Designing targeted mass spectrometry assays for membrane proteins in complex matrices using a peptide immuno-enrichment strategy

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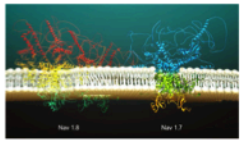
1 – Introduction

a/ Background

- The quantitative measurement of targeted protein(s) in treated tissue provides essential information to gauge effectiveness of a new treatment, especially if a new modality is employed for the first time.
- Targeted mass spectrometric assays are well positioned to deliver initial quantitative data as specific immuno-directed protein reagents may not be readily available or may be too costly to develop for early investigations.



b/ The target

- SCN9A (NAV1.7), a member of the multi-pass transmembrane voltage-gated sodium channel receptor family, has been shown to modulate pain in peripheral nerves, in particular, in dorsal root ganglions (DRGs)[1].
- Initial PRM-MS-based assays were developed to monitor protein knock-down of the two channel receptors SCN9A and SCN10A after administration of a SCN9A-specific LNA. However, protein quantification remained below LLOQ in DRG.

Fig. 1 ***Schematic representation of the NAV1.8 (SCN10A) and NAV1.7 (SCN9A) integral sodium channel 24-pass transmembrane proteins embedded in the membrane bilayer of a neuronal cell.***

c/ Assay development and context of use

- Request for an immune-enrichment targeted MS assay to **quantify SCN9A in bulk DRG tissue** collected in a dose-range finding study. The data would be **supportive** of **functional target engagement** if protein knock-down is demonstrated.
- The described mass spectrometric-based assay development included the following steps:

1) **Build assay and define assay conditions** for optimal immune-enrichment and measurement of SCN9A (the target), SCN10A (a non-targeted related protein) and TFR (a potential protein normalizator) peptides. Selected peptides should be conserved across species.

2) **Establish LOD/LLOQ and linearity of assay** in assay-relevant tissue (among them, DRGs)

3) **Detect and quantify** SCN9A, SCN10A and TFR peptides in DRG samples collected in the dose-range finding study.

2 – Assay Analytical Design

Cell and tissue samples were largely processed according to published protocols.[2,3] The tryptic digestion was performed using an "Addition Only" protocol performed on an Agilent Bravo robot. Peptide enrichment (endogenous and spiked SIS) was achieved using specific rabbit monoclonal antibodies covalently cross-linked to magnetic beads. Peptides were chromatographed on an HALO Peptide-ES C18 column (2.7 µm Fused-Core® column, 0.3x50 mm; 5-min gradient; buffer A: 0.1 % formic acid/5% DMSO in water; buffer B: 90 % acetonitrile/5 % DMSO in 0.1 % formic acid in water) and analyzed using a Sciex QTRAP 6500 mass spectrometer. The peak areas were analyzed using MultiQuant™ software (Sciex) and/or Skyline. (https://skyline.ms/project/home/software/Skyline/begin.view).

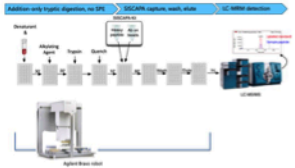


Fig. 2 ***Sample processing, immune-enrichment of selected peptides, and targeted MS measurement.***

References

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2. Whiteaker, J.R., Zhao, L., Anderson, L. *et al.* An automated and multiplexed method for high throughput peptide immunoaffinity enrichment and multiple reaction monitoring mass spectrometry-based quantification of protein biomarkers. *Molecular and Cellular Proteomics* **9,** 184–196 (2010) http://dx.doi.org/10.1074/mcp.M900254-MCP200
3. Razavi, M., Anderson, N.L., Pope, M.E. *et al.* High precision quantification of human plasma proteins using the automated SISCAPA immuno-MS workflow. *N. Biotechnol.* **33(5, pt A),** 494-502 (2016). https://doi.org/10.1016/j.nbt.2015.12.008.

3 – Initial characterization of the SCN9a/SCN10a anti-peptide antibodies

- 30 µL of a pooled DRG lysate (approx. 90 µg total protein) were processed and the SCN9a/SCN10a peptides were enriched using 5 µg of either antibodies and analyzed using SRM-MS. A prototype LLOD/LOQ was achieved in the same protein background using spiked heavy peptides in increasing concentrations:

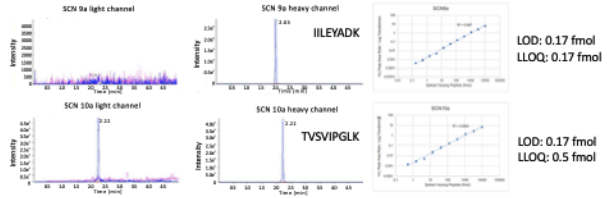

Fig. 3 ***Prototype SRM assays in DRG lysate.*** Measurements were performed in triplicate. Peptide enrichment increased sensitivity of the assay by a factor of 50-fold compared to the original setup without the need for fractionation. However, whilst SCN10a was readily measurable in DRGs, SCN9a was not, which triggered a second round of optimization prior to assay finalization. LLOQ was defined as the lowest measurement for which CV<20%.

