

Proteome Sciences

An update on CSF Tau for the GBSC Workgroup



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Chief Technology Officer
4th October 2012

- What forms of Tau do we need to measure in CSF?
- Total Tau?
- Specific phosphosites?
- Truncation products?
- Other PTM's?

- Over 30 pathological phosphorylation sites known in brain
- Glycosylation is increasingly recognised as an important Tau PTM
- The lack of antibodies with required site specificity will make multiplex immunoassay development difficult
- Mass spectrometry may provide an efficient and cost-effective alternative

Characterisation of Tau and phospho-Tau populations within cerebrospinal fluid - The relevance to Alzheimer's Disease biomarker development

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Introduction

We first developed Phospho-Tau SRM to quantify Tau phosphorylation sites in preclinical material. We currently endeavor to extend these measurements to peripheral fluids such as CSF and blood. Adapting the Phospho-Tau SRM approach towards the analysis of such clinical material is attractive because other phosphorylated epitopes of CSF Tau may provide further informative biomarker utility. Here we report the findings of a two-dimensional gel electrophoresis and Western blotting study to analyse the population of various forms of Tau within CSF. We also describe further characterisation of CSF Tau and phospho-Tau populations using mass spectrometry and the insights gained to guide the set up and utility of the Phospho-Tau SRM method for CSF Tau.

Tau, Phospho-Tau and Alzheimer's Disease

A pathological feature of AD is the accumulation in brain neurons of hyperphosphorylated Tau. This results in its dissociation from microtubules and polymerisation into tangles of paired helical filaments (PHF). These aggregates are responsible for neuronal cell death (Figure 1, Panel A).

Currently there are two Tau-related biomarkers that have been shown to be elevated in human CSF as a marker of AD disease progression, namely total Tau (C-Tau) and phospho-Tau (P-Tau) (Figure 1, Panel B, courtesy of Jack et al. 2010).

Phospho-Tau SRM Assays have been developed to quantify Tau phosphorylation at multiple key sites within the protein. These assays are currently utilised to analyse pre-clinical material to support Tau directed drug development programmes (Lippner et al. 2012).

However, Tau analysis in the brain are not necessarily the same as those found in the peripheral fluids such as CSF and plasma and this presents significant challenges (Figure 1, Panel C, taken from Ward et al. 2009).

Hence, a detailed characterisation of the Tau molecules present in CSF would provide valuable insights to guide the set up and utility of the Phospho-Tau SRM methods for the analysis of clinical CSF specimens.

Methods

For two-dimensional gel electrophoresis, a pooled CSF sample (Serabab Laboratories International Ltd (UK), depleted of abundant proteins (DMS103) depletion column, Agilent), was prepared as previously described by Hanisch et al., 2010, with modifications. Two-dimensional gel electrophoresis and Western blotting was performed using pH 5-10 non linear immobilized gradient for first dimension and 10% SDS-PAGE for the second dimension separation. Western blotting was performed using a selection of antibodies to highlight particular epitopes.

To increase Tau peptide recovery from CSF samples, for characterisation by mass spectrometry, CSF (fmL) aliquots were precipitated with 2.5% perchloric acid prior to immunoprecipitation with BT2 as described by Podleski et al., 2008, with minor modifications. Immunoprecipitated proteins were eluted in Laemmli sample buffer and concentrated on a spinning disk prior to tryptic digestion. The overnight stained protein bands of interest were reduced, alkylated and digested in-gel with trypsin prior to analysis by MS. Samples were either analysed via LC-MS/MS using a Tryptic collision induced dissociation (CID) method (Orbitrap-Volvo, ThermoFisher Scientific) or a LC-SRM (ESI-Vantage, ThermoFisher Scientific). In both cases peptides were resolved over a 2hr LC gradient using a Proxeon nanoLC (ThermoFisher Scientific).

Results & Discussion

Two-dimensional gel electrophoresis and Western blotting highlights numerous features, with distinct isoelectric points and molecular weights (Figure 2). Interestingly, low molecular weight species are detected in CSF and at least one of these molecules is found to originate from the central region of the Tau sequence. A C-terminal fragment of Tau encompassing the full G4181 epitope is also observed. Efforts to obtain mass spectrometry data involved 'on-bead' tryptic digestion of the material within each spot. To date, the MS analysis of the individual 2DE spots did not reveal any Tau peptides and further experiments are required to confirm the presence of Tau and to provide further annotation of the individual Tau moieties in CSF.

