.1	437.74	18.2	674.36	775.42
20	1185 C4BP_HUMAN (P04003) C4b-binding protein alpha chain precursor	GYILVGQAK	22.1	474.78	19.5	615.38	728.47
21	20293 Q96QZ4 (Q96QZ4) Leucine-rich alpha-2-glycoprotein precursor	VAAGAFQGLR	22.2	495.28	20.2	748.40	819.44
22	1187 CFAH_HUMAN (P08603) Complement factor H precursor	IDVHLVPDR	22.2	532.29	21.4	486.27	599.35
23	3413 ITIH1_HUMAN (P19827) Inter-alpha-trypsin inhibitor heavy chain	GPDVLTATVSGK	22.3	572.81	22.8	663.36	776.45
24	5826 FA10_HUMAN (P00742) Coagulation factor X precursor	TGIVSGFGR	22.3	447.25	18.5	523.26	622.32
25	6597 IC1_HUMAN (P05155) Plasma protease C1 inhibitor precursor	LLDSLPSDTR	22.3	558.80	22.3	575.29	890.43
26	1192 CO3_HUMAN (P01024) Complement C3 precursor	GYTQQLAFR	22.5	542.28	21.8	762.42	1083.55
27	6595 PEDF_HUMAN (P36955) Pigment epithelium-derived factor precursor	ELLDTVTAPQK	22.5	607.84	24.0	544.29	744.42
28	4629 HEMO_HUMAN (P02790) Hemopexin precursor	VWVYPPEK	22.6	509.27	20.6	470.26	732.38
29	6591 ANT3_HUMAN (P01008) Antithrombin-III precursor	DDLYVSDAFHK	22.8	655.31	25.6	704.34	803.40
30	5804 CFAI_HUMAN (P05156) Complement factor I precursor	IVEYVDR	22.9	503.78	20.4	681.31	794.40
31	3023 A1BG_HUMAN (P04217) Alpha-1B-glycoprotein	LLELTGPK	23.0	435.76	18.1	644.36	757.44
32	5802 CFAB_HUMAN (P00751) Complement factor B precursor	EELLPAQDIK	23.0	578.32	23.0	671.37	784.46
33	241 VTDB_HUMAN (P02774) Vitamin D-binding protein precursor	YTFELSR	23.1	458.23	18.9	652.34	752.39
34	3624 TETN_HUMAN (P05452) Tetranectin precursor (TN)	NWETEITAQPDGGK	23.3	773.37	29.6	672.33	1116.54
35	4385 APB_HUMAN (P04114) Apolipoprotein B-100 precursor	ATGVLYDYVNK	23.4	621.82	24.5	801.38	914.45
36	5861 THRB_HUMAN (P00734) Prothrombin precursor	ETAASLLQAGYK	23.5	626.33	24.6	679.37	879.49
37	6571 AACT_HUMAN (P01011) Alpha-1-antichymotrypsin precursor	EQLSLLDR	23.5	487.27	19.9	603.36	716.42
38	7385 VTNC_HUMAN (P04004) Vitronectin precursor	DWHGVPGQVDAAMAGR	23.6	556.26	22.2	576.29	691.32
39	370 APOH_HUMAN (P02749) Beta-2-glycoprotein I precursor	EHSSLAFWK	23.7	552.78	22.1	838.44	975.49
40	5802 CFAB_HUMAN (P00751) Complement factor B precursor	YGLVTYATYPK	23.9	638.34	25.0	843.42	942.43
41	3023 A1BG_HUMAN (P04217) Alpha-1B-glycoprotein	SGLSTGWTQLSK	23.9	632.83	24.8	920.48	1007.51
42	5170 SAMP_HUMAN (P02743) Serum amyloid P-component precursor	VGEYSLYIGR	24.1	578.81	23.0	871.46	1057.53
43	1194 CO5_HUMAN (P01031) Complement C5 precursor	FQNSAILTIQPK	24.1	680.39	26.4	699.44	812.52
44	6572 KAIN_HUMAN (P29622) Kallistatin precursor (Kallikrein inhibitor)	IAPANADFAFR	24.1	596.81	23.6	504.75	840.41
45	1327 KNG_HUMAN (P01042) Kininogen precursor	TVGSDTFYSFK	24.3	626.3	24.6	994.46	1051.46
46	241 VTDB_HUMAN (P02774) Vitamin D-binding protein precursor	HLSLLTTLSNR	24.3	627.86	24.7	590.32	1004.57
47	3700 RETB_HUMAN (P02753) Plasma retinol-binding protein precursor	FSGTWYAMAK	24.6	581.28	23.1	927.45	1014.47
48	5826 FA10_HUMAN (P00742) Coagulation factor X precursor	MLEVPYVDR	24.6	561.29	22.4	649.33	877.44
49	6571 AACT_HUMAN (P01011) Alpha-1-antichymotrypsin precursor	EIGELYLPK	24.9	531.29	21.4	633.39	819.46
50	5170 SAMP_HUMAN (P02743) Serum amyloid P-component precursor	AYSLFSYNTQGR	24.9	703.84	27.2	825.39	972.46
