*Supplemental Experimental Procedures for:*

# Highly Multiplexed and Reproducible Ion Current-Based Strategy for Large-Scale Quantitative Proteomics and the Application to Protein Expression Dynamics Induced by Methylprednisolone in 60 Rats

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***1. Precipitation/on-pellet digestion***

Protein disulfide bond reduction was carried out in 4 mM Tris(2-carboxyethyl) phosphine at 37$^{o}$C for 20 min in an Eppendorf Thermomixer, and alkylation was performed with 20 mM iodoacetamine at 37$^{o}$C for 30 min in darkness. The proteins were precipitated by a two-step procedure: first, one volume of chilled organic solvent cocktail (at -20$^{o}$C) containing 89% acetone, 1% acetic acid and 10% chloroform was added so that the mixture turned cloudy but no visible particulate was observed. The mixture was vortexed thoroughly to extract the detergents and non-protein matrix components into the solvent phase. Second, 8 volumes of chilled acetone/acetic acid/chloroform mixture was added step-wise with vigorous vortexing, and then incubated at -20$^{o}$C overnight. After centrifugation at 20,000 g for 30 min, supernatants were gently removed and the pellets washed with 800 µL of chilled mixture (85/15, v/v %) followed by solvent removal. Instead of pellet suspension, we performed a two-step on-pellet-digestion procedure. In step one (*digestion-aided pellet dissolution*), 100 µL Tris buffer (50 mM, pH 8.5) containing trypsin at an enzyme/substrate ratio of 1:30 (w/w) was added and incubated at 37°C for 2 h with vigorous vortexing at 120 rpm using an Eppendorf Thermomixer; the solution should be free of visible particulates after this step; in step two (*complete cleavage*), another batch of trypsin solution was added at an enzyme/substrate ratio of 1:25 (w/w). The mixture was incubated at 37°C overnight (12 h) while vortexing to achieve a complete digestion. Digestion was terminated by adding 1 µL formic acid followed by centrifugation at 20,000 g for 20 min at 4°C. Finally, supernatant containing 6 µg tryptic peptides was injected into the LC/MS for each sample. Precipitation/on-pellet-digestion of the 60 samples was carried out in 3 cohorts (20 samples per cohort) to minimize the effect of peptide degradation over the 20-day analysis.

### *2. Nano-LC gradient conditions and MS parameters*

Mobile phases were A, 0.1% formic acid in 2% acetonitrile and B, 0.1% formic acid in 82% acetonitrile. Digests containing 6 µg of peptides were loaded onto a large-ID trap (300 µm ID x 1 cm, packed with Zorbax 3-µm C18 material) with 1% B at a flow rate of 10 µL/min, and the trap was washed for 3 min before being brought in-line with the nano-LC flow path. A series of nanoflow gradients (flow rate: 250 nL/min) was used to back-flush the trapped samples onto the nano-LC column for separation. The column was heated at 52°C in the above-mentioned heating sheath. A 7-h shallow gradient was used to achieve sufficient peptide separation. The optimized gradient profile was as follows: 3 to 8% B over 15 min; 8 to 24% B over 215 min; 24 to 38% B over 115 min; 38 to 63% B over 55 min; 63 to 97% B over 5 min, and finally isocratic at 97% B for 15 min.

An LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA) was used for detection. Programmed tip washing (described above) was employed prior to every run to maintain reproducible ionization efficiency. The instrument was operated under data-dependent product ion mode. One scan cycle included an MS1 survey scan (m/z 310-2000) at a resolution of 60,000 to acquire precursor peak of peptides, followed by seven MS2 scans at CID activation mode, to fragment the top seven most abundant precursors in the survey scan. The target value for MS1 by Orbitrap was $8\times10^6$, under which the Orbitrap was calibrated for mass accuracy and FT transmission. The use of a high target value on the Orbitrap enabled highly sensitive detection without compromising the mass accuracy (<5 ppm) and resolution. The activation time was 30 ms, isolation width was 3 Da for ITMS; the normalized activation energy was 35% and the activation q was 0.25.