Mass spectrometry experiments involved the analysis of CSF aliquots (100) precipitated with 2.5% perchloric acid prior to an IP with SpA-BT2. A single phosphorylated Tau peptide (TPSLPFFPR, Figure 3, Panel A) was identified when approximately half of the digested, immunoprecipitated sample (equivalent to 500µL) was analysed by LC-MS/MS (Orbitrap-Volvo mass spectrometer) using a Top-10, 10 min, data dependent method. The remaining half of the sample was also analysed (LC-MS/MS) but the time saving an include list of sequenced and known tryptic Tau peptides and phosphopeptides, to maximise the opportunity of detecting Tau peptides. The same single peptide (TPSLPFFPR) was identified. The MS/MS spectra matched the theoretical spectra within our database with an increased peak and, since no other peptides were detected, this peptide is likely to be the most abundant Tau epitope available for mass spectrometry analysis within the hospital remnant CSF sample analysed. Clearly however, the current data fully representative of endogenous Tau population in CSF. No phosphorylated Tau peptides were detected.

The sample preparation was repeated, again using 1mL Serabab hospital remnant CSF. The whole sample was loaded onto the Orbitrap-Volvo for analysis. Two tryptic peptides were detected as shown (Figure 3, Panel B and C). Interestingly, the peptide STPTAEADVYAPLDEGAPDK is specific to the 1N Tau isoform.

In order to further explore the possibility of detecting phosphorylated Tau peptides, Serabab hospital remnant CSF was prepared as described above and analysed using an LC-SRM method, monitoring for known Tau peptides and phosphopeptides previously observed in our in-house Tau databases. These Tau peptides, STPTAEADVYAPLDEGAPDK, IOSTENLK and SPVAVGDTSPR were detected (Figure 4). No evidence of phosphorylation was seen in this control sample.

Results present here demonstrate that Tau is detectable by mass spectrometry from as little as 1mL hospital remnant CSF. However, further improvements in sample preparation are required to fully characterise the nature of Tau in CSF from control and Alzheimer Disease patients.

Figure 2: 2DE Western Blot probed with three distinct anti-Tau antibodies:
 A) BT2 mAb (MN1010, Thermo)
 B) HTT mAb (MN1000, Thermo)
 C) DKO 204 antibody (100:100) against C-terminal epitope in Tau

Figure 3: LC/MS/MS detection of Tau peptides using the Orbitrap-Volvo.
 A) TPSLPFFPR B) STPTAEADVYAPLDEGAPDK C) IOSTENLK

Figure 4: SRM spectra of three endogenous Tau peptides enriched from 1mL Serabab hospital remnant CSF and analysed using the TSQ Vantage.

Conclusions

- Multiple forms of Tau are present in hospital remnant CSF.
- Tau peptides can be detected by Mass Spectrometry from as little as 1mL CSF.
- No phospho-peptides were identified in this study.
- Further improvements in sample preparation are required to fully characterise the nature of Tau in control and Alzheimer Disease CSF.

References

Hanisch et al., J Proteome Res 2010; 9: 1476-1482.
 Jack et al., JAMA 2010; 303: 1138-1147.
 Lippner et al., J Biol Chem 2012; 287: 1162-1170.
 Podleski et al., J Proteome Res 2008; 7: 2174-2177.
 Ward et al., Mol Cell Proteomics 2009; 8: 1914-1924.

- Proteome Sciences has developed several MS methods for quantitation of Tau and site-specific phosphorylation
- Tau phosphorylation 6plex – AQUA SRM with tryptic digestion for human/murine Tau in brain tissue and neuronal cell culture
- Tau phosphorylation 7plex – AQUA SRM with AspN digestion for R406W mutant human Tau in brain tissue and neuronal cell culture
- TMTcalibrator – isobarically labelled multi-point peptide standards and MS3 for highest sensitivity and precision



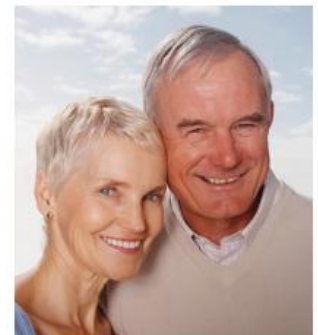
Tau Phosphoprotein Assays for Tauopathies & Alzheimer's Disease

