Proteome Sciences

Candidate verification of iron-sensitive meningococcal proteins using TMT SRM

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Overview

- Isobaric TMT reagents are a suitable tool for discovery studies in biomarker studies or to identify candidates for therapeutic targets [1],[2].
- In combination with single reaction monitoring (SRM), an isotopic set of structurally identical TMT reagents can be utilised for the targeted verification of protein regulations from a discovery study.
- This new approach, TMT SRM, allows for a rational and time-saving transfer of discovery data into verification studies.

Introduction

- The effect of iron-depletion on the protein regulation of the Gram-negative bacterium *Neisseria meningitidis* was analysed by making use of the isobaric TMTduplex reagents[2]. This reflects the response of this opportunistic pathogen upon the iron-limited environment during human infection.
- Of the 35 proteins found to be regulated by iron-depletion in the discovery study, a selected set was chosen for verification by targeted SRM analyses. SRM has increased sensitivity and specificity in both identification and quantitation compared to intensity-dependant MS/MS acquisition as utilised in global discovery studies.
- For candidate verification samples were labeled with an isotopic set of TMT reagents being structurally similar to the TMTduplex reagents as used in the discovery study. Here LC, MS and MS/MS characteristics of the target analytes are maintained across discovery and verification phases of biomarker development. This allows both for a seamless setup of SRM experiments and for the usage of an internal reference sample.
- When using isobaric TMTduplex reagents in discovery studies, quantitation is achieved by comparison of the intensities of the m/z values of the reporter groups: 126.13 and 127.13 Da, respectively (Figure 1A).
- The two isotopic reagents light TMT and heavy TMT used in the verification phase have a mass difference of 5 Da. This mass difference gives minimal cross-contamination in the targeted SRM analyses (Figure 1B). As utilised in SRM experiments, quantitation is based on the use of structural b and/or y ions generated after collision-induced fragmentation, the tag functions here to introduce a mass difference into the peptides from different samples.

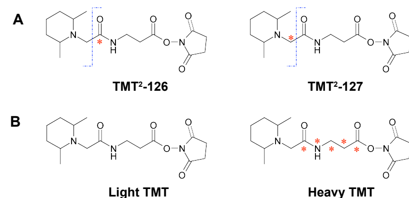


Figure 1 Structure of the different sets of TMT label reagents. A) The isobaric TMTduplex set. B) The isotopic set of light TMT and heavy TMT. Red asterisks mark ^{13}C and ^{15}N atoms. The blue dotted lines mark the fragmentation site to release the reporter ion upon MS/MS.

Methods

- *N. meningitidis* cells (strain MC58) from two independent growth experiments were cultured under iron-rich and iron-limited conditions. Proteins were extracted by ultrasonication, reduced, alkylated and digested in triplicate with trypsin using standard methods. Following digestion, equal aliquots of each of the 12 samples were combined to generate a reference sample. The reference sample was labeled with light TMT, the remaining aliquots of the experimental samples were labeled with heavy TMT (Figure 2A)
- Peptides of the candidate proteins were pre-selected for targeted analysis following identification in the TMT discovery study and if the following criteria was fulfilled: fully labeled with TMT and digested with trypsin, proteotypic and contained no known modifications (either *in vivo* or experimental). Knowledge of the LC, MS and MS/MS characteristics of the peptides further enabled the selection of the most suitable peptides for targeted quantitation. The final set of peptides taken forward for quantitative analysis were selected following assessment of SRM sensitivity and specificity for each target analyte by SRM-triggered MS/MS.
- Experimental and reference samples were mixed 1:1. SRM analyses were performed on a 4000 QTrap with on-line RP chromatography (Ultimate3000) with 1µg total protein load on column. Q1 and Q3 resolution was set to unit and default DP and CXP voltages applied. SRM scheduling (Analyst v1.5) enabled the measurement of several hundred transitions in one run.
- Following the exclusion of transitions that were affected by the background matrix (Figure 2B), peak areas were summed to give a total intensity for all light TMT and heavy TMT transitions for each peptide. The peak area for each experimental sample was measured relative to the peak area for the reference sample. These ratios for each peptide were averaged across analytical and technical repeats. Ratios of all peptides relating to a particular protein were then averaged. Finally, protein averages for each biological replicate were then expressed as the geometric mean ratio of Fe-:Fe+.

