# Phosphoproteomics Studies using dia-PASEF and short gradients

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## Introduction

Protein phosphorylation is one of the most important post-translational modifications as it can rapidly change a protein's function. Mass spectrometry-based phosphoproteomics has identified >150,000 post-translational phosphorylation sites in the human proteome. To disentangle their functional relevance increasing the throughput and sensitivity of phosphoproteomics approaches is highly desirable. Here, we demonstrate how key features of dia-PASEF translate to high-throughput and sensitivity phosphoproteomics.

## Methods

HeLa cells were prepared as described (Oliinyk et al., 2022). 200 µg of the proteome digest were used to enrich phosphorylated peptides according to the 'EasyPhos' protocol. Peptides were separated within 7- and 21-minute ACN gradients on an 8cm x 75µm column (PepSep) using a nanoElute LC. The LC system was connected via a CaptiveSpray source to a timsTOF Pro (Bruker Daltonik GmbH & Co KG). The MS was operated in dia-PASEF mode, for which method has been optimized for phosphopeptides using the py_diAID software. The raw files were processed in Spectronaut (v17, Biognosys) in library-free mode ('directDIA'). PTM localisation mode was activated defining a threshold of 0 for all phosphopeptides, 0.75 for class I phosphopeptides and 0.99 for the analysis of phosphopeptide positional isomers. To report only unique phosphosites, we used Peptide Collapse (v.1.4.4) plugin tool for Perseus.

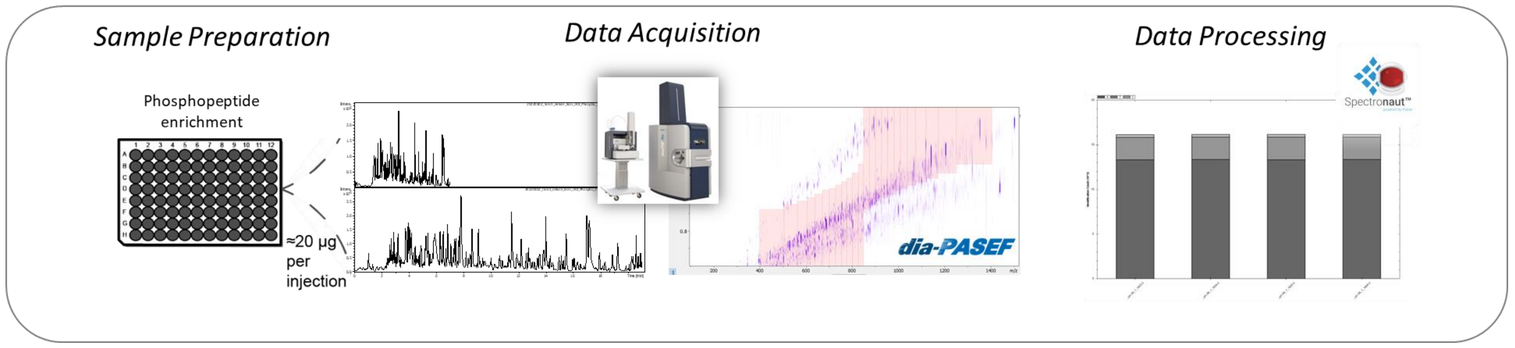
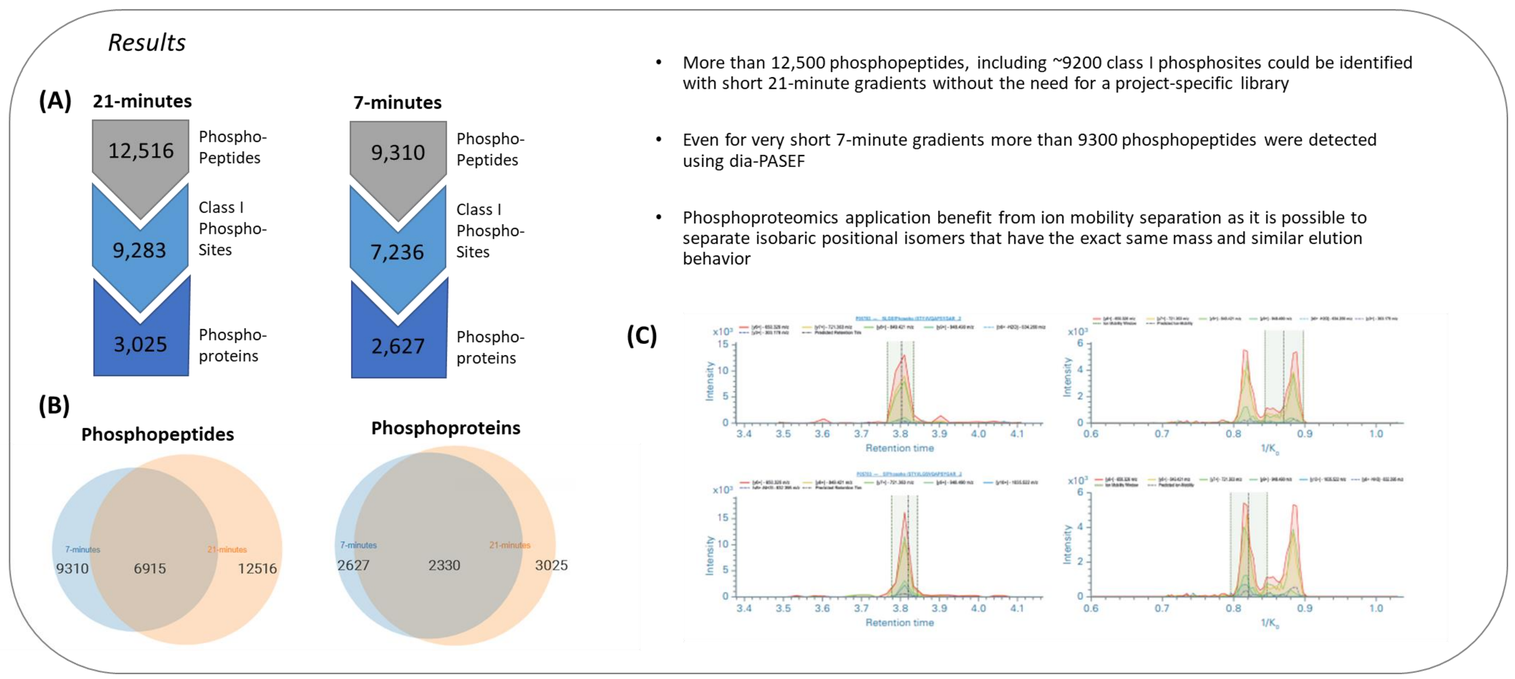