Measure multiple phospho-Tau sites simultaneously

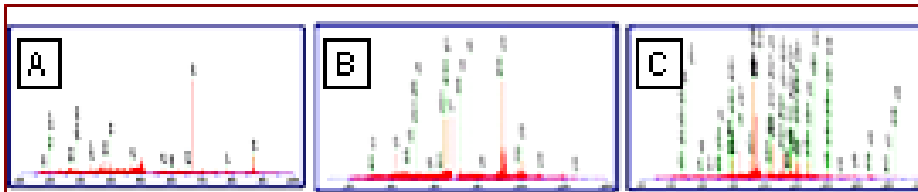
Proteome Sciences offers unique phospho-Tau biomarker panels for simultaneous mass spectrometry measurement of total Tau protein and 6 specific phosphorylation sites that have been strongly linked with Tau pathology. Both human and murine Tau isoforms may be analyzed from either transgenic murine brain tissue or human and murine cultured cells. The assay is available in 2 versions to enable measurement of phospho-Tau for different Tau transgene variants.

Tau Phosphorylation 6-plex
Most commonly used Tau transgenic variants

Tau R406W Phosphorylation 7-plex
Use with mutant Tau R406W transgene



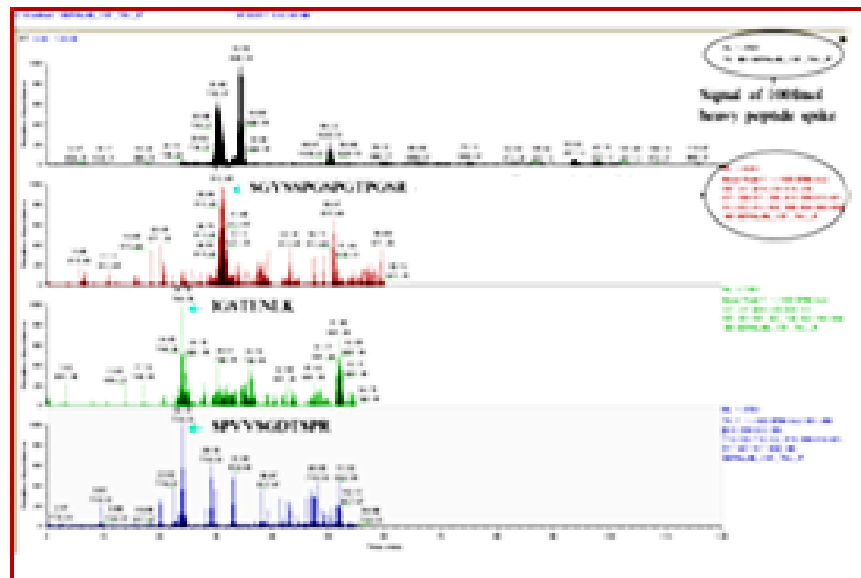
Detection of endogenous CSF Tau by MS



Orbitrap Velos

Figure 3: LC/MS/MS detection of Tau peptides using the Orbitrap

A. TPSLPTPPTR B. SGYSSPGSPGTPGSR
C. STPTAEAE EAGIGDTPSLEDEAAGHVTQAR



TSQ Vantage

Figure 4: SRM spectra of three endogenous Tau peptides enriched from 1mL Seralab hospital remnant CSF and analysed using the TSQ Vantage

