ABSTRACT

Traditionally, quantitative phosphoproteomics strategies were limited to the use of stable-isotope labeling of cell cultures, which is the most commonly used sample type. Recent developments have been made using label-free techniques which make the analysis applicable to biological fluids and tissues, as well as those not amenable to SILAC-based approaches. Here, we describe a label-free strategy that achieves the robust quantitative reproducibility essential for the success of label-free workflows.

For the phosphoprotein enrichment column, a fixed-coul lozically separated with anisotropic portions to the appropriate dimensions for the bound phosphopeptide to be enriched. The column was placed in-line with a Waters QpC pump, autosampler, and UV detector. Enrichment of phosphopeptides was performed through a series of injections of sample, wash buffer, and elution buffer. Quantitative reproducibility of the procedures was determined using on-chip RCVC measurements after accurate mass measurement and robust now normalized of identified phosphopeptides from four replicates enriched analysis by LC/MS/MS on a Waters Synapt G1. The average CV of the 10 identified phosphopeptides was 8.1%, which is a quantitative enrichment specificity of 90.6%

In addition to the enrichment, the described platform also includes improvements to the LC/MS/MS analysis of enriched samples. This strategy employs the high/low pH separation of phosphopeptides that has been recently used to increase the coverage of unenriched samples in a reasonable time frame. By adjusting the fraction to compensate for the more acidic nature of phosphopeptides, a 3‐dimensional analysis was collected in LC/MS/MS using a Waters nanoAcquity with a Waters Synapt G1. To achieve this, a Waters nanoAcquity was coupled to a Synapt G1 to increase the total number of spectra assigned to phosphopeptides in a rat brain lysate from 473 to 212 over a single‐dimension analysis while only increasing the total runtime by 70%. Additionally, 90% of phosphopeptides were identified in a single 2D fraction, highlighting the orthogonality of the separation.

Improvements in Enrichment of Phosphopeptides with Automated, Capillary Column Format

Evaluation of Enrichment Flow Rate and Identification of Phosphopeptides Throughout Each Stage of Enrichment

Experiment Details
- Replicate enrichments of 700 µg rat brain lysate were performed at both 5 and 20 µg sample inputs.
- Fractions were collected throughout the enrichment.
- Flow through from sample injection
- Wash with glycine buffer
- Wash without glycine buffer
- Elution
- Flowthrough fraction was concentrated and re‐enriched a second time. The elution fraction from this was analyzed as the ‘re‐enriched flowthrough’

In addition to the high‐level of specificity found in the Elution fractions, the absence of phosphopeptide signal in the re‐enriched flowthrough highlights the completeness of the enrichment in a single pass.

Effect of Modifier on LC/MS Peak Shape and Reproducibility

Intensity Ratio Plot of Phosphopeptides GluAc‐enriched vs. DHB‐enriched

Difference in LC/MS peak width of 100 phosphopeptides

Resulting Quantitative CVs of Identified Phosphopeptides

Overall, the distribution of phosphopeptides within the two enrichments are similar as shown in the ratio plot. However, the peak widths are significantly broader in the DHB‐enriched samples, which results in a greater number of phosphopeptides with larger degrees of quantitative variability.

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