

Overview

- Single and multiple reaction monitoring (SRM and MRM) is emerging as a highly selective and sensitive approach for the quantitation of proteins¹.
- By combining MRM with peptide labeling using Tandem Mass Tags (TMT), quantitation of the experimental sample to a reference sample is possible, whilst maintaining the selectivity of MRM. Here structurally identical but isotopically different labels are utilised (TMTzero and TMTsixplex).
- This approach has application for the validation of candidate 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. Plasma contains a number of medium/high abundant proteins which have emerged as candidate AD biomarkers from discovery exercises². Assays are required for the validation of these candidates; MRM-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. Accuracy in the quantitation of the analytes of interest by MRM can be improved by combining with TMT as this allows the incorporation of an internal reference into the analysis. Here we present experiments to demonstrate the approach for application to the validation of candidate biomarkers implicated in AD.

Methods

Two plasma samples (Dade Behring) were digested with trypsin and labeled with TMT⁰ or TMT⁶-127 (Figure 1). Samples were combined in ratios of 1:1, 3:1, 9:1 and 27:1. LC/MS analysis was performed using an Ultimate 3000 nano LC (Dionex) and a 4000 QTRAP (Applied Biosystems). Peptides (600ng total protein o/c) were resolved by reversed-phase chromatography over a 30min ACN gradient. An IDA analysis was performed on the TMT⁰ and TMT⁶ labeled samples (1:1 mix) to define the optimal Q1 and Q3 transitions for a set of ten arbitrarily chosen plasma peptides (Table 1). MRM quantitation was performed at unit resolution for both Q1 and Q3 with a dwell time of 100msec per transition (total cycle time 2.3 sec; 6-9 data points FWHM). The quantitative measurement was based on the integrated peak area from this scan mode. All peak areas were extracted using the Analyst automated peak integration tool. All measurements were performed in triplicate.

Results

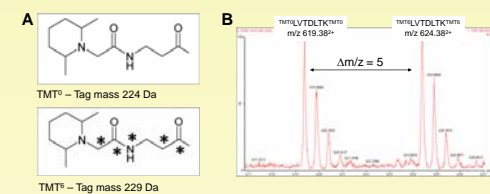


Figure 1 A Structure of TMT⁰ and TMT⁶-127. TMT⁰ has ¹³C and ¹⁵N isotopic labels (*). B MS spectrum of LVTDLTK labeled with TMT⁰ and TMT⁶-127. The peptide is labeled at the N-terminus and lysine giving a mass difference of 10 Da between the labeled peptides. The doubly charged precursor ions have a $\Delta m/z$ of 5.

Peptide sequence	Charge state	RT mins	CE	TMT ⁰		TMT ⁶	
				Q1	Q3	Q1	Q3
A ¹⁵ N ¹³ LHVHEEPHTETVR	3	6.8	38	590.66	839.44 (y7)	592.33	839.44 (y7)
B ¹⁵ N ¹³ LTVDK ¹⁵ MT	2	9.0	29	512.29	653.39 (b4)	517.29	658.39 (b4)
Ci ¹⁵ N ¹³ DLMSR	2	10.0	40	530.30	906.60(pseudo y)	532.80	909.60(pseudo y)
Cii ¹⁵ N ¹³ DLMSR	2	10.0	40	530.30	685.40 (b4)	532.80	690.40 (b4)
D ¹⁵ N ¹³ ILGGHDAK ¹⁵ MT	3	10.5	32	457.94	670.42 (y4)	461.31	676.42 (y4)
E ¹⁵ N ¹³ ALPAPIEK ¹⁵ MT	2	29.3	35	643.90	878.55 (y6)	648.90	883.55(y6)
F ¹⁵ N ¹³ LVTDLTK ¹⁵ MT	2	31.9	34	619.38	585.40 (y3)	624.38	590.40 (y3)
Gi ¹⁵ N ¹³ FQNALLR	2	13.9	43	592.90	1031.76(pseudo y)	595.40	1034.7(pseudo y)
Gii ¹⁵ N ¹³ FQNALLR	2	13.9	43	592.90	798.50 (b5)	595.40	803.50 (b5)
H ¹⁵ N ¹³ SASDLWDNLK ¹⁵ MT	2	34.1	46	849.47	598.40 (y3)	854.47	603.40 (y3)
Ii ¹⁵ N ¹³ LIVNEVEFAK ¹⁵ MT	2	35.3	43	799.48	819.46 (y5)	804.48	824.46 (y5)
Iii ¹⁵ N ¹³ LIVNEVEFAK ¹⁵ MT	2	35.3	43	799.48	589.38 (y3)	804.48	594.38 (y3)
J ¹⁵ N ¹³ SLSVPPVYVPLK ¹⁵ MT	2	20.7	60	925.55	581.41 (y3)	930.55	586.41 (y3)

Table 1 TMT⁰- and TMT⁶-labeled plasma peptides selected for MRM quantitation. For each peptide (A-J) the number of TMT labels per peptide is indicated along with the peptide's retention time and precursor ion charge state. The collision energy is given for peptide fragmentation, optimised for the detection of higher mass structural ions (Q3 transitions) for improved MRM sensitivity. The Q1 and Q3 transitions for the specific detection of both the TMT⁰- and TMT⁶-labeled versions of the peptides are also listed.

