

Overview

- Single reaction monitoring (SRM) is emerging as a highly selective and sensitive approach for the quantitation of proteins¹.
- By combining SRM with peptide labeling by Tandem Mass Tags (TMT), quantitation of the experimental sample to a reference sample is possible, whilst maintaining the selectivity of SRM. Here, structurally identical but isotopically different TMT reagents are utilised (light TMT and heavy TMT; Figure 1).
- This approach has application for the verification of candidate AD biomarkers from discovery experiments.

Introduction

Highly specific and sensitive biomarkers for Alzheimer's disease (AD) are required to assist in the effective diagnosis and monitoring of disease progression. Such markers may relieve the dependency on cognitive testing to track AD and may ultimately evolve into surrogate endpoints for the disease. The plasma proteins clusterin, complement c3/c3a, alpha-2 macroglobulin (A2M), serum-amyloid protein (SAP), gamma-fibrinogen, gelsolin and complement factor H (CFH) have emerged as candidate AD biomarkers from discovery exercises². Assays are required for the validation of these candidates; SRM-based approaches are an attractive alternative to ELISAs due to the sensitivity and selectivity of the technique, the capacity to multiplex and the limited availability of antibodies. Here, signature peptides unique to the protein of interest are measured to provide quantitative information of that protein in the sample. The incorporation of a TMT-labeled internal reference into the analysis is beneficial as this alleviates the need for the production of heavy isotope-labeled peptides and has the potential to generate an internal reference from all endogenous analytes. Initial results have demonstrated TMT SRM as an accurate and reproducible method of peptide quantitation. We now move to provide a TMT SRM assay allowing the evaluation of eight candidate biomarkers in AD and control plasma samples.

Methods

Using existing MS/MS data, at least three peptides per protein were selected for quantitation (Table 1). Criteria for selection were; no missed cleavages with trypsin, no variable modifications (*in-vivo* or experimental) and a unique sequence in the human transcribed genome (proteotypic). Synthetic versions of each peptide were prepared and labeled with light TMT to act as a reference for quantitation. Peptides were infused into a 4000 QTRAP (Applied Biosystems) and MS/MS data was acquired. Optimal fragment ions were chosen for all peptides to facilitate maximum detection of each in Q3. Corresponding heavy TMT-labeled fragment ion masses were calculated and MS instrument parameters optimised for individual Q1 and Q3 transition pairs. A pooled plasma sample was digested, labeled with heavy TMT and combined with the light TMT-labeled reference peptides. LC/MS/MS analysis was performed using an Ultimate 3000 nano LC (Dionex) and a 4000 QTRAP. Peptides were resolved by reversed-phase chromatography over a 90min ACN gradient. Using accurate retention times for each peptide, SRM scheduling was applied to the method (+/- 3min detection window; 2.5sec cycle time; 37.8msec dwell time and 21 data points at FHMW).

Methods

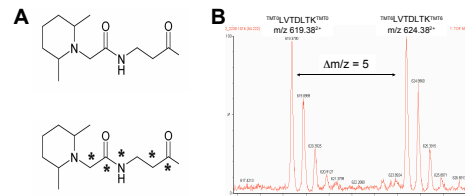


Figure 1 A) The isotopic set of light TMT and heavy TMT. Asterisks mark ¹³C and ¹⁵N atoms. B) MS spectrum of LVTDLTK labeled with light TMT and heavy TMT. The peptide is labeled at the N-terminus and lysine giving a mass difference of 10 Da between the labeled peptides. The doubly charged precursor ions have a Δ m/z of 5.

