

Introduction

- The establishment of highly specific and sensitive validation assays is a key task to transfer candidate biomarkers into validation studies. In response to the limited availability of ELISA's, single and multiple reaction monitoring (SRM, MRM) is emerging as a highly selective and sensitive approach for the quantitation of proteins.¹
- Here we present a validation approach which combines the selectivity and sensitivity of MRM with isotopic peptide labeling (Tandem Mass Tags²). In this approach Tandem Mass Tag products are utilised, namely the structurally identical but isotopically different TMTzero (added mass per label: 224 Da) and TMTsixplex (added mass per label: 229 Da).

Methods

Two aliquots of a generic plasma sample 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

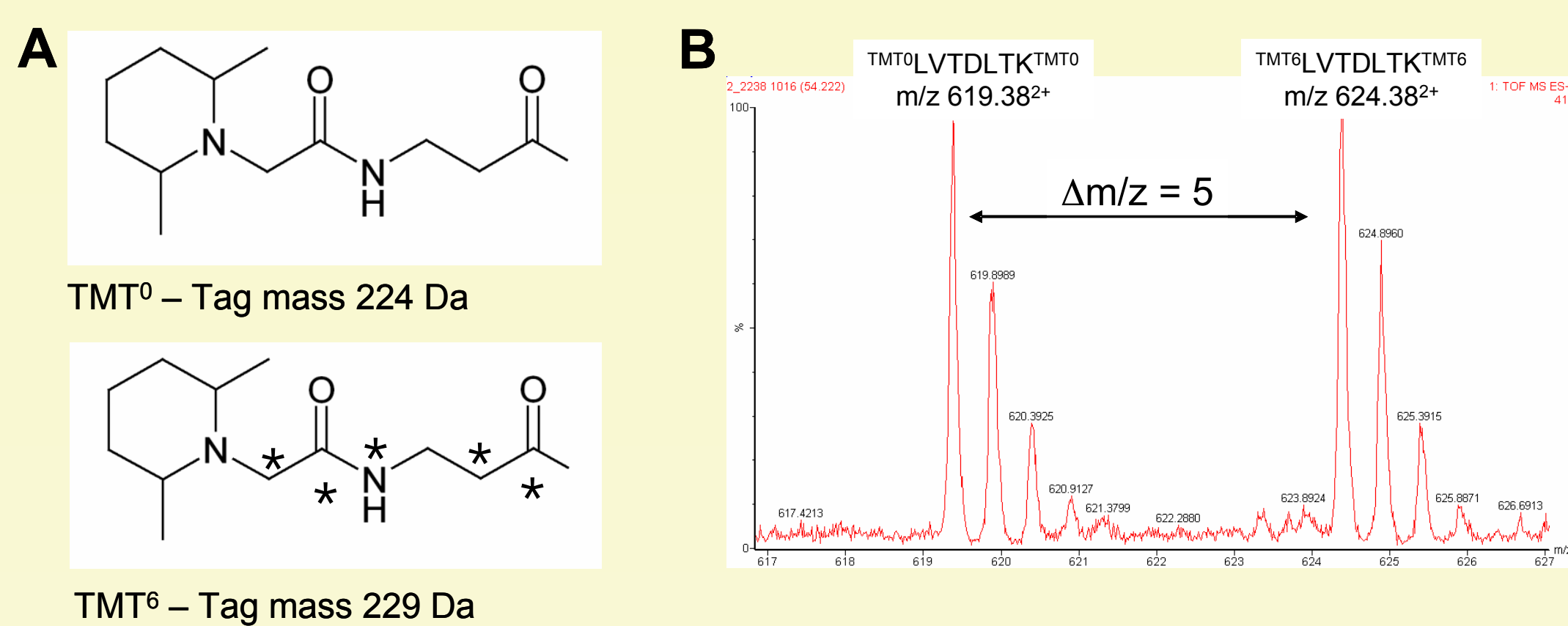


Figure 1 A Structure of TMT⁰ and TMT⁶-127. TMT⁶ has ¹³C and ¹⁵N isotopic labels (*). B MS spectrum of LVTDLTK labeled with TMT⁰ and TMT⁶-127. The peptide is labeled at the N-terminus and lysine giving a mass difference of 10 Da between the labeled peptides. The doubly charged precursor ions have a $\Delta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