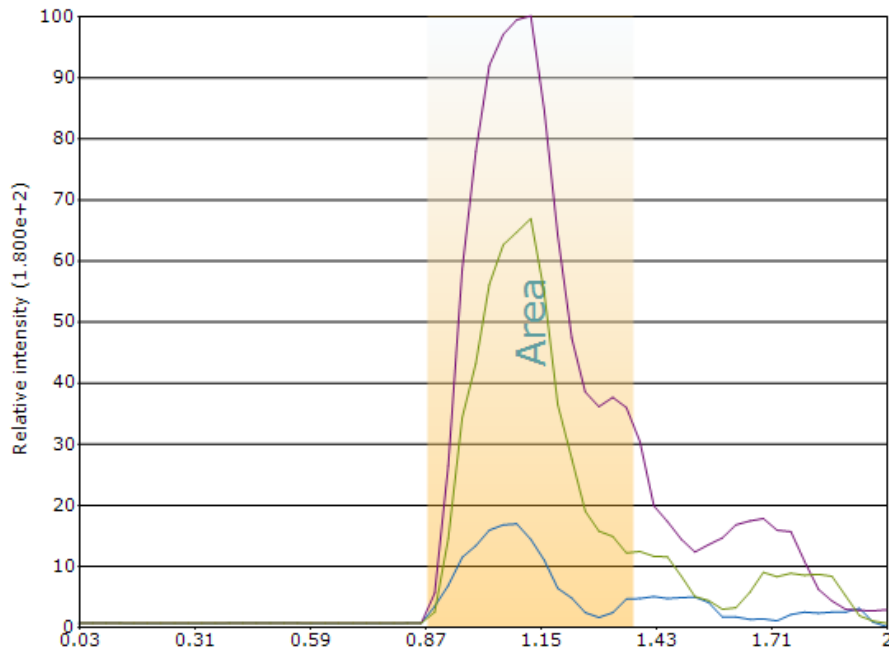
Starting
volume
of CSF

= 1ml

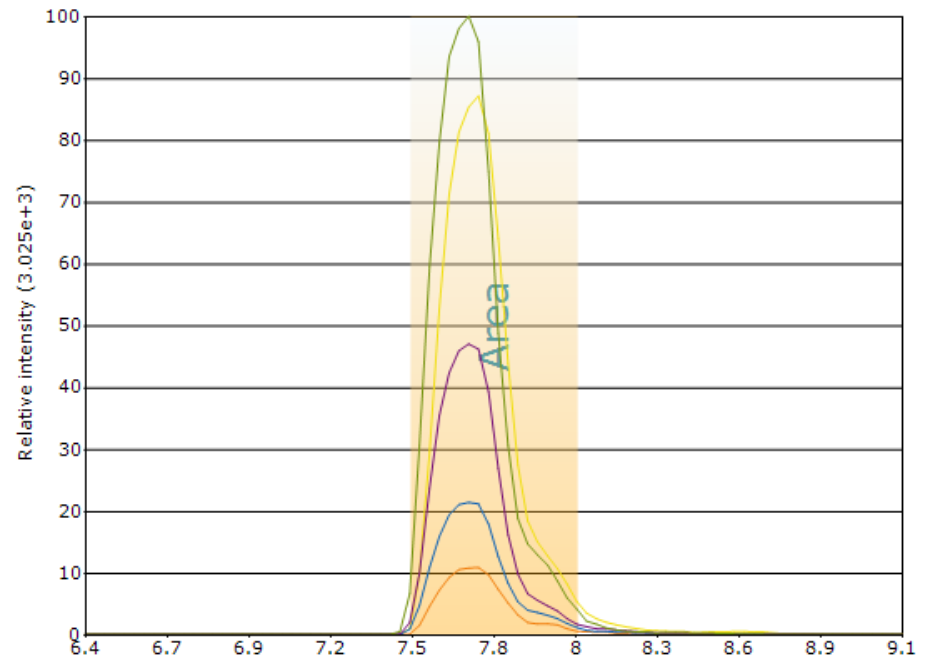
- **Development of Quantitative CSF Tau MS assays using synthetic reference peptides**
- **First needed to improve the analytical performance of the Total Tau surrogate peptide**
 - Retention time
 - Sensitivity
 - Precision
- **Combine the use of TMTcalibrator and MS3**
 - Trigger initiated MS/MS and MS3 acquisitions
 - Reduce/eliminate interference from background

Comparison of the elution times of 100fmol total peptide analysed by SRM after micro flow (100µL/min) rate chromatography (TSQ Vantage)

