Translating Proteomics Methods from Discovery to MRM-based Quantitation

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Introduction

Peptide quantitation using multiple reaction monitoring (MRM) has emerged as an important methodology for biomarker verification. Such assays are typically multiplexed, multiple reaction monitoring (MRM) analyses which can provide the high-throughput required. To maximize the effectiveness of MRM analysis, retention time scheduling allows acquisition of the peptide transitions only when the peptide is eluting from the LC. This reduces the number of concurrent MRM channels and improves peak symmetry and sensitivity. However, developing these methods can be time-consuming as discovery data is often obtained on a high resolution mass spectrometer using nanoflow LC. In this study, we have explored rapid translation from discovery to routine LC/MS analysis using a simple retention-time marker approach.

Experimental

Sample preparation

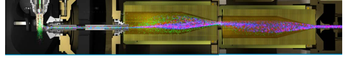
An *E. coli* lysate (2.7 mg, BioRad) was reduced, alkylated (carbamidomethyl) and digested with trypsin using a 2,2,2-trifluoroethanol based protocol.

Instruments

The HPLC-Chip/MS system consisted of 1200 Series nanoflow and capillary HPLC pumps, microdegassers, micro wellplate autosampler with thermostat, HPLC-Chip/MS interface, and a 6550 Q-TOF mass spectrometer. The standard flow system consisted of a 1290 Infinity Series UHPLC coupled to a 6490 QQQ using the JetStream source. MassHunter Acquisition B.05.00 was used for instrument control.

Both the 6550 Q-TOF and 6490 QQQ incorporate iFunnel technology which is a combination of three fundamental innovations:

- Agilent Jet Stream technology – ESI with thermal gradient ion focusing confinement
- Hexabore sampling capillary with 6 parallel bores to enable sampling a much larger fraction of the ions
- Dual-stage ion funnel for efficient removal of large gas volumes and ion transfer to Q1 optics



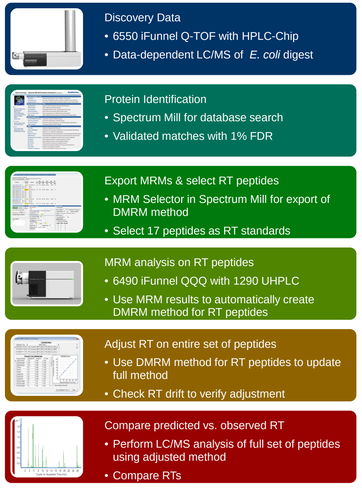
Experimental

Protein Database Search

Protein/peptide identifications were done via database searching against an *E. coli* subset of the SwissProt data base using Spectrum Mill MS Proteomics Workbench B.04.00. Spectral matches were autovalidated using a peptide and protein global false discovery rate of 1%.

Workflow

Translating from discovery to targeted methods can be a bottleneck as a transitions must be determined for peptides from each protein of interest. As shown in the workflow below, we have used a set of software tools to facilitate this process. In addition to automatically creating MRM methods based on data-dependent data, an algorithm in the QQQ MassHunter acquisition software will automatically adjust retention times (RTs) based on user selected RT standard transitions. When changing LC conditions, a single MRM analysis of these RT standards allows the algorithm to adjust RTs in any number of designated dynamic MRM (retention-time scheduled MRM) methods.



Conditions Tested for RT Correction

Six different experiments were done to test the applicability of this approach across different column phases, inner diameters, column lengths and gradient conditions. Table 1 (below) summarizes the experiments

	A	B	C	D	E	F	G
LC	Chip LC	**UHPLC #1**	**UHPLC #2**	UHPLC #2	UHPLC #2	UHPLC #2	UHPLC #2
Column type	Zorbax 300SB C18, 5 µm	**Zorbax 300SB C18, 1.8 µm**	Zorbax 300SB C18, 1.8 µm	**Poroshell EC C18, 2.7 µm**	**BEH C18 1.7 µm**	**Zorbax 300SB C18, 1.8 µm**	**BEH C18, 1.7 µm**
Column length	150 mm	**100 mm**	100 mm	**150 mm**	**50 mm**	50 mm	50 mm
Column ID	75 µm	**2.1 mm**	2.1 mm	2.1 mm	2.1 mm	2.1 mm	2.1 mm
Gradient time	60 min	**40 min**	**25 min**	25 min	**15 min**	15 min	15 min
Flow rate	300 nL/min	**400 uL/min**	400 uL/min	400 uL/min	400 uL/min	400 uL/min	400 uL/min
Solvent	ACN	ACN	ACN	ACN	ACN	ACN	**MeOH**

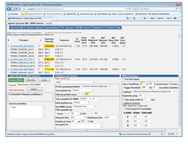
RT Correction

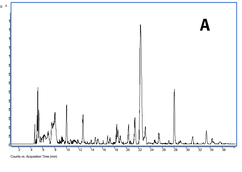
1. A → B
2. B → C
3. C → D
4. D → E
5. E → F
6. F → G

Translating From Discovery to Targeted

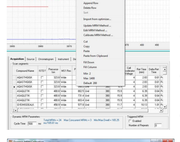
Mining discovery data for targeted analysis.
Export from discovery data:

- HPLC-Chip/Q-TOF data collected using 75 µm id column (far right)
- For top 200 proteins identified, selected 5 peptides with three transitions each (right)
- Exported dynamic MRM (DMRM) list includes RTs from nanoflow LC/MS
- 17 peptides (51 MRMs) selected for study





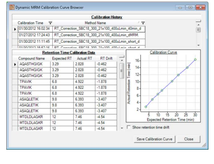
Converting retention time scheduled MRM method from nanoflow to standard flow LC/MS.