	Peptide sequence	Charge state	RT mins	CE	TMT ⁰		TMT ⁶	
					Q1	Q3	Q1	Q3
A	TMTLVHVEEPTETVR	3	6.8	38	590.66	839.44 (y7)	592.33	839.44 (y7)
B	TMTLTVDKTMT	2	9.0	29	512.29	653.39 (b4)	517.29	658.39 (b4)
Ci	TMTDTLMISR	2	10.0	40	530.30	906.60(pseudo y)	532.80	909.60(pseudo y)
Cii	TMTDTLMISR	2	10.0	40	530.30	685.40 (b4)	532.80	690.40 (b4)
D	TMTILGGHLDAKTMT	3	10.5	32	457.94	670.42 (y4)	461.31	675.42 (y4)
E	TMTALPAPIEKMT	2	29.3	35	643.90	878.55 (y6)	648.90	883.55(y6)
F	TMTLVTDLTKTMT	2	31.9	34	619.38	585.40 (y3)	624.38	590.40 (y3)
Gi	TMTFQNALLR	2	13.9	43	592.90	1031.76(pseudo y)	595.40	1034.7(pseudo y)
Gii	TMTFQNALLR	2	13.9	43	592.90	798.50 (b5)	595.40	803.50 (b5)
H	TMTSASDLWDNLKTMT	2	34.1	46	849.47	598.40 (y3)	854.47	603.40 (y3)
li	TMTLVNEVTEFAKTMT	2	35.3	43	799.48	819.46 (y5)	804.48	824.46 (y5)
lii	TMTLVNEVTEFAKTMT	2	35.3	43	799.48	589.38 (y3)	804.48	594.38 (y3)
J	TMTSLSPYVIVPLKTMT	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).

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 TMT⁶ 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.