Protein	Peptide Sequence	I.D.	Light TMT Q1	Heavy TMT Q1	Q3
Clusterin	TMTLLSNLEEAQ ^{TMT}	1	783.5	788.5	b7, b8, y7
	TMTASSIIDELFQDR	2	809.4	811.9	b7, b8, b11
	TMTIDSLLENDR	3	649.8	652.3	b5, b8, y7
	TMTVTTVASHTSDSDVPSGVTEVVK ^{TMT}	4	921.5	924.8	y6, y10, y11
	TMTALQEYR	5	502.3	504.8	b3, b4, b5
	TMTYNELK ^{TMT}	6	614.4	619.4	b4, b5, y5
Complement c3/c3a	TMTIYTPGSTVLVYR	7	532.3	534.0	b4, y7, y8
	TMTFYIYNEK ^{TMT}	8	794.4	799.4	b4, b7, y5
	TMTLVAYYTLIGASGQR	9	868.5	871.0	y11, y12, y13
CFH	TMTASHLGLA	10	447.2	449.7	b4, y4, y5
	TMTSPDVINGSPISQK ^{TMT}	11	895.5	900.5	y8, y9, y10
	TMTIDVHLVPDR	12	429.9	431.6	b3, b4, y5
A2M	TMTVGEVLK ^{TMT}	13	546.9	551.9	b4, b5, y5
	TMTAIGYLVNTGYQR	14	740.4	742.9	y8, y10, y11
	TMTGTHGLLVK ^{TMT}	15	687.4	692.4	b7, b8, y5
Gamma-fibrinogen	TMTLLIYAVLPTGDVIGDSAK ^{TMT}	16	765.1	768.4	b7, y6, y11
	TMTGEAFTLK ^{TMT}	17	607.4	612.4	b6, y4, y5
	TMTYLQEIYNSNNQK ^{TMT}	18	981.5	986.5	y7, y8, y9
SAP	TMTLDGSDVDFK ^{TMT}	19	664.9	669.9	b6, y6, y7
	TMTVGPEADK ^{TMT}	20	582.3	587.3	y5, y6, b6
	TMTVLGQEQDQSYGK ^{TMT}	21	921.5	926.5	b8, y10, y11
APOE	TMTVGEYSYIGR	22	690.9	693.4	b7, y7, y9
	TMTAYSLFSYNTQGR	23	815.9	818.4	y8, y10, y11
	TMTLGPLVEQGR	24	596.9	599.4	b5, b6, b8
Gelsolin	TMTLQAEAFQAR	25	629.3	631.8	b5, b6, b7
	TMTSELEEQLTPEAEETR	26	978.0	980.5	b7, b8, y11
	TMTQTVSVLPEGETPLFK ^{TMT}	27	760.1	763.4	b6, b7, y10
	TMTAGALNSNDAFVLK ^{TMT}	28	884.5	889.5	b8, b9, b12
	TMTASDFITK ^{TMT}	29	665.9	670.9	b6, y4, y7
	TMTAVEVLPK ^{TMT}	30	602.4	607.4	b5, y5, y6

Table 1 Plasma peptides from each candidate protein selected for SRM quantitation. For each protein, the sequences of each peptide is indicated, along with the peptide's TMT labeling properties. The Q1 transitions for the specific detection of the light TMT and heavy TMT-labeled versions of the peptides are listed along with the fragment ions selected for both in Q3, respectively.

Results

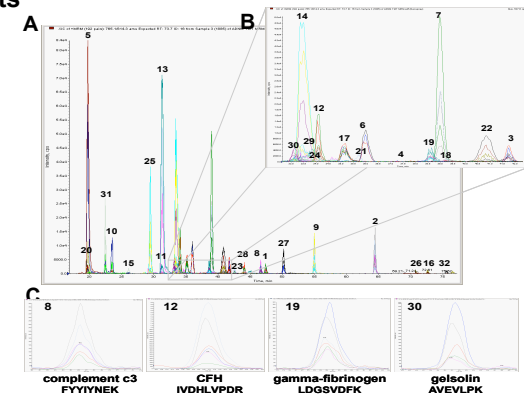


Figure 2 A) An SRM XIC for light TMT- and heavy TMT-labeled plasma peptides 1-32. All transitions listed in Table 1 were assessed. It can be seen that light TMT- and heavy TMT-labeled peptide pairs co-elute. B) SRM XIC showing the peptides which elute over the busiest part of the LC gradient (32min – 42min). C) SRM XIC for a selected peptide of the proteins complement c3, CFH, gamma-fibrinogen and gelsolin. Peptide I.D. is displayed in the top left-hand corner. Light TMT transitions for each peptide are coloured in blue, red and green, while their heavy TMT labeled counterparts are coloured in grey, cyan and pink.

All peptides were observed in the plasma sample by TMT SRM at varying intensities. To achieve accurate quantitation of each candidate peptide, it was necessary to establish whether there was significant contribution of non-specific signal from the plasma, when no reference peptides were added. Heavy TMT-labeled plasma was analysed over all light TMT and heavy TMT transitions. Signals observed from any light TMT SRM transitions represented non-specific background from the plasma matrix. Those transitions which had significant background (S:N ratio of less than 10:1) were subsequently removed from the method.

Conclusions and future work

- Performance criteria for the TMT SRM assay are currently being defined (precision, LOD, LOQ and linearity).
- Once the assay parameters have been defined the candidate proteins will be quantitated in a longitudinal clinical sample cohort. Expression levels will be monitored in AD and age and sex-matched controls and correlations made to clinical measures.

References

1 Andersen L and Hunter CL. (2006) Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol Cell Proteomics*. 5(4):573-88
 2 Hye A. et al (2008) Proteome-based plasma biomarkers for Alzheimer's disease. *Brain* 129:3042-3050