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Differential quantification of isobaric phosphopeptides using data-independent acquisition in mass spectrometry

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Materials and methods

Extraction of Total Proteins from Cells – Serum-starved HeLa cells were grown in suspension and treated with EGF for 5 min and 20 min. Total proteins were extracted from cells as follows: briefly, cell pellet was lysed using a buffer with 6 M urea/2 M thiourea in 50 mM ammonium bicarbonate solution (pH 8.2). After the centrifugation at 600 x *g* for 5min, an aliquot of supernatant containing about 3mg of proteins was diluted in 50 mM ammonium bicarbonate solution (pH 8.2) to reduce urea concentration. The sample solution was incubated for 1h after the addition of 5 mM DTT with subsequent incubation with 20 mM iodoacetamide for 30 min in the dark. Proteins were digested with trypsin overnight with an enzyme (µg) to substrate (µg) ratio of approximately 1:50. All the incubations were done at room temperature to avoid reaction between urea and the protein sample.

Enrichment of phosphopeptides by titanium dioxide (TiO_2) chromatography – Phosphopeptides were enriched using TiO_2 chromatography. Briefly, the tryptic peptide sample was concentrated to 100 µl by lyophilization and TiO_2 enrichment of phosphopeptides was performed as previously described[1]. The lyophilized phosphorylated peptide samples were reconstituted in 0.1% trifluoroacetic acid (TFA) and desalted using Poros Oligo R3 RP micro-columns. The peptide samples were subsequently lyophilized and stored at −80 °C for further analysis

Nano liquid chromatography – tandem mass spectrometry (nLC-MS/MS) – Sample separation and introduction into the mass spectrometer was performed using a reversed phase high performance nLC with a two component mobile phase system: 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A Thermo Scientific™ Easy nLC™ 1000 nano-uHPLC system was equipped with a 75 µm x 20 cm in-house packed column using Reprosil-Pur C18-AQ (3 µm; Dr. Maisch GmbH, Germany). Peptides were eluted using a gradient from 0 % to 26% B over 120 minutes, and then to 80% over the next 5 min, at a flowrate of 300 nL/min. Each sample was run in three technical replicates. DDA was performed in an Orbitrap Fusion™ (Thermo Scientific, San Jose, CA) as follows: spray voltage was set at 2.3 kV and capillary temperature was set at 275 °C. Full scan MS spectrum (*m/z* 350−1200) was performed in the Orbitrap with a resolution of 120,000 (at 200 *m/z*) with an AGC target of 5x10e5. By using the Top Speed MS/MS option set to 2.5 sec the most intense ions above a threshold of 5x10e4 counts were selected for fragmentation. Fragmentation was performed with higher-energy collisional dissociation (HCD) with normalized collision energy of 27, an AGC target of 10e4 and a maximum injection time of 120 msec. MS/MS data were collected in centroid mode in the ion trap mass analyzer (normal scan rate). Only charge states 2-5 were included. The dynamic exclusion was set at 60 sec. DIA was performed using mostly the same settings. The differences were: two MS scans were performed in the orbitrap at 60,000 resolution (at 200 *m/z*) using a scan range of 400-1100 *m/z* with an AGC target of 2x10e5. The two scans were performed one as first scan event and one after 14 MS/MS events, in order to have two full MS scans per cycle at equal intervals. MS/MS was performed in the orbitrap at a resolution of 15,000 (at 200 *m/z*) using subsequent isolation windows of 25 *m/z*. The AGC

target was set to 5x10e4 and a maximum injection time of 50 msec. All raw files are available at the Chorus database (https://chorusproject.org/, project ID: 1037).

Data analysis – DDA runs were searched using Proteome Discoverer (v1.4, Thermo Scientific, Bremen, Germany). Mascot (v2.5) was used as database searching engine. The database selected was downloaded from UniProt (Homo sapiens). Search parameters were as follows: MS tolerance: 10 ppm; MS/MS tolerance: 0.5 Da; enzyme: trypsin; static modifications: carbamidomethyl (C); dynamic modifications: phosphorylation (STY). Peptides were filtered for high confidence (FDR<1%) using Percolator for validation. Proteome Discoverer results and DIA raw files were uploaded in Skyline[2], where XIC was automatically performed. The selection of isobaric peptides was manually performed, and all calculations of differential quantification using unique fragment ions was performed using Excel. In case multiple fragment ions could be adopted to estimate the ratio between two isobaric species we used the average of the ratios.

References

1. K. Engholm-Keller, P. Birck, J. Storling, F. Pociot, T. Mandrup-Poulsen and M. R. Larsen, *Journal of proteomics*, 2012, **75**, 5749-5761.
2. B. MacLean, D. M. Tomazela, N. Shulman, M. Chambers, G. L. Finney, B. Frewen, R. Kern, D. L. Tabb, D. C. Liebler and M. J. MacCoss, *Bioinformatics*, 2010, **26**, 966-968.