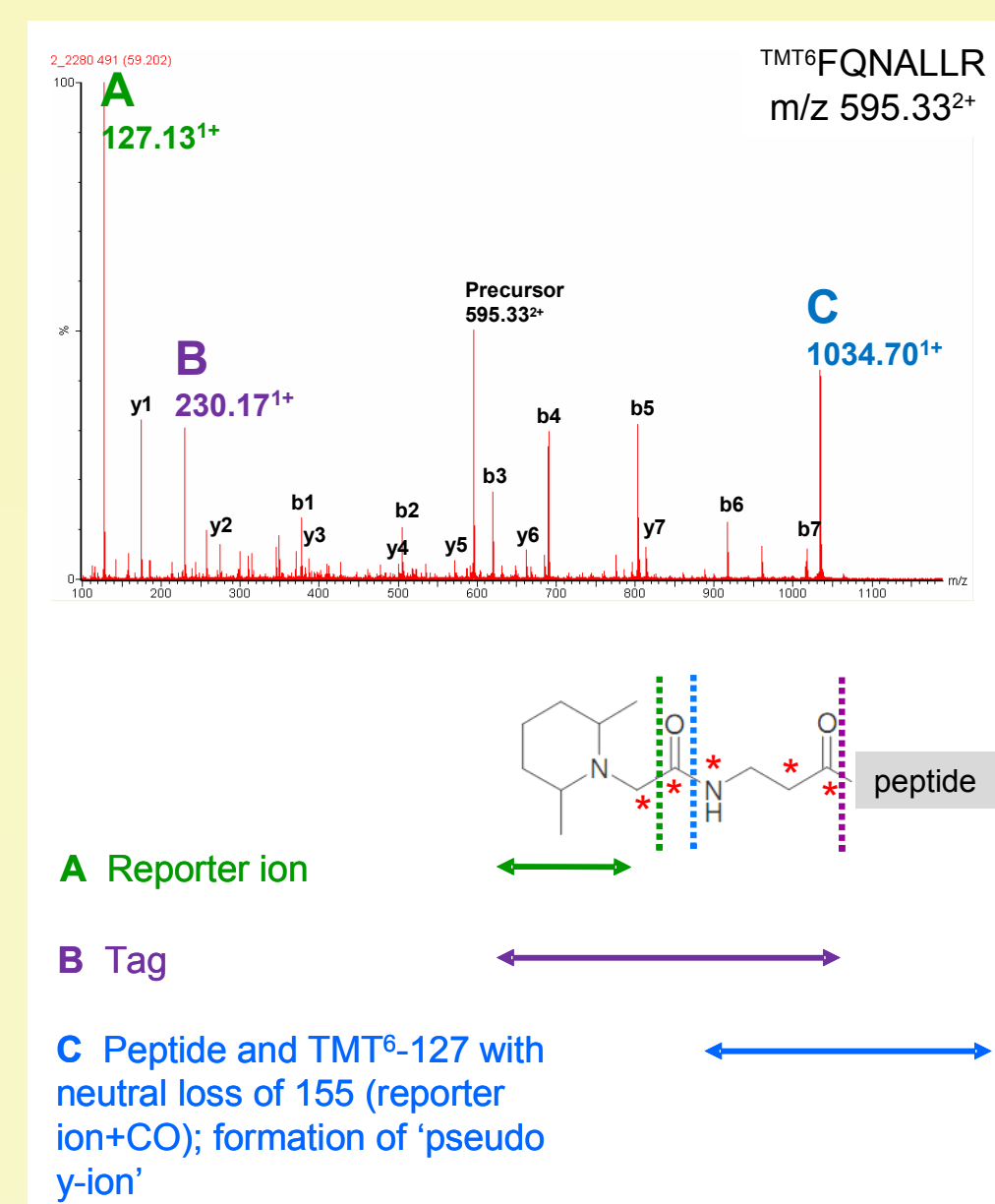


Figure 2 TMT MS/MS fragment ions. MS/MS spectra of FQNALLR (m/z 595.332⁺) labeled with TMT⁶-127. The fragment ions of TMT⁶-127 are highlighted. In addition to the reporter ion at m/z 127 the intact tag at m/z 230 is observed and an ion representing the intact peptide plus a remaining portion of the tag. 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 maybe compromised if an ion of the same m/z co-elutes.