4 – Optimization and assay qualification

- Optimal tryptic digestion performed with 10% deoxycholate/overnight incubation at room temperature.
- Determination of an optimal antibody binding / sample ratio for each studied analyte to generate a 3-plex assay.
- Determination of LOD/LLOQ in a pipetting scheme reflecting the expected analyte concentration in the study.

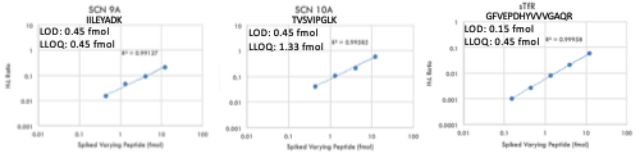

Fig. 4: LOD and LLOQ determination in DRG lysate. Measurements were performed in triplicate in 30 µL DRG lysate pool (approx. 90 µg total protein) prepared in the same buffer as for the DRF study (injecting 1/3 of the immune-enriched peptides on column). LLOQ was defined as the lowest measurement for which CV<20%. With these optimizations, endogenous SCN9A was measurable in DRG at ≈LLOQ while SCN10A and was measured at ≈10-fold above LLOQ. TFR was measured at considerably higher levels that SCN9A and SCN10A.

- Generation of low and high QC samples for the determination of day-to-day variability using sample and volume conditions used in the study and for assay performance monitoring. A lysate of the neuronal cell line SK-N-A p75 were spiked with low (500 amol) and high (15000 amol) levels of the light peptides was prepared to investigate within- and day-to-day (3 consecutive days) reproducibility. All measurements were well below 10% CV for all conditions.
- In anticipation of low levels of SCN9A levels in the DRF study, a lysate volume of up to 100 µL (300 µg total protein) was found acceptable for processing without detrimental effect on reproducibility or signal interference.

5 – DRF sample measurement

- 108 DRGs of various weights (8—65 mg) collected in a dose-ranging finding study (Covance Study Number 8418426 approved by the local German Institutional Animal Care and Use Committee) were lysed in 500 µL ThermoFisher Paris® kit lysis buffer using Presellys beads. 100 µL of the lysate volume were processed as described for determination of SCN9A, SCN10A and TFR levels.
- Sample processing order and LC-MS injection were randomized to avoid obvious batch effect. Pooled DRG samples were used to evaluate plate reproducibility and consistency. Sample processing and LC-MS instrument performance were monitored using the low/high QC samples.

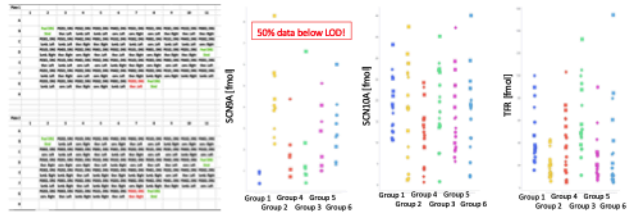

Fig. 5 LEFT, Sample plate position and injection order. Control DRG pools (to calculate NCVs per plate) are labelled in green while known lost DRG samples (negative controls) are labelled in red. **RIGHT, SCN9a, SCN10A and TFR peptide quantification.** All peptide amounts in [fmol] after normalization to the heavy standard. Groups 1-6 encompass four subjects each x 3 areas (lumbar (square), thoracic (circle) and cervical (diamond), left or right) x 6 treatment groups.

5 – Increased sensitivity using a nanoflow LC setup

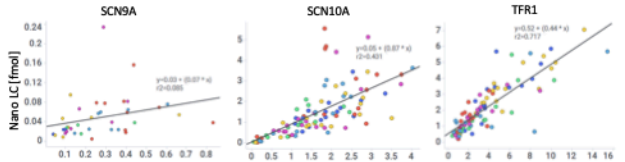

Fig. 6 Comparison of a microflow LC (horizontal axis) with a nanoflow LC (vertical axis) setups. Half of the samples injected on the Sciex Q-Trap 6500 instrument was frozen and re-analyzed onto a nanoflow LC setup equipped with an online enrichment pre-column (100 µm i.d x 20 mm, 5 µm Acclaim C18 ThermoFisher) using a vented Tee design (analytical column: 75 µm i.d x 150 mm; 3 µm Reprosil AQ pure (Dr. Maisch AG), 15 min gradient, using a ThermoScientific QE-HFX mass spectrometer in PRM mode (300 ms accumulation, 60,000 resolution in MS1 and MS2 mode). Sample coloring correspond to the treatment groups (Fig. 5). In the nanoflow LC setup, all SCN9A data points were above LOD.

6 – Conclusions

- **Quantitative assessment of membrane proteins** in tissue lysates was made possible using an peptide immune-enrichment strategy.
- **A nanoflow LC setup was shown to increase analyte sensitivity** by a factor of 10-20 at the cost of throuput and increased variability.
- Quantitative treatment-related effects were not measurable due to the absence of a suitable protein normalizator.