Results: *SRM set-up and method validation*

- Thirty seven peptides were taken forward for targeted quantitation (Figure 3) following an iterative selection process of the most suitable peptides/ transitions: a) candidate peptides selected from TMT discovery data that satisfied criteria for robust SRM quantitation b) sensitive and selective detection of each transition by SRM c) transitions that were unaffected by the background matrix (Figure 2B).

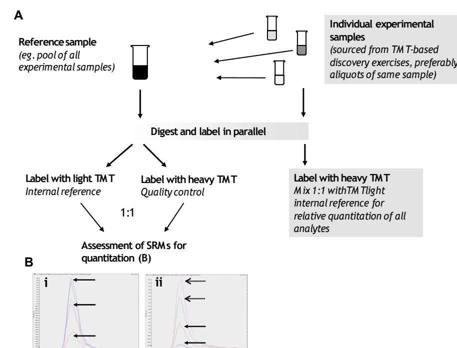


Figure 2 TMT SRM for the verification of iron-sensitive proteins. A) TMT labeling strategy for the experimental sample set and the generation of a biological reference for use as an internal standard and for the quality control of transitions suitable for quantitation. Bi) An example of a peptide where the ratios for all the paired transitions are equal, the three transitions pairs are indicated by a solid arrow. ii) An example of a peptide where one of the three transition pairs is not observed in a 1:1 ratio (dashed arrows). These transitions are likely to be affected by the background matrix where non-specific ions contribute to the signal. Such transitions are excluded from subsequent quantitative analysis.

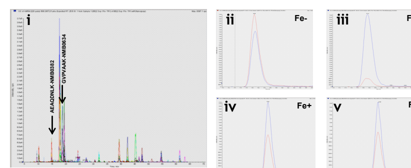
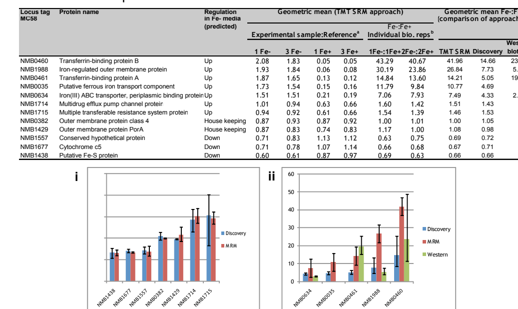


Figure 3 i) Extracted TMT SRM chromatogram for all 37 peptides. Three transition pairs per labeled peptide were measured making a total of 222 transitions measured. For peptide GVPVAAK (upregulated protein NMB0634) the TMT SRM transition pair Q1 550.35, Q3 714.50 (heavy TMT, experimental sample) and Q1 545.35, Q3 709.50 (light TMT, reference) has been extracted in both an Fe- (ii) and an Fe+ experimental sample (iii). The SRM for the experimental and reference samples are highlighted in red and blue respectively. The reference is constant in both samples, this peptide is shown to be upregulated in Fe- conditions. For peptide AEAQDNLK (housekeeping protein NMB0382) the TMT SRM transition pair Q1 673.88, Q3 744.37 (heavy TMT) and Q1 668.88, Q3 739.37 (light TMT) has been extracted in both an Fe- (iv) and Fe+ (v) conditions. This peptide is shown not to be regulated by iron availability.

Performance of TMT SRM

- In the discovery study proteins could be identified and quantitated only following cellular/SCX fractionation. All target peptides were verified by TMT SRM in parallel using cell lysate without application of SCX chromatography. This demonstrates the sensitivity of the approach.
- Protein regulations were consistant across TMT SRM and TMTduplex discovery studies (Figure 4). Western blot data independently confirmed the TMT-based quantitation results.



