**Improved PhosphoTau SRM assay sensitivity enables multi-site Tau phosphorylation quantitation in a preclinical model of AD treated with novel small molecule inhibitors of Casein Kinase 1 delta**

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**Introduction**

The Phospho-Tau SRM 6plex and 7plex assays are fit-for-purpose mass spectrometry assays that enable multiplexed site-specific quantitation of total Tau levels and five and six phosphorylation sites respectively in preclinical models of AD.1-5 (Figure 1 and Tables 1-4). In their original assay format, prior to SRM analysis, tau peptides and phosphopeptides were resolved by microflow (100μL/min) reversed phase (RP) chromatography. The linear working range of the microflow-Phospho-Tau SRM 6plex and 7plex assays was 5-1000mM on column (g%), with CVs ranging from 5-20%. Analysis up to 10μg total protein per sample was consummated. Herein we describe how miniaturisation of the Phospho-Tau SRM assays to nanoflow (300nL/min) improved assay sensitivity by up to two orders of magnitude as well as reducing the amount of sample consumed per analysis five fold. The utility of the nanoflow-Phospho-Tau SRM assays, both preclinical and downstream phosphorylation events, will be demonstrated in a tau transgenic model mouse treated with novel Casein Kinase 1 delta inhibitors.

**Methods**

Phospho-Tau SRM 6plex and 7plex assay microflow and nanoflow assay

The Phospho-Tau SRM 6plex and 7plex assays quantify site specific tau phosphorylation levels in pre-clinical material using a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific). Prior to SRM analysis which monitored up to three SRM transitions per analyte, phosphopeptides and pre-clinical samples were resolved by RP-chromatography (XBridge C18 3.5μm 1 x 100mm, Waters) over a 9 minute gradient 0-30% ACN flowing at 100μL/min (buffer A, 0.1% TFA; buffer B, ACN, 0.1% TFA). Time-aligned SRM peaks were visualised, verified and the endogenous analyte levels calculated using Peakview as described above and endogenous analyte levels reported as pg amount of peptide/phosphateopeptide in 2μg. Statistical analysis was applied to the dataset to establish any statistically significant differences between analytical and biological replicates.

**Results**

**Assessment of assay performance characteristics**

- Nanoflow Phospho-Tau SRM 6plex and 7plex assay calibration curves showed consistent improvements in sensitivity across all analytes within each assay of up to two orders of magnitude.
- The majority of analytes measured in the two assay versions achieved a lower LOD of less than 40pg on column.
- QC samples acquired at regular intervals during the acquisition of the sample cohort typically achieve CV values less than 10% for the majority of analytes in both the 6plex and 7plex assays.

**Assessment of performance characteristics**

- 16 point calibration curve (in buffer) for microflow assay 0.25-1000 fmol heavy peptide spiked with 100 fmol heavy peptide in triplicate.
- 15 point calibration curves (in buffer) for nanoflow assay 0.05-150 fmol heavy peptide spiked with 15 fmol heavy peptide in triplicate.
- A quality control (QC) containing a 1:1 mix of light and heavy peptides (n=5) was used to monitor assay performance on a routine basis.
- Analytical Limits of Detection (LOD) and Limits of Quantitation (LOQ) were determined by calculating the light peptide signal in a buffer sample containing only heavy peptides.
- LOD = average light signal of n=10 x 3 x STDev
- LOQ = average light signal of n=10 x 10 x STDev

**Application of the Phospho-Tau SRM 6plex and 7plex nanoflow assays to the measurement of phosphorylated tau levels in an AD mouse model treated with novel Casein Kinase 1 delta inhibitors**

Cortex and hippocampal tissue dissected from TMT mice that had been treated for 8 weeks with either vehicle, PS278 & PS110 (n=8 per treatment group) were homogenised at 100 mg/mL in a TIR buffer containing 1mM EGTA, 1mM PMSF, 10mM Naf, 1mM Na2VO3 and Roche Protease inhibitor cocktail. The sample lysate was then partitioned into four fractions (F1 = soluble Tau, F2 = heat treated Tau, F3 = Sarkosyl soluble Tau; F4 = Sarkosyl insoluble Tau; Figure 2) to provide an analysis of distinct populations of phosphorylated Tau. Following fractonation the total protein concentration for each fraction was determined by Bicinchoninic acid and 10μg total protein digested in gel using either Trypsin or AspN according to the standard operating procedures (SOP) for Phospho-Tau SRM 6plex and 7plex assays. Each sample was analysed in triplicate with 2μg total protein per sample loaded on column i.e. n=3 analytical replicates. Samples were randomised for analysis and analysed with the Phospho-Tau SRM 6plex and 7plex assays according to the SOP. Time-aligned SRM peaks were visualised and the endogenous analyte levels calculated using Peakview as described above and endogenous analyte levels reported as pg amount of peptide/phosphateopeptide in 2μg. Statistical analysis was then performed on the dataset to establish any statistically significant differences between analytical and biological replicates.

**Conclusions**

**The utilisation of Nanoflow RP chromatography improved sensitivity of the Phospho-Tau SRM 6plex and 7plex assays by up to 2 order of magnitude.**

**Low picogram tau levels can be detected in as little as 2μg total protein.**

**No statistically significant differences were observed between analytical replicates (n=3) of the same sample**

**Nanoflow chromatography enabled phosphorylation sites that are only four residues apart to be partially resolved and individually quantified.**

**References**

3. Application of Phospho-Tau SRM assays to the measurement of soluble tau (pT) phosphorylation levels in a mouse model of AD treated with novel Casein Kinase 1 delta inhibitors. JIR 13 844-854

**Figure 1: The Phospho-Tau SRM Assay Workflow**

**Figure 2: Analyse measurement in the Phospho-Tau SRM Assay**

**Figure 3: Comparison of Microflow (black line) and Nanoflow (red line) Calibration Curves for Phospho-Tau (4S) and (5T) 1**

**Figure 4: Treatment with novel casein kinase 1 delta inhibitors regulates the overall phosphorylation levels in a tau transgenic mouse model.**

**Figure 5: Specific time-aligned SRM transitions for pSer46 and pThr 50.** Using microflow chromatography the two singly phosphorylated peptides elute as one peak (A), however in nanoflow chromatography the two singly phosphorylated peptides partially resolve to enable quantification of each peptide separately (B-D). Panel D represents the Y3 SRM transition which is common to both pSer46 and pThr 50. Whereas panels C and D show SRM transitions that are specific phosphorylation at pSer46 and pThr 50 respectively.