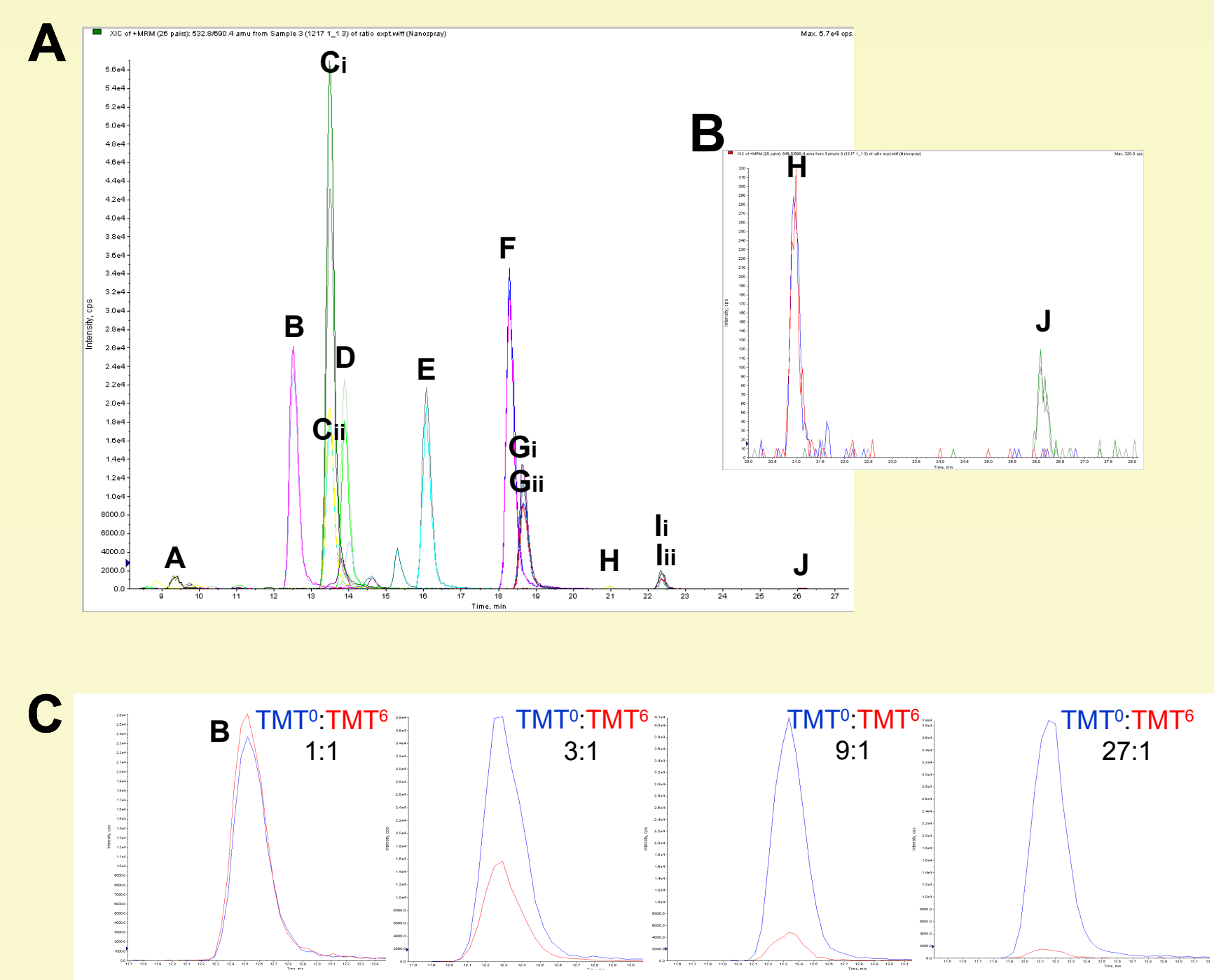


Figure 3 A MRM XIC for TMT⁰- and TMT⁶-labeled plasma peptides A-J. All transitions listed in Table 1 were assessed. It can be seen that TMT⁰- and TMT⁶-labeled peptide pairs co-elute. B MRM XIC showing the lower intensity peptides, H and J. C MRM XIC for Peptide B. TMT-labeled samples were mixed in 1:1,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