Locus tag MC58	Protein name	Regulation in Fe- media (predicted)	Geometric mean (TMT SRM approach) Experimental sample:Reference[a]				Fe-:Fe+ Individual bio. reps[b]		Geometric mean Fe-:Fe+ (comparison of approaches[c])		
			1 Fe-	3 Fe-	1 Fe+	3 Fe+	1Fe-:1Fe+	2Fe-:2Fe+	TMT SRM	Discovery	Western blotting
NMB0460	Transferrin-binding protein B	Up	2.08	1.83	0.05	0.05	43.29	40.67	41.96	14.66	23.54
NMB1988	Iron-regulated outer membrane protein	Up	1.93	1.84	0.06	0.08	30.19	23.86	26.84	7.73	5.40
NMB0461	Transferrin-binding protein A	Up	1.87	1.65	0.13	0.12	14.84	13.60	14.21	5.05	19.59
NMB0035	Putative ferrous iron transport component	Up	1.73	1.54	0.15	0.16	11.79	9.84	10.77	4.69	
NMB0634	Iron(III) ABC transporter, periplasmic binding protein	Up	1.51	1.51	0.21	0.19	7.06	7.93	7.49	4.33	2.89
NMB1714	Multidrug efflux pump channel protein	Up	1.01	0.94	0.63	0.66	1.60	1.42	1.51	1.43	
NMB1715	Multiple transferable resistance system protein	Up	0.94	0.92	0.61	0.66	1.54	1.39	1.46	1.53	
NMB0382	Outer membrane protein class 4	House keeping	0.87	0.93	0.87	0.92	1.00	1.01	1.00	1.05	
NMB1429	Outer membrane protein PorA	House keeping	0.87	0.83	0.74	0.83	1.17	1.00	1.08	0.98	
NMB1557	Conserved hypothetical protein	Down	0.71	0.83	1.13	1.12	0.63	0.75	0.69	0.72	
NMB1677	Cytochrome c5	Down	0.71	0.78	1.07	1.14	0.66	0.68	0.67	0.71	
NMB1438	Putative Fe-S protein	Down	0.60	0.61	0.87	0.97	0.69	0.63	0.66	0.66	

Figure 4 Comparison of mean protein regulations in Fe- media between TMTduplex discovery, western blot and TMT SRM verification experiments. The table displays: [a] Means of heavy TMT (expt.):light TMT (ref.) at the protein level for each biological replicate under Fe+ and Fe- conditions, [b] Means of Fe-:Fe+ for each biological replicate, [c] Means of Fe-:Fe+ across the biological replicates for each quantitation approach. For the discovery and western blot data mean values are based on three biological replicates. i) Proteins with regulations between the Fe- and Fe + cultures of close to 1:1 (housekeeping proteins) and slightly up- and down-regulated ones. ii) Proteins strongly upregulated under Fe- conditions. Error bars represent the 95% confidence limits for the mean values across the biological replicates for each approach.

Conclusions

- Using isobaric and isotopic sets of structurally identical TMT reagents, we identified both well-known and previously unreported regulations of iron-modulated proteins in a global discovery study and verified these regulations in subsequent, SRM-based verification studies. This combined workflow, applied to the identification of iron-sensitive meningococci proteins has the potential to find new anti-microbial targets.
- Further, this rational usage of TMT allows for a seamless and time-saving linkage between the early stages of biomarker development.

References

[1] Dayon, L. *et al.*, (2008) Relative quantification of proteins in human CSF by MS/MS using 6-plex isobaric tags. *Anal. Chem.*, 80: 2921-31.

[2] van Ulsen P., et al., (2009) Identification of proteins of *N. meningitidis* induced under iron-limiting conditions using the isobaric tandem mass tag (TMT) labeling approach. *Proteomics*, 9: 1771-81.