Fig. 1 Phosphoprotemic workflow using dia-PASEF. TiO2 enriched phosphopeptides were separated on a nanoLC system (7- and 21-min gradient length) coupled to a timsTOF Pro using dia-PASEF. Data processing was done using Spectronaut (v17, Biognosys).




- More than 12,500 phosphopeptides, including ~9200 class I phosphosites could be identified with short 21-minute gradients without the need for a project-specific library
- Even for very short 7-minute gradients more than 9300 phosphopeptides were detected using dia-PASEF
- Phosphoproteomics application benefit from ion mobility separation as it is possible to separate isobaric positional isomers that have the exact same mass and similar elution behavior

Fig. 2 dia-PASEF phosphoproteomics with fast chromatographic separation. (A) Total number of identified phosphopeptides and phosphorylated proteins in quadruplicate analysis with 21- and 7-minute LC gradients, injecting aliquots equivalent to 20 µg of starting material. (B) Overlap of identified phoshopeptides and -proteins between 7- and 21-minute LC gradients. (C) Two-dimensional separation of isobaric phosphopeptides. Extracted ion chromatograms (left) and mobilograms (right) for an example of isobaric positional isomers (SLGpSVQAPSYGAR (top) and pSLGSYQAPSYGAR (bottom)) not resolved by chromatography but ion mobility separation.

## Results

Enabling high-throughput phosphoproteomics necessitates reducing the amount of input material and shortening the analysis time per sample. Here, we report results from improved LC and MS methods in combination with optimized data processing applied to low amounts of starting material for phosphoproteomics analysis. To assess the performance of dia-PASEF, we injected amounts of HeLa phosphopeptides equivalent to the enrichment from 20 µg starting material (the protein mass of ~100,000 cells) in short gradients of 21- and 7-minutes. With library-free directDIA data processing, we quantified over 12,500 phosphopeptides including ~9,200 class I phosphosites in 21-minute gradient (Fig. 2A). Decreasing gradient time to 7-min we still quantified about 80% of the class I sites with a median coefficient of variation <10% in quadruplicates. DIA approaches can result in a very high reproducibility as they are not relying on stochastic precursor selection. Reassuringly, our data also revealed a high overlap between the two gradients with about 78% of the protein group identifications in the 7-minute gradient being shared with the 21-minute gradient (Fig. 2B).

Accurate identification and quantitation of phosphopeptides strongly depends on resolving peptides of similar mass and retention times. TIMS separation compensates for higher peptides density per time unit in shorter gradients and allows separation of positional isomers of nearby phosphosites that remained unresolved with fast chromatography (Fig. 2C).

## Summary

Increasing the throughput and sensitivity of phosphoproteomics is highly desirable. Here we show the application of dia-PASEF for short-gradient phosphoproteomics from low sample amounts without the need for a spectrum library. This enables rapid and accurate quantification of major cellular signaling pathways.

## Conclusion

- dia-PASEF on the timsTOF platform is well suited for high-throughput and high-sensitivity phospho-proteomics. More than 12,500 phospho-peptides could be reproducibly identified with short 21-min gradients from low input material.
- Presented results show high degree of reproducibility and data completeness for short gradients using low sample input material.

timsTOF Pro