51	1187 CFAH_HUMAN (P08603) Complement factor H precursor	SSNLIILEEHLK	25.0	465.93	19.2	768.41	881.51
52	4385 APB_HUMAN (P04114) Apolipoprotein B-100 precursor	TEVIPPLIENR	25.1	640.86	25.1	741.41	838.48
53	7367 GELS_HUMAN (P06396) Gelsolin precursor	EPGLQIWR	25.1	499.78	20.3	602.34	772.44
54	6576 ANGT_HUMAN (P01019) Angiotensinogen precursor	ALQDQLVLVAAK	25.2	634.89	24.9	956.58	1084.63
55	4869 PRTS_HUMAN (P07225) Vitamin K-dependent protein S precursor	FSAEFDFR	25.2	509.74	20.6	713.31	784.36
56	4629 HEMO_HUMAN (P02790) Hemopexin precursor	NFPSPVDAAFR	25.3	610.81	24.1	775.41	959.49
57	6572 KAIN_HUMAN (P29622) Kallistatin precursor (Kallikrein inhibitor)	FYYLIASETPGK	25.5	694.86	26.9	802.44	1078.57
58	6592 HEP2_HUMAN (P05546) Heparin cofactor II precursor	YEITTIHNLFR	25.6	469.58	19.3	686.37	1001.54
59	5828 FA12_HUMAN (P00748) Coagulation factor XII precursor	LHEAFSPVSYQHDLALLR	25.6	699.36	27.1	585.40	1259.56
60	6591 ANT3_HUMAN (P01008) Antithrombin-III precursor	TSDQIHFFFAK	25.7	670.84	26.1	659.36	796.41
61	6596 A2AP_HUMAN (P08697) Alpha-2-antiplasmin precursor	QEDDLANINQWVK	26.3	786.89	30.1	901.47	972.51
62	6576 ANGT_HUMAN (P01019) Angiotensinogen precursor	SLDFTELDVAAEK	26.3	719.36	27.8	745.42	975.49
63	20293 Q96QZ4 (Q96QZ4) Leucine-rich alpha-2-glycoprotein precursor	DLLLPQPDLR	26.5	590.34	23.4	725.39	838.47
64	3848 ZA2G_HUMAN (P25311) Zinc-alpha-2-glycoprotein precursor	YSLTYIYTGLSK	26.6	704.87	27.3	668.36	944.49
65	5805 CFAI_HUMAN (P05156) Complement factor I precursor	VFSLQWGEVK	26.7	596.82	23.6	859.46	946.48
66	1185 C4BP_HUMAN (P04003) C4b-binding protein alpha chain precursor	LSLEIEQLELQR	26.9	735.91	28.3	545.30	915.48
67	5181 CO9_HUMAN (P02748) Complement component C9 precursor	LSPIYNLVPVK	26.9	621.88	24.5	521.82	832.48
68	6574 CBG_HUMAN (P08185) Corticosteroid-binding globulin precursor	WSAGLTSSQVDLYIPK	26.9	882.96	33.3	1062.58	1250.65
69	6597 IC1_HUMAN (P05155) Plasma protease C1 inhibitor precursor	FQPTLLTLPR	26.9	593.35	23.5	712.48	910.57
70	1327 KNG_HUMAN (P01042) Kininogen precursor	YFIDFVAR	27.0	515.77	20.9	607.32	720.40
71	5181 CO9_HUMAN (P02748) Complement component C9 precursor	AIEDYINEFSVR	27.2	728.36	28.1	508.28	1142.53
72	1135 CLUS_HUMAN (P10909) Clusterin precursor	LFDSDPITVTVPVEVSR	27.4	937.50	35.2	886.49	1296.76
73	3413 ITIH1_HUMAN (P19827) Inter-alpha-trypsin inhibitor heavy chain	LGVYELLLK	27.4	524.32	21.1	778.46	934.55
74	1192 CO3_HUMAN (P01024) Complement C3 precursor	DFDFVPPVVR	27.7	595.81	23.6	567.36	666.43
75	3624 TETN_HUMAN (P05452) Tetranectin precursor (TN)	LDTLAQEVALLK	27.7	657.39	25.7	871.51	941.49
76	3700 RETB_HUMAN (P02753) Plasma retinol-binding protein precursor	YWGVASFLQK	27.8	599.82	23.7	693.39	849.48
77	3848 ZA2G_HUMAN (P25311) Zinc-alpha-2-glycoprotein precursor	EIPAWVPFDPAAQITK	29.1	891.97	33.6	728.43	1087.58
78	1328 HRG_HUMAN (P04196) Histidine-rich glycoprotein precursor	DGYLFQLLR	29.7	562.81	22.4	676.41	789.50
79	4372 ALS_HUMAN (P35858) Insulin-like growth factor binding protein	LAYLQPALFSGLAELR	30.8	881.49	33.3	658.39	1173.66
80	4372 ALS_HUMAN (P35858) Insulin-like growth factor binding protein	VAGLLEDTFPGLLGLR	31.5	835.98	31.7	725.48	1330.72

