2D-LC MS/MS Conditions

The SCX column (BioSCX Series II 0.8 x 50 mm, 3.5 µm particle size, 300 Å pore size), was upstream of a RP trapping cartridge (ZORBAX 300SB-C18 column 0.3 x 5 mm, 5 µm particle size, 300 Å pore size) and an analytical column, (ZORBAX 300SB-C18 column 0.1 x 150 mm, 3.5 µm particle size, 300 Å pore size) on a model 1100 Capillary and Nano LC system (Agilent Technologies, Palo Alto, CA, USA).

Each biological replicate was first run with 7 SCX fractions and then 2h RP-LC-MSMS analysis, and then a longer 2DLC run using 8 SCX fractions and 3h RP-LC-MSMS. The optimised conditions of the second 2D-LC experiment allowed maximal analysis of the moderately complex, highly enriched glycopeptide sample. For the first analysis, samples were eluted from the SCX column using a step-gradient of 2.5, 5, 7.5, 10, 15, 20 and 100% salt buffer (500 mM ammonium formate, 5% acetonitrile, 1% formic acid). The salt step-gradient was 0 – 3 min gradient from 0% salt buffer to the previous fraction salt percentage, 12 min linear gradient to the current salt buffer set, 2 min constant salt buffer, linear reduction of salt over 5 min, then washing of the SCX and trap columns with 5% ACN, 0.1% formic acid for 8 min before switching the flow to the RP pump for analytical separation of peptides on the RP column to the mass spectrometer. The reverse phase gradient of acetonitrile with 0.1% formic acid for each fraction was, 5 – 15% for 3 min, 15 – 30% linear gradient for 57 min and then 30 – 60% for 15 min to elute peptides at a flow rate of 0.8 µl/min.

For the second analysis, the buffers, sample injection and elution parameters remained the same, except the step gradient fractions were preceded by an additional RP fractionation of the SCX column injection flow-through (0% buffer B). The reverse

phase separation was also prolonged to 3 h to optimise the identification of each SCX elution fraction:, 5 – 15% for 5 min, 15 – 30% linear gradient for 95 min and 30 – 60% for 35 min to elute peptides.