

In-depth analysis of the HeLa phosphoproteome using specific phosphoprotein purification chromatography and MALDI chip based IMAC phosphopeptide enrichment

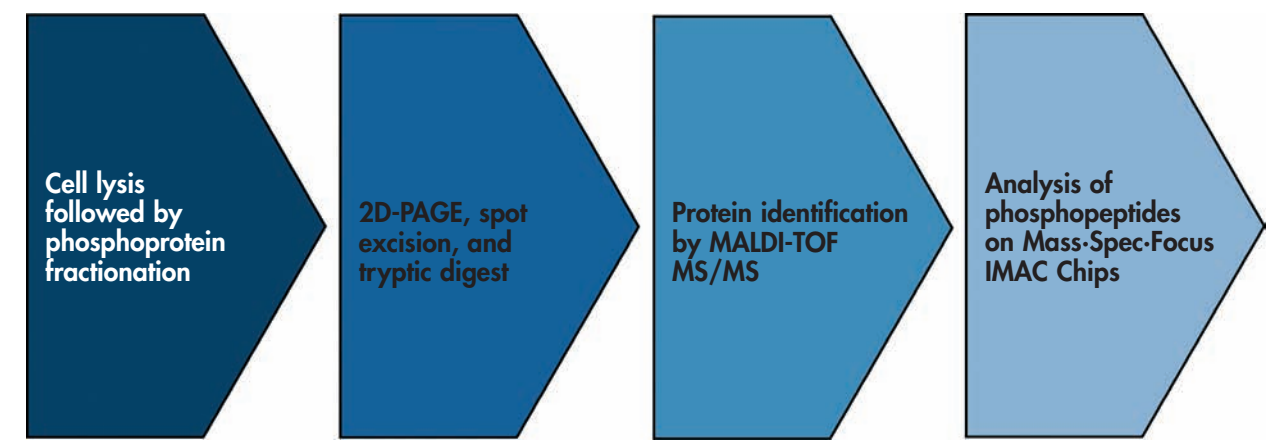


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Introduction

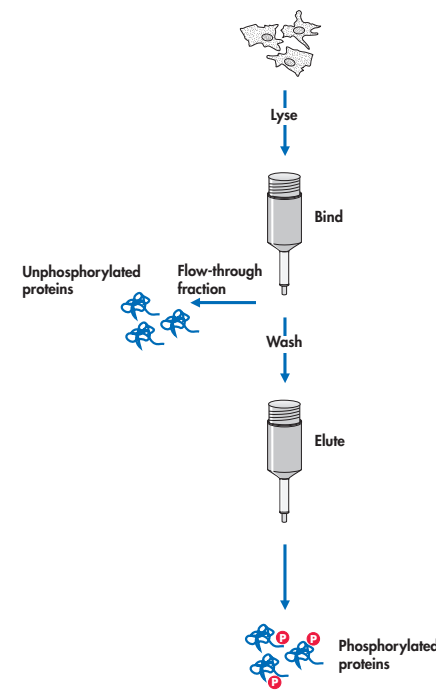
- The specific phosphorylation of serine, threonine, or tyrosine residues is the most common mechanism for the regulation of cellular protein activity.
- Reversible protein phosphorylation controls a wide range of cellular processes and activities such as transmembrane signaling, intracellular amplification of signals, and cell-cycle control.
- The analysis of phosphorylated residues forms the core of signal-transduction studies.
- The major problem facing proteomics researchers investigating phosphorylation is the removal of background proteins and efficient isolation of phosphopeptides for MS analysis.
- Here we present a workflow for efficient purification and MALDI-MS analysis of phosphopeptides.