Table I. *Complete list of 80 serum peptides targeted by the MRM assay, arranged according to their elution time.*

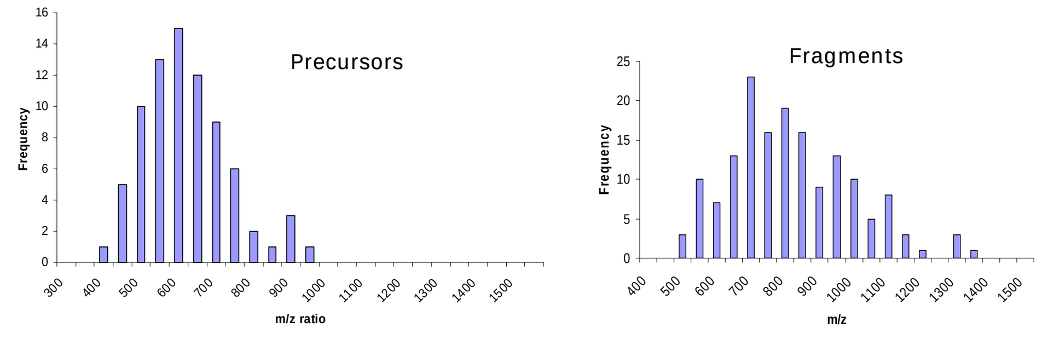

Figure 2. *MW distribution of the peptide precursors and fragments selected for MRM.*

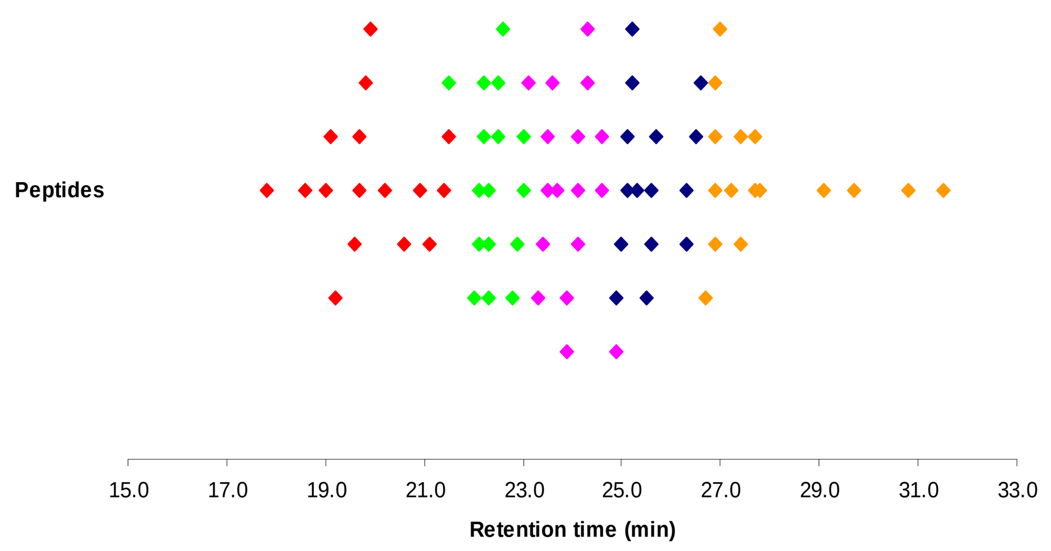

Figure 3. *Peptide elution profile during the LC gradient. The retention time axis was divided into 0.3 min bins and peptides were grouped according to their retention times. Co-eluting peptides, contained in the same 0.3 min retention time bin, were spread across the y-axis for better visualization. 16 peptides (32 MRMs) were monitored simultaneously during each of the 5 retention time windows employed for the entire run.*

