

Urea-based Plasma Sample Preparation and Data Analysis Protocol

Solution and Sample Preparation

The digests were prepared through simultaneous denaturation and reduction of the homogenate with 9M urea/20mM dithiothreitol for 30 min at 37°C. Denatured proteins were alkylated with iodoacetamide (40 mM final concentration) for 30 min at room temperature, and then samples were diluted to reach a final urea concentration of 0.55 mM prior to tryptic digestion. Digestion was carried out at a 20:1 substrate:enzyme ratio using TPCCK-treated trypsin (Worthington) for 18 hrs at 37°C. After digestion, samples were acidified with aqueous 1% formic acid (FA), and a chilled SIS peptide mixture was added. Samples were concentrated *via* solid phase extraction (10 mg Oasis HLB cartridges; Waters), using the manufacturer's recommended protocol. The SPE column was conditioned with 100% methanol (1 mL), followed by washing with 100% H₂O/0.1% FA (1 mL), the sample (diluted to 1 mL using 100% H₂O/0.1% FA) was then loaded onto the column, followed by washing 2 times with water (1 mL each). Finally, the sample was eluted with 60% ACN/0.1% FA (300 µL) and lyophilized to dryness. The dried samples were rehydrated in 0.1% formic acid to a 1 µg/µL concentration for LC/MRM-MS analysis.

LC/MRM-MS Equipment and Conditions

The samples were separated on-line with a RP-UHPLC column (150 x 2.1 mm i.d., 1.8 µm particle diameter; Agilent) maintained at 50°C. Peptide separations were performed at 0.4 mL/min over a 43 min run, *via* a multi-step LC gradient (1.5-81% mobile phase B; mobile phase B: 0.1% FA in ACN). The exact gradient was as indicated in the peptide information table. A post-column equilibration of 4 min was used after each sample analysis.

The LC system was interfaced to a triple-quadrupole mass spectrometer (Agilent 6495/Agilent 6490) via a standard-flow ESI source, operated in the positive ion mode. The MRM acquisition parameters employed for the quantitation were as follows: 3500 V capillary voltage, 300 V

nozzle voltage, 11 L/min sheath gas flow at a temperature of 250°C, 15 L/min drying gas flow at a temperature of 150°C, 30 psi nebulizer gas pressure, 380 V fragmentor voltage, 5 V cell accelerator potential, and unit mass resolution in the first and third quadrupoles. For optimal peptide collision induced dissociation, peptide-specific CE values had previously been determined experimentally.

Quantitative Analysis

The MRM data was visualized and examined with MassHunter Quantitative Analysis software (version B.07.00; Agilent) and/or was analysed with Skyline software Version 3.1 (see [1]). For each peptide, the relative peak area ratios of the natural (NAT) to SIS were calculated. This ratio and the known concentration of SIS peptide was used to calculate the concentration of the NAT peptide in the sample by comparison to a standard curve. This involved peak inspection to ensure accurate selection, integration, and uniformity (in terms of peak shape and retention time) of the SIS and NAT peptide forms. Thereafter, the processed response (*i.e.*, peak area) data was input into the software tool – Qualis-SIS – for quantitative analysis. After defining the regression analysis criteria (*i.e.*, $1/x^2$ regression weighting, <20% deviation in a given level's precision and accuracy) for each concentration level of the standard curve, the tool automatically generates and extracts assay-related information from each standard curve. The endogenous protein concentrations in the mouse samples are determined through linear regression (see [2, 3] for additional information).

References

- [1] MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., *et al.*, Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* 2010, 26, 966-968.
- [2] Percy, A. J., Chambers, A. G., Yang, J., Hardie, D. B., Borchers, C. H., Advances in multiplexed MRM-based protein biomarker quantitation toward clinical utility. *Biochim. Biophys. Acta* 2014, 1844, 917-926.
- [3] Mohammed, Y., Percy, A. J., Chambers, A. G., Borchers, C. H., Qualis-SIS: Automated Standard Curve Generation and Quality Assessment for Multiplexed Targeted Quantitative Proteomic Experiments with Labeled Standards. *J. Proteome Res.* 2015, 14, 1137-1146.