1. Introduction

Protein activity is mainly modulated by dynamic reversible post-translational modifications (PTMs) such as site-specific phosphorylation, which regulates all cellular processes. Despite continuous improvements, global analysis of protein phosphorylation is still challenging due to its sub-stoichiometric nature and low abundance. Off-line high pH reversed-phase peptide fractionation has shown great promise to overcome this challenge and provides a basic framework for performing in-depth phosphoproteomics studies. However, deep phosphoproteome analyses typically require several days of instrument time. This is not feasible for large-scale clinical studies with many experimental conditions, where deep phosphoproteomes from low amounts of starting material with rapid single-shot analyses are preferred. Here we describe, in detail, how to setup an efficient and reproducible workflow with automated PAC digestion and Ti-IMAC phospho-enrichment on a Kingfisher Flex robot and utilize the short and sensitive separation methods on the Evosep One in combination with fast scanning MS/MS methods on an Orbitrap Exploris 480 MS.

Figure 1: Experimental overview of phospho-enrichment workflow
2. Automated PAC digestion

We used HeLa cells cultured in DMEM media, which were harvested in boiling lysis buffer (5% sodium dodecyl sulfate (SDS), 5 mM tris-(2-carboxyethyl)phosphine (TCEP), 10 mM chloroacetamide (CAA), 100 mM Tris, pH 8.5). The protein digestion was automated on a Kingfisher Flex robot in 96-well format by protein aggregation capture (PAC)\(^1\) on magnetic microparticles, followed by on-bead trypsin digestion.

Volumes and protocol setup is outlined in Figure 2. Protease activity was quenched by acidification with trifluoroacetic acid (TFA) to a final concentration of 1%, and the resulting peptide mixture was concentrated on Sep-Pak, where it can be stored at 4°C until further processing. A fraction of the peptide mixture can in parallel be loaded directly onto Evotips for downstream full proteome analysis.

![Figure 2: Detailed overview of the automated PAC digestion](image)

3. Automated phosphoenrichment

Peptides were eluted with 75 µl 80% Acetonitrile into the phosphopeptide binding plate. Additional volumes and protocol setup is outlined in Figure 3\(^2\). Phosphopeptides were acidified with trifluoroacetic acid (TFA) and purified directly on Evotips according to manufacturer’s protocol. Ensure that the Evotips are continuously wet during loading.

![Figure 3: Detailed overview of the automated phosphoenrichment](image)
4. LC-MS/MS

The phosphopeptides were eluted by running the Evosep One® pre-formed 21 min gradient on a 150µm ID column packed with 1.9µm beads in front of an Orbitrap Exploris 480 MS4, (Figure 4) allowing analysis of 60 samples per day. Spray voltage were set to 2 kV, funnel RF level at 40, and heated capillary temperature at 275°C. The mass spectrometer was operated in data dependent acquisition mode with full MS resolution set at 60,000 at m/z 200 and full MS AGC target at 300% with an injection time (IT) of 25 ms. Mass range was set to 350–1400. AGC target value for fragment spectra was set at 200% with a resolution of 15,000 and injection times of 22 ms and Top12. Intensity threshold was kept at 2E5. Isolation width was set at 1.3 m/z. Normalized collision energy was set at 30%.

Figure 4: Total ion current chromatogram from phosphopeptide enriched sample

5. Results

To evaluate the performance of the Evosep One for phosphoproteomics, we measured single shot HeLa phosphoproteomes in triplicates with the 60 samples per day method. The total ion current chromatogram shows a well distributed elution profile over the gradient, which translates into more than 400 unique phosphopeptides identified per min. during the gradient. A challenge for phosphoproteomics is not only identifying as many phosphopeptides as possible, but also on providing confident site localization. Both are obtained with the fast acquisition methods and short gradient, where we on average identified 7860 phosphopeptides with an enrichment efficiency of 92% and more than 5000 localized sites from ~200 µg of peptide. Reassuringly, the reproducibility between replicates is good with a Pearson correlation of 0.97 (Figure 5).

Figure 5: Identifications and reproducibility of single-shot phosphoproteomes
6. Conclusion

By employing a streamlined and automated workflow for phosphopeptide enrichment in combination with fast and robust chromatographic performance delivered by the Evosep One, we offer a workflow for routine use in large clinical studies, which can also be easily extended to tissue samples. The use of automation in the entire workflow eliminates the hands-on challenges and ensures excellent reproducibility when handling a large number of samples.

The workflow can be extended to data-independent acquisition as well as tandem mass tags (TMT) in combination with high pH reversed phase fractionation. We have previously shown that the best compromise between MS instrument time used and sequencing depth obtained is when running short gradients. With an effective usage time of almost 90%, the Evosep One is the ideal LC system for this type of large-scale analysis.

References