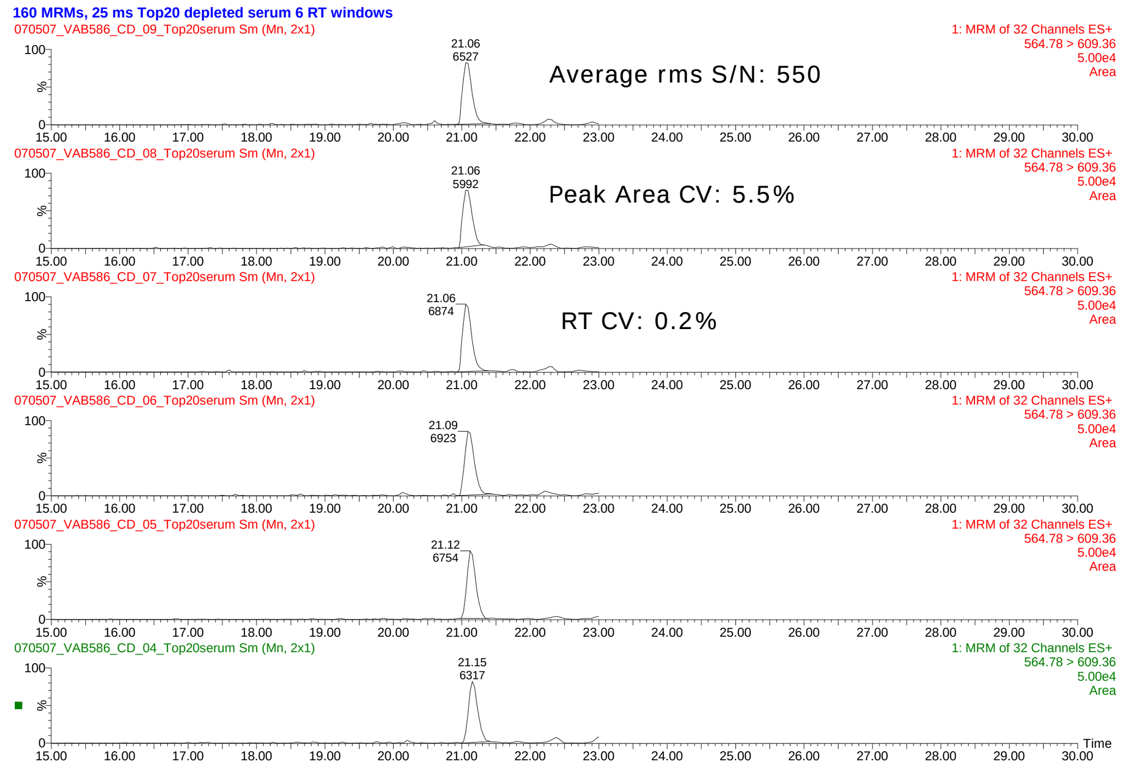

Figure 4. *Example of retention time and peak area reproducibility for the peptide ESDTSYVSLK from C-reactive protein.*

