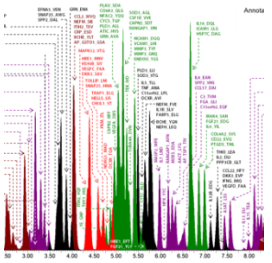
MRM-LC-MS/MS Assay for inflammatrory associated proteins in serum



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Abstract

Multiplex targeted proteomic assay to measure human proteins associated with inflammation in serum. A unique peptide is selected to represent a protein and is measured using multiple reaction monitoring (MRM) mass spectrometry.

Materials

MATERIALS

- Waters Acquity CSH C18 2.1×10 0mm 1.7 µm Column
- Waters Acquity VanGuard CSH C18 1.7 µm Precolumn
- Promega trypsin **Promega Catalog #**V5113
- Enolase from bakers yeast (S. cerevisiae) **Sigma Aldrich Catalog #**E6126

Sample preparation

1 samples are prepared for targeted mass spectrometric analysis utilising 20 µL serum.

2 Prior to preparation, 5 pmol yeast enolase 1 protein (ENO1) (sigma UK) were added to each 20 µL serum sample to act as an internal standard, accounting for variation in sample preparation and instrumental analysis.

3 To reduce the complexity of the sample matrix and enable the detection of low- and medium abundant proteins, the serum was precipitated with 40 µL 3M ammonium sulphate.

4 This was followed by incubation at room temperature for 10 minutes before centrifugation at 16900 x g for 10 minutes to separate supernatant from protein pellet.

5 The supernatant was discarded and the protein pellet was washed with 50 µL 1.8M ammonium sulphate.

6 The proteins were thereafter solubilised in 20 µL digestion buffer containing 6M urea, 2M thiourea, 2% ASB-14 and 100mM Tris, pH7.8, and shaken until all protein pellet had been re-suspended.

7 To reduce sulphide bonds, 45 µg dithioerythriol was added and the samples were shaken for 60 minutes.

8 To prevent the sulphide bonds from reforming, 108 µg iodoacetic acid were added and the samples were shaken for another 60 minutes.

9 165 µl MilliQ water was added to dilute the concentration of urea

10 add 1 µg trypsin (Promega, UK) was added to digest the proteins into tryptic peptides.

11 The samples were incubated in a +37 °C water bath for 16 hours.

12 In order to remove salts, a solid phase extraction (SPE) was performed on a 100 mg Bond Elut C18 96 well plate (Agilent, Santa Clara, CA, United States). Prior to SPE clean up, the samples were adjusted to a concentration of 0.1% trifluoroacetic acid (TFA).

13 The SPE beds were washed with 1 mL 60% acetonitrile (ACN), 0.1% TFA before equilibration by two 1 mL aliquots of 0.1% TFA.

14 The samples were loaded onto the beds and salts were washed away by the addition of 1 mL 0.1% TFA before elution of the desalinated samples by two 250 µL aliquots of 60% ACN, 0.1% TFA.

15 Solvents were evaporated using a SpeedVac (Eppendorf, Hamburg, Germany).

LC-MS/MS Analysis

16 Before mass spectrometric analysis, the samples were reconstituted in 50 µL 3% ACN, 0.1% TFA.

17 Utilising a Waters Acquity Ultra Performance Liquid Chromatography system (Waters, Manchester, UK), 4 µL of sample was injected onto a Waters 50 mm CORTECS® UPLC® C18 1.6 µm column fitted with a 5 mm VanGuard column of the same chemistry, operating at 45 °C, for chromatographic separation. The mobile phase consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in ACN, pumped at a flow rate of 0.6 mL min-1. The starting conditions of 3% B were kept static for 0.1 minutes, before initialising the linear gradient utilised to elute and separate the peptides over 7.7 minutes to 40% B. B was thereafter linearly increased to 80% over 0.2 minutes and held for 1 minute to wash the column before returning to the initial conditions followed by equilibration for 1 minute prior to the subsequent injection. The LC system was coupled to a Waters Xevo-TQ-S triple quadrupole mass spectrometer for multiple reaction monitoring (MRM) detection in positive electrospray ionisation mode. The capillary voltage was set to 2.8 kV, the source temperature to 150 °C, the desolvation temperature to 600 °C, the cone gas and desolvation gas flows to 150 and 1000 L hour-1 respectively. The collision gas consisted of nitrogen and was set to 0.15 mL min-1. The nebuliser operated at 7 bar. The cone energy was set to 35 V and the collision energies varied depending on the optimal settings for each peptide. Three injections were performed for each sample.

Data Processing

18 After acquisition, the data were imported to Skyline open source software (https://skyline.ms/project/home/software/Skyline/begin.view). The quantifier and qualifier ions were inspected and compared to a pooled sample spiked with increasing amounts of synthetic peptides to ensure that the correct peaks were identified and integrated. The relationships between quantifier and qualifier ions were evaluated for consistency across the samples and the most abundant peptide ions were chosen for quantitation. The integrated areas were exported to Microsoft Excel and the analyte quantifier areas were normalised to yeast enolase 1 to render ratios. The relative response of each analyte to the response of the known concentration of the internal standard was used to estimate the concentration of the proteins in the samples.

19 To evaluate the stability of the assay, five repeated injections of 1 pmol synthetic standards were performed and the coefficients of variation were calculated. The coefficients of variation for the most significant compounds were 14% for protein NDRG1 (NDRG1), 20% for carbamoyl-phosphate synthase [ammonia], mitochondrial (CPSM), 9% for cystatin C (CST3), 3% for progranulin (GRN), 5% for collagen triple helix repeat-containing protein 1 (CTHRC1) and 4% for thioredoxin-dependent peroxide reductase, mitochondrial (PRDX3).

20 Table 1 Protein -peptide list.docx

List of proteins and corresponding peptides included in the multiplex inflammation panel assay