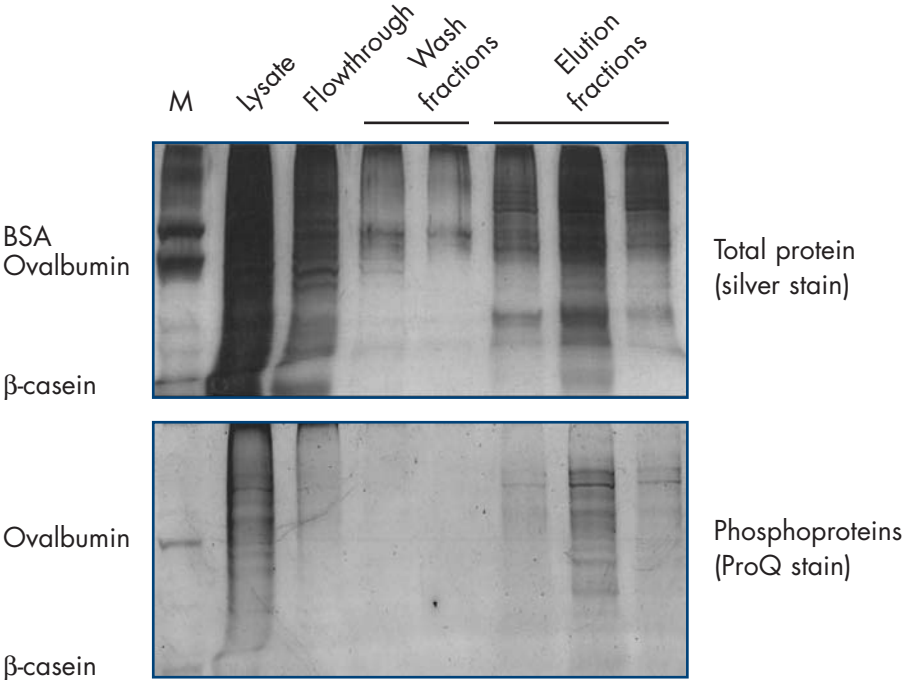
Methods

- The phosphorylation level of 2x10⁷ HeLa-S3 cells was increased by incubation with the phosphatase inhibitors Calyculin A (100 nM) and NaVO₃ (1 mM) for 20 min. After a washing step (2 x PBS) cells were lysed and phosphoproteins were purified using the QIAGEN PhosphoProtein Purification kit according to the manufacturer's instructions. Eluted phosphoproteins and crude cell lysate protein were precipitated with TCA/sodium de-oxycholate and resolubilized in a 2D-PAGE compatible buffer system. Proteins were then separated on a 2D gel (pH 3–10 non-linear gradient) and phosphoproteins specifically visualized using ProQ Diamond (Invitrogen). To control purification efficiency, gels were also stained for total protein with colloidal Coomassie or silver.
- MS analysis: Spots of interest were picked with the help of the Bruker Proteiner sp robot and prepared for digestion according to standard protocols. Digestion was carried out with trypsin (Sigma) overnight at RT. After incubation, the digests were brought to 10 µl by addition of TFA (1%) and 0.5–1 µl was spotted on a stainless steel plate or a

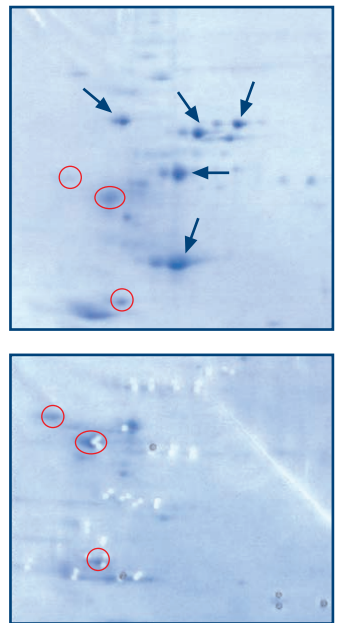
Mass-Spec-Turbo Chip. The remaining sample was transferred to a Mass-Spec-Focus IMAC chip and processed as specified in the handbook using CHCA as matrix.



Efficient separation of phosphoproteins

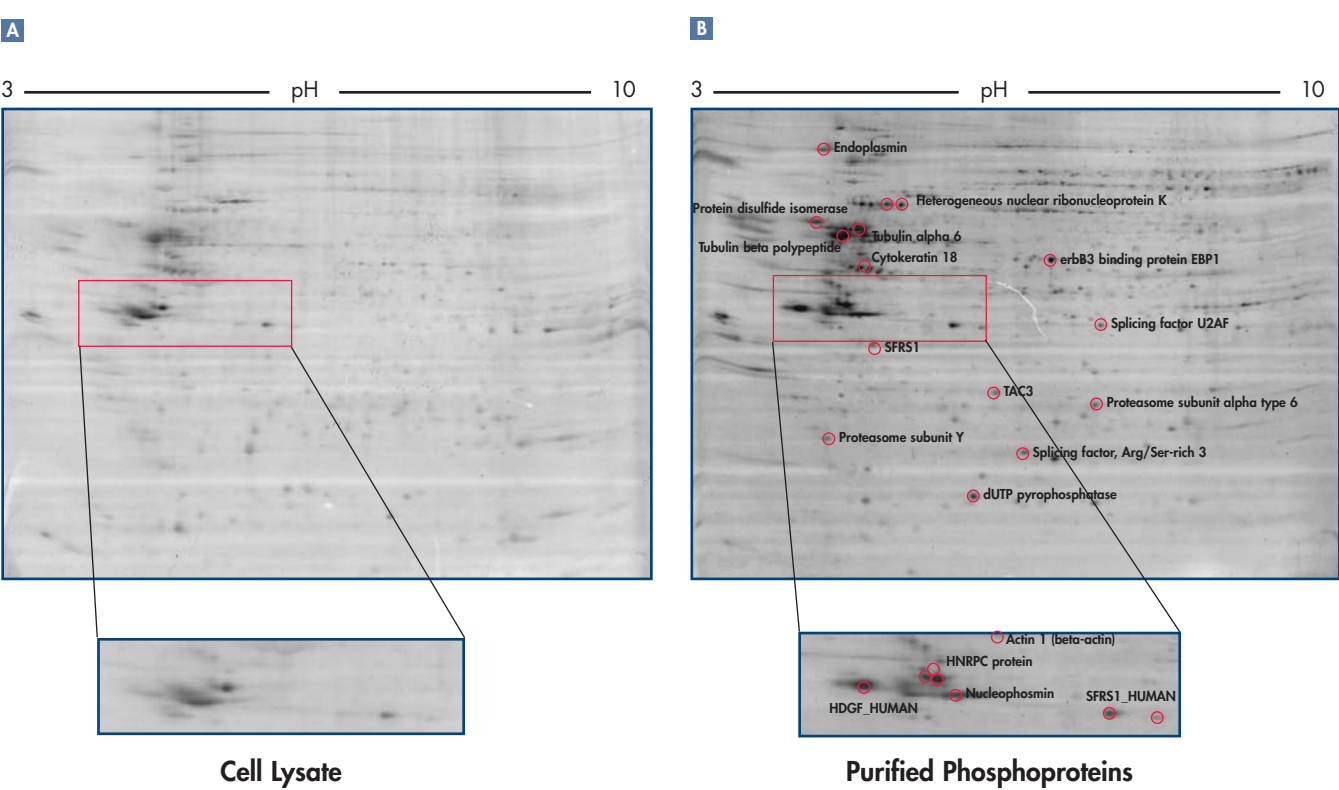


Fractions from the phosphoprotein purification procedure were separated by SDS-PAGE and stained with silver or the phosphoprotein stain ProQ. The marker proteins (M) were BSA and the phosphoproteins ovalbumin and β -casein.



