Deoxycholate -based Plasma Sample Preparation and Data Analysis Protocol

Solution and Sample Preparation

In the sample preparation, 10 µL of raw or pooled plasma (for samples or control, respectively) was subjected to 10% deoxycholate, 20 mM dithiothreitol, and 0.5 M iodoacetamide (all in Tris buffer at pH 8.0). Denaturation and reduction occurred simultaneously at 37°C for 30 min, with alkylation occurring thereafter in the dark at room temperature for 30 min. Proteolysis was initiated by the addition of TPCK-treated trypsin (35 µL at 1 mg/mL; Worthington) at a 20:1 substrate:enzyme ratio. After overnight incubation at 37ºC, proteolysis was quenched with 1% FA. The balanced SIS peptide mixture (150 to 0.2 fmol/µL for the standard curve of the pooled control sample, 10 fmol/µL for the individual samples) was then spiked into a digest aliquot

The concentration of the SIS mixture used in the control measurements varied across 6 concentration levels in a dilution series of 1:3:5:2:5:5, which correspond to concentrations designated F to A, where F is the highest and A is the lowest SIS concentration used. In this dilution series the standard D is balanced to the endogenous concentration, which is estimated from a single-point measurement of a pooled sample from the sample set/cohort being measured. Samples were then concentrated by SPE (Oasis HLB, 2 mg sorbent; Waters), using the manufacturer's recommended protocol. After solid phase extraction, the concentrated eluate was frozen, lyophilized to dryness, and rehydrated in 0.1% FA (final concentration: 1 µg/µL digest) for LC/MRM-MS.

LC/MRM-MS Equipment and Conditions

Fifteen-µL injections of the plasma tryptic digests were separated with a Zorbax Eclipse Plus RP-UHPLC column (2.1 x 150 mm, 1.8 µm particle diameter; Agilent) that was contained within a 1290 Infinity system (Agilent). Peptide separations were performed at 0.4 mL⁄min over a 27 min run, via a multi-step LC gradient (1.5-81% mobile phase B; mobile phase compositions: A was 0.1% FA in H_2O while B was 0.1% FA in ACN). The exact gradient was as indicated in the peptide information table. The column and autosampler were maintained at 50 and 4˚C, respectively. A post-gradient 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 general MRM acquisition parameters employed were as follows: 3.5 kV 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 quadrupole mass analysers. The high energy dynode (HED) multiplier was set to -20 kV for improved ion detection efficiency and signal-to-noise ratios. Specific LC-MS acquisition parameters were employed for optimal peptide ionization/fragmentation and scheduled MRM. Note that the peptide optimizations had previously been empirically optimized by direct infusion of the purified SIS peptides. In the quantitative analysis, the targets were monitored over 500-ms cycles and a 1-min detection windows.

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.