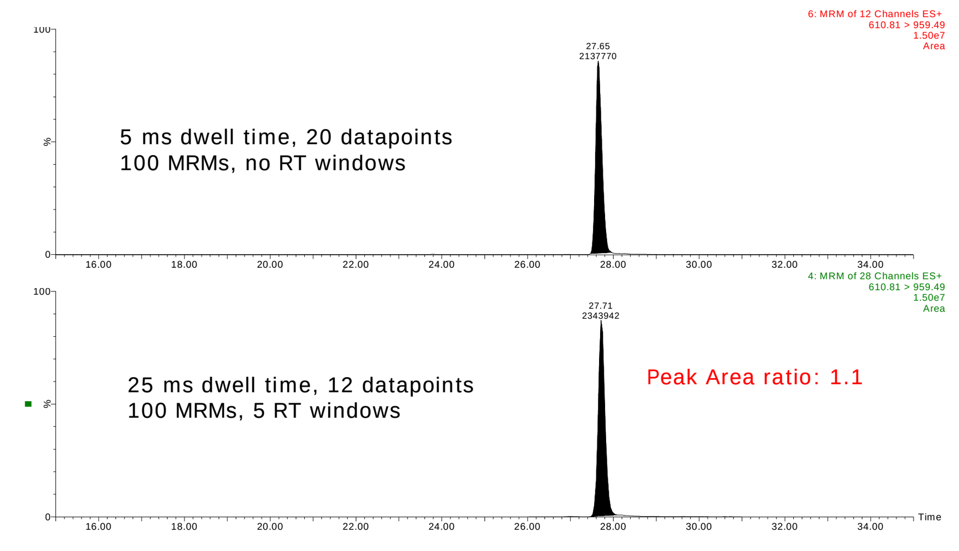

Figure 5. *The effect of dwell time on a Hemopexin peptide.*

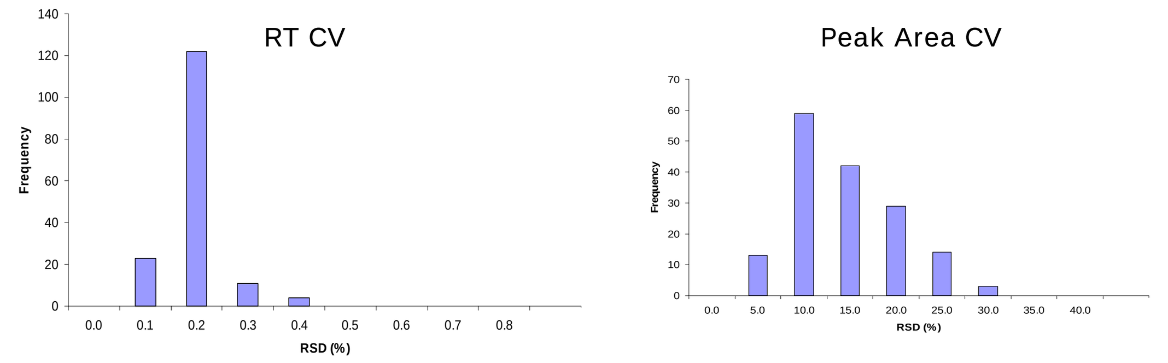

Figure 6. *Retention time and peak area reproducibility for 160 chromatographic peaks generated by the MRM assay. 70% of the transitions monitored had peak areas CV's below 15%.*

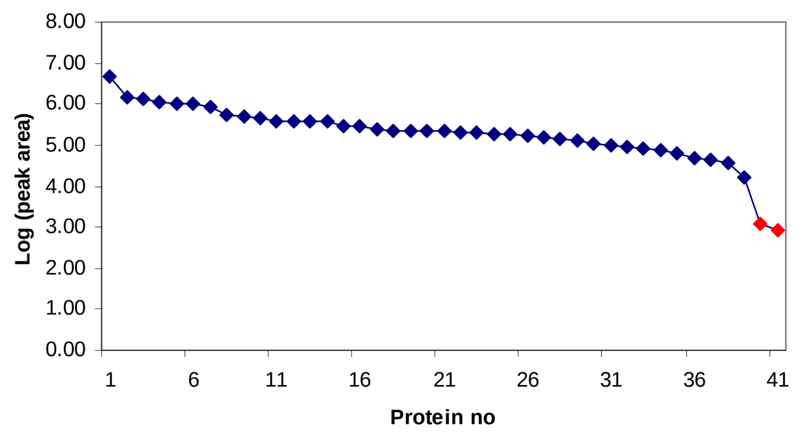

Figure 7. *Dynamic range of measured serum proteins. Peak areas corresponding to all 4 MRM transitions of each protein were summed.*

CONCLUSIONS

- The MRM assay monitored 80 peptides from 41 serum proteins, using two peptides/protein (except for CRP and L-selectin), and two transitions/peptide, for a total of 160 MRM transitions.
- By using the MS^E data to design the MRM experiment one can achieve very high success rates (approaching 100%) for finding the best MRM transitions for each protein.
- Peak area reproducibility (CV < 15% for 70 % of MRMs) indicates that the matrix interference due to ion suppression is not a significant problem when using 30 min gradients (60 min total runtime);

References

1. Anderson L, Hunter CL *Moll Cell Proteomics* **2006**, 5.4, p573.
2. Wolf-Yadlin A, Hautaniemi S, Lauffenburger D, White FM *PNAS* **2007**, 104p5860.
3. Unwin RD, Griffiths JR, Leverentz MK, Grallert A, Hagan IM, Whetton AD *Moll Cell Proteomics* **2005**, 4.8, p1134.
4. Craig R, Cortens JP, Beavis RC *Rapid Comm Mass Spectrom* **2005**, 19, p1944.

50 YEAR LEGACY — VISION • CONTRIBUTION • ADVANCEMENT

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS

720002661EN