To demonstrate the approach, following IDA (MS/MS) analysis of a TMT⁰- and TMT⁶-labeled plasma sample (mixed 1:1), a range of plasma peptides were selected that had either one or two tags attached, were doubly or triply charged and had varying hydrophobicities and intensities (Table 1). Three of the peptides C, G and I were measured using two transitions. As an example Peptide G has one tag attached with the doubly charged precursors (Q1 transition) having a m/z 592.90²⁺ (TMT⁰) and m/z 595.40²⁺ (TMT⁶; $\Delta m/z=2.5$). The b5 ion (Q3 transition) has one tag still attached, therefore the singly charged fragment ions have a m/z difference of 5 (798.51⁺ and 803.51⁺). As a second transition the pseudo y-ion was selected (Figure 2).

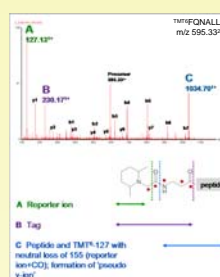


Figure 2 TMT MS/MS fragment ions. MS/MS spectra of FQNALLR (m/z 595.33²⁺) labeled with TMT⁰-127. The fragment ions of TMT⁰-127 are highlighted. In addition to the reporter ion at m/z 127 the intact tag at m/z 230 is observed and an ion representing the intact peptide plus a remaining portion of the tag. These ions are characterised as 'pseudo y-ions'. Pseudo y-ions may serve as Q3 transitions without prior insight into fragmentation patterns for peptide detection. Furthermore, the pseudo y-ion has use as an additional transition along side more selective structural fragment ions for peptide quantification. Selectivity of pseudo y ions may be compromised if an ion of the same m/z co-elutes.

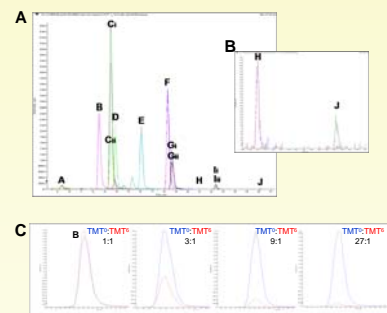


Figure 3 A MRM XIC for TMT⁰- and TMT⁶-labeled plasma peptides A-J. All transitions listed in Table 1 were assessed. It can be seen that TMT⁰- and TMT⁶-labeled peptide pairs co-elute. B MRM XIC showing the lower intensity peptides, H and J. C MRM XIC for Peptide B. TMT⁰-labeled samples were mixed in 1:1, 3:1, 9:1 and 27:1 ratios (TMT⁰:TMT⁶)

To assess the selectivity of the transitions for the detection of each labeled version of the peptide, two MRM analyses were performed (TMT⁰-labeled plasma with TMT⁰ specific transitions only and vice versa). No cross-talk was observed between transitions. For precursors of higher charge state with one tag attached, selectivity can still be achieved (eg. Peptide A). Here, the approach discriminates between transitions with a m/z difference of 1.66 (Q1); whilst Q3 transitions remain identical.

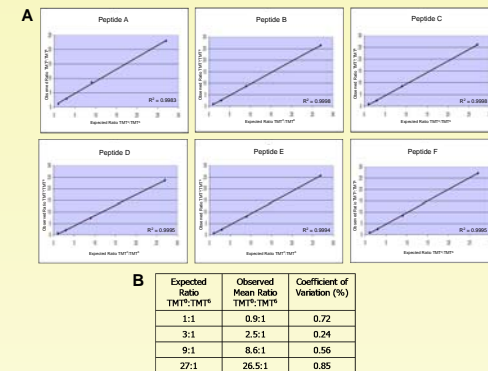


Figure 6 A Plots of 'observed vs expected' ratios for Peptides A-F. The mean of three measurements is plotted for each ratio (error bars are ± 1 sd). B The observed mean ratio for peptide B along with the CVs calculated for each ratio. Considering all peptides, 83% of CVs were <5%. Peptides which exhibited higher CVs were the more hydrophobic, lower intensity peptides (eg. H and J).

Conclusions

- A useful approach for peptide quantitation is combining MRM and peptide labeling (TMT). Using the structurally identical but isotopically different TMT's a reference can be included across validation experiments whilst maintaining the selectivity of MRMs.
- Excellent correlation between observed and expected ratios for most peptides. For all peptides quantitated 83% of CVs were <5%.
- Rapid transition into biomarker qualification assays from TMT-based discovery studies where LC, MS and MSMS characteristics of proteotypic peptides are already defined.
- Alleviates the need for immunoassay assay development and the production of heavy isotope-labeled peptides.
- Future experiments will focus on the validation of candidate biomarkers implicated in AD.

References

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