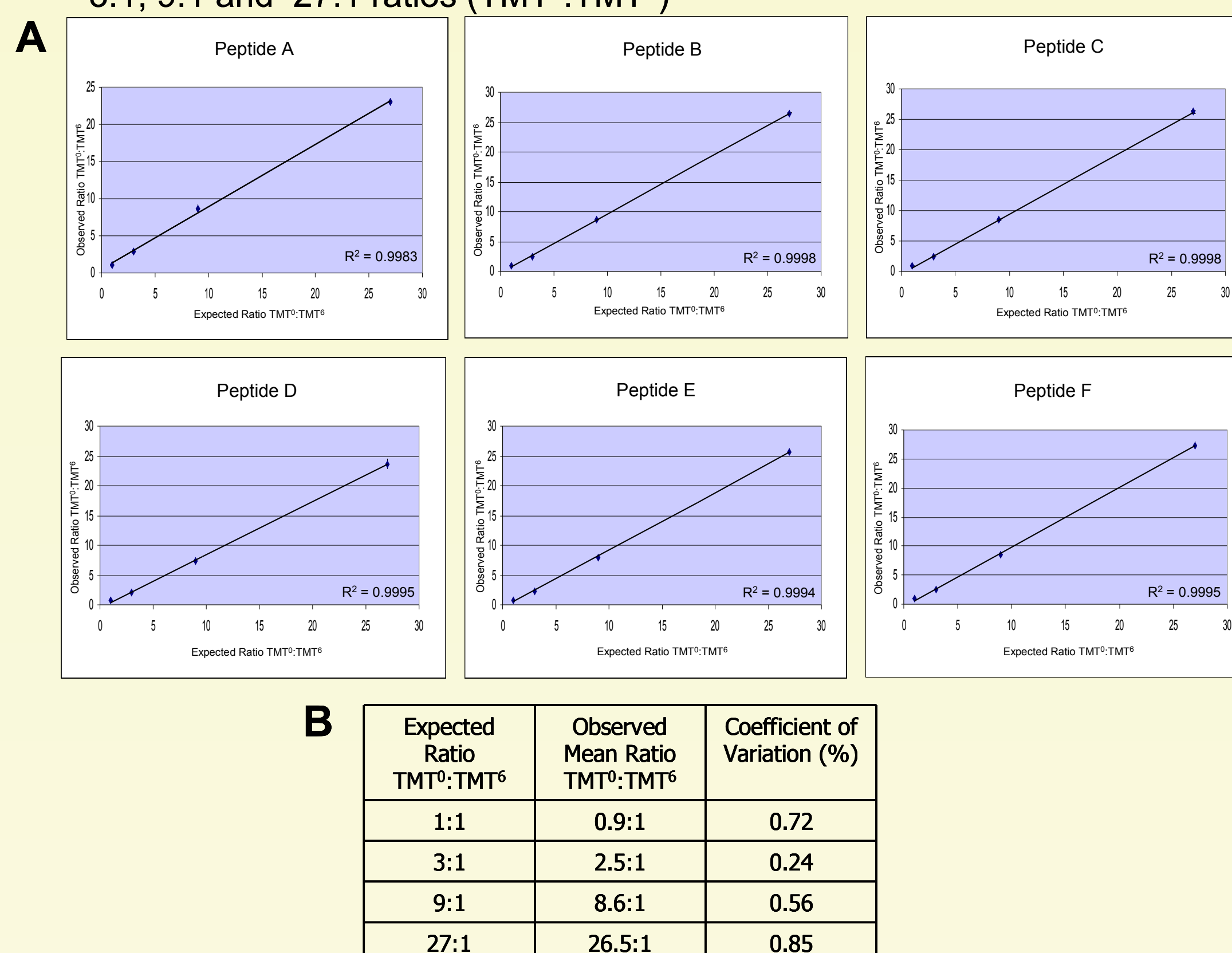


Figure 6 A Plots of 'observed vs expected' ratios for Peptides A-F. The mean of three measurements is plotted for each ratio (error bars are ± 1 sd). B The observed mean ratio for peptide B along with the CVs calculated for each ratio. Considering all peptides, 83% of CVs were <5%. Peptides which exhibited 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. Using the structurally identical but isotopically different TMT's a reference can be included across validation experiments whilst maintaining the selectivity of MRMs.
- Utilising TMT, integration between discovery and validation phases of biomarker development can now be achieved. In this workflow TMT-labeled proteotypic peptides are selected, which represent candidate biomarkers, identified as being differentially regulated by discovery phase. The elution characteristics and MS/MS properties of the target peptides are established during the discovery phase for streamlined transition into an MRM quantitation format.

References

- Anderson L. and Hunter CL. (2006) Quantitative mass spectrometric MRM assays for major plasma proteins. *Mol Cell Proteomics*, 5(4):573-88
- Dayon L. et al. (2008) Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. *Anal. Chem.*, 15;80(8):2921-31