Unlabeled Total Tau peptide elutes at 0.87 mins



TMT labeled Total Tau peptide elutes at 7.5 mins

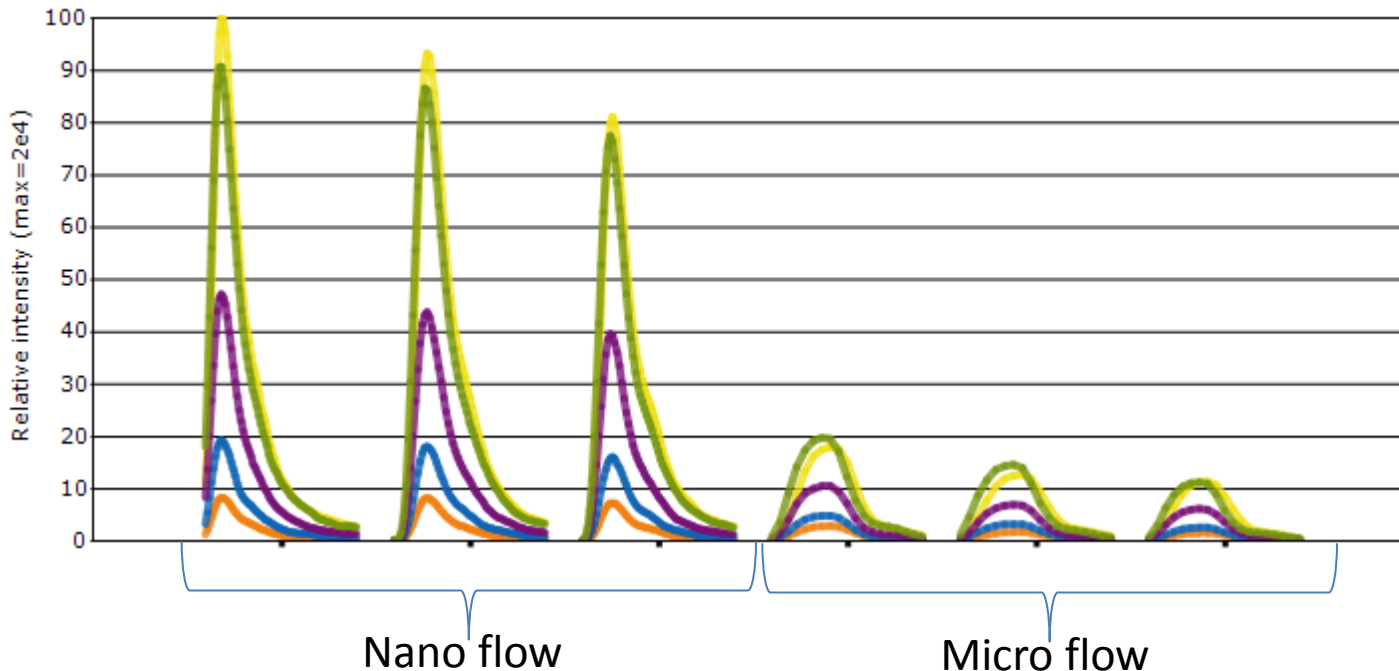


TMT labeling improves retention of Total Tau Surrogate

TMT labelled Total Tau peptide

Moving from micro flow (100µL/min) rate to nano flow rate (300nL/min) improves sensitivity and precision

	Nano	Micro
Amount	25 fm	100 fm
Average signal intensity	5.5E4	1.65E4
Flow rate	300 nL/min	100 µL/min
Average total file area	2.01E6	1.394E5
CV	10%	23%
Retention time	17.63 min	7.67 min

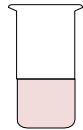


TSQ Vantage

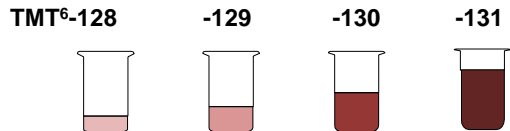
- We have seen endogenous Tau peptides in 1ml CSF run on Orbitrap (LC/MS/MS) and TSQ Vantage (SRM)
- We can accurately quantify total Tau and several phosphosites in brain tissue by AQUA-SRM
- The AQUA-SRM method may be applicable to CSF but concerns regarding robustness and overall sensitivity for phosphopeptides
- A more sensitive and comprehensive method is desirable
- TMTcalibrator with MS3 quantitation run on the Orbitrap has the potential to reach required levels of sensitivity and can theoretically cover all relevant species and PTM's

TMT Calibrator: Multipoint Calibration

Reference sample



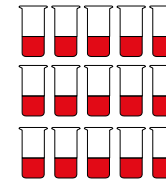
Reduce/alkylate
Digest
Split 1:2:4:8 ratio



Label with TMT⁶-128 to -131 to generate TMT Calibrator

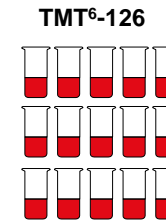


Mix & analyse



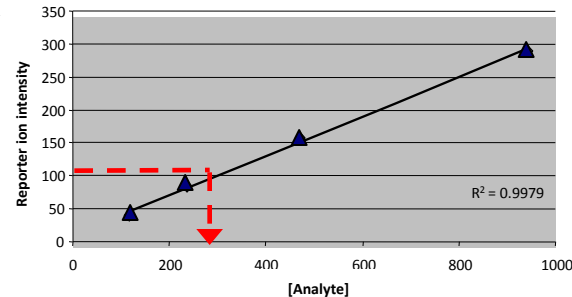
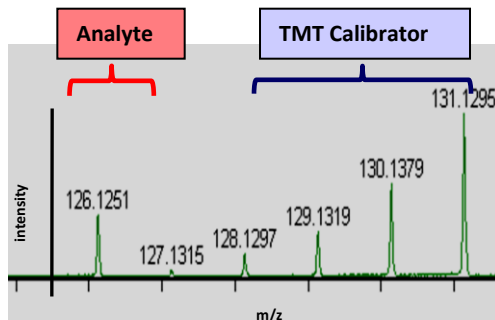
Individual experimental samples (analyte)