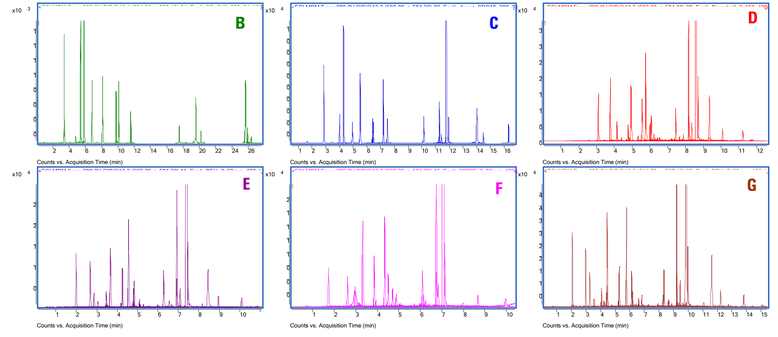
Adjust RTs

- 1290 UHPLC /6490 QQQ with 2.1 mm column
- RT standard set of 8 peptides was selected.
- Did single MRM analysis with the 8 RT peptides
- Used "Calibrate MRM Method" option in acquisition software to adjust full DMRM methods

From the single data file, an automatic algorithm was used to recalibrate retention times for all 17 peptides (51 transitions) in the full DMRM method. The results can be inspected in the RT calibration screen (below) before accepting the adjusted method.



Optimizing DMRM Methods



A → B			B → C			C → D			D → E			E → F			F → G		
Real	Cali	dRT	Real	Cali	dRT	Real	Cali	dRT	Real	Cali	dRT	Real	Cali	dRT	Real	Cali	dRT
2.855	2.828		2.855	2.828		3.530	3.530		1.990	1.948		1.730	1.731		6.700	6.885	
4.000	3.175	0.825	4.000	4.087	-0.087	4.510	4.940	-0.430	2.670	2.583	0.087	2.620	2.474	0.146	6.900	2.986	3.914
4.300	4.222	0.078	4.300	4.313	-0.013	5.000	5.240	-0.240	2.870	2.902	-0.032	2.930	2.305	0.625	7.200	4.363	2.837
4.930	4.922		4.930	4.922		5.060	5.700	-0.640	3.470	3.459	0.011	2.980	2.980		8.500	4.050	4.450
5.510	5.373	0.137	5.510	5.559	-0.049	5.860	5.840		3.670	3.659		3.330	3.204	0.126	8.500	11.668	-3.168
6.420	6.393	0.027	6.420	6.393	0.027	6.160	6.330	-0.170	4.190	4.111	0.079	3.860	3.864	-0.004	8.600	8.204	
6.464	5.956	0.508	6.464	6.529	-0.065	6.840	7.070	-0.230	4.260	4.271	-0.011	3.920	3.920	0.000	10.600	11.455	
7.180	6.976	0.204	7.180	7.251	-0.071	7.080	7.110		4.310	3.940	0.370	3.940	3.786	0.154	10.700	7.141	3.559
7.487	7.460	0.027	7.487	7.460	0.027	7.460	7.655	-0.195	4.560	4.524	0.036	4.350	4.188	0.162	10.800	5.302	5.498
10.139	10.131		10.139	10.131		7.890	7.880	0.010	4.840	4.819	0.021	4.520	4.501	0.019	11.700	11.146	0.554
11.271	11.176	0.095	11.271	11.272	-0.001	10.020	10.040		6.270	6.279		6.100	6.109		11.700	11.710	
11.735	11.706	0.029	11.735	11.609	0.126	11.070	11.170	-0.100	6.920	7.041	-0.121	6.750	6.758	-0.008	11.900	7.632	4.268
11.750	11.615	0.135	11.750	11.715	0.035	11.640	11.648	-0.008	7.380	7.454	-0.074	7.060	7.224	-0.164	13.100	12.164	0.936
11.937	11.925	0.012	11.937	11.925	0.012	11.830	11.838	-0.008	7.470	7.592	-0.122	7.140	7.135	0.005	13.300	12.928	
14.020	14.014		14.020	14.014		13.040	13.105	-0.065	8.450	8.468		8.306	8.306		13.400	12.395	1.005
14.470	14.729	-0.259	14.470	14.128	0.342	13.970	13.570	0.400	8.950	9.049	-0.099	8.690	8.812	-0.122	14.300	5.742	8.558
16.320	16.313		16.320	16.307		15.670	15.690		10.100	10.134		9.970	9.975		15.900	7.039	8.861
Avg. abs dRT		0.195	Avg. abs dRT		0.071	Avg. abs dRT		0.208	Avg. abs dRT		0.089	Avg. abs dRT		0.128	Avg. abs dRT		3.967

Adapting RTs during LC method development

During routine method development, it is not uncommon to test many conditions. From the parameters tested, we found RT adjustment worked well for the following:

- Column id and length changes
- Different column type (only C18 tested, though)
- Changes in the gradient
- Switching standard LC systems

The solvent change caused selectivity differences (as would be expected) and thus only some of the peptide RT changes could be correctly recalibrated.

Conclusions

- Demonstrated a simple algorithm for RT adjustment that was effective for typical method development changes
- Method changes that cause shifts in selectivity (organic solvent change, large column temperature change) were less successful as was anticipated.
- The ability to rapidly adapt methodology will facilitate the development of routine quantitative methods
- RT adjustment is also useful with quality control and column replacement for routine methods
- This approach also works for small molecules