Reduce/alkylate
Digest



Label with TMT⁶-126

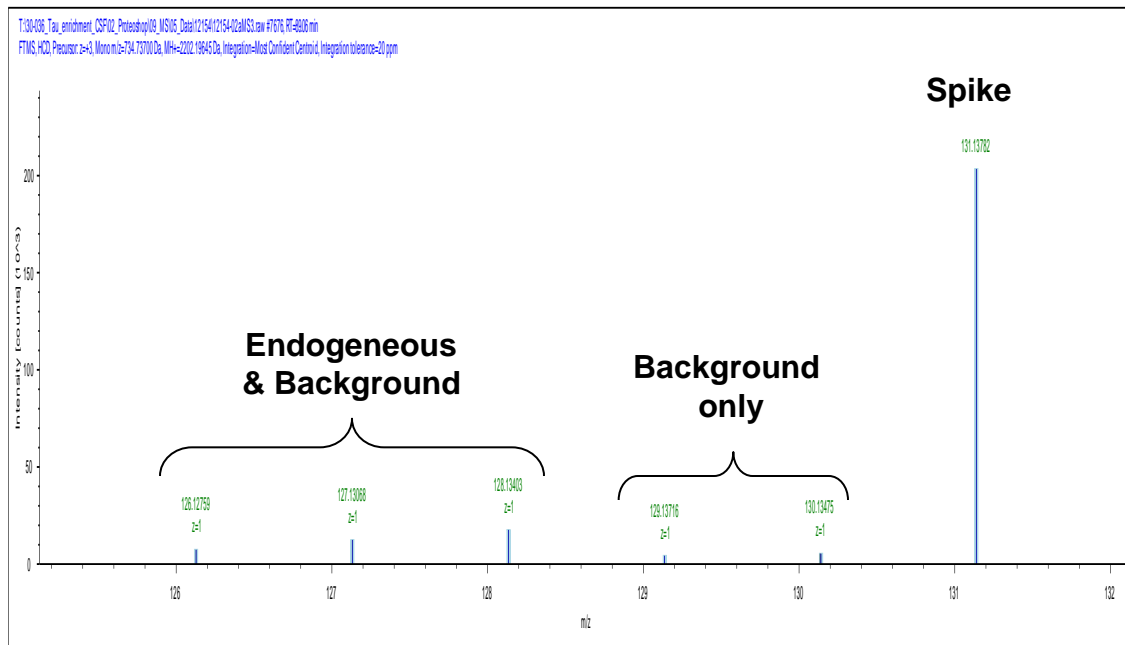
Four point calibration curve generated from TMT Calibrator reporter ion intensities



- Analysis of TMT-labelled synthetic Tau reference peptides and phosphopeptides for method development
 - Confirm detection of phosphorylated tau peptides in buffer
 - Creation of inclusion list
- Analysis of CSF spiked with TMT-labelled synthetic Tau reference peptides and phosphopeptides
 - Use of spiked peptide and include list ensures precursor ions are amongst top 10 selected for MS/MS
 - For PoC study endogenous Tau peptides identified in same MS/MS spectrum as equivalent reference peptide spiked in at high concentration (2ng/ml)
 - Potential influence of contaminating peptides in MS/MS generating TMT reporter ions – switch to MS3 method

TMT⁶-131 labelled Tau peptides spiked into CSF MS3 results

- 14 Phosphopeptides
- 10 unphosphorylated peptides



100ul CSF

- Low abundance of Tau in CSF requires enrichment prior to MS
 - Chromatography with depletion column
 - Perchloric acid precipitation*
- For ultra-sensitive detection use trigger concept
 - Isotopic or isobaric peptide spiking at high concentration
 - Using TMTcalibrator can make spike from multi-point standard curve
- Must use MS3 quantitation to ensure precision for ultra-low abundance species due to contaminant peptide co-isolation

*Portelius et al. 2008 J. Proteome Res. 7, 2114–2120

- We have demonstrated multiple isoforms of Tau in CSF
- Three unmodified peptides seen from 1ml CSF using Orbitrap
- SRM methods lack sufficient sensitivity for robust Tau quantitation in CSF
- TMTcalibrator has potential to detect and quantify multiple Tau phosphorylation sites and total Tau to deliver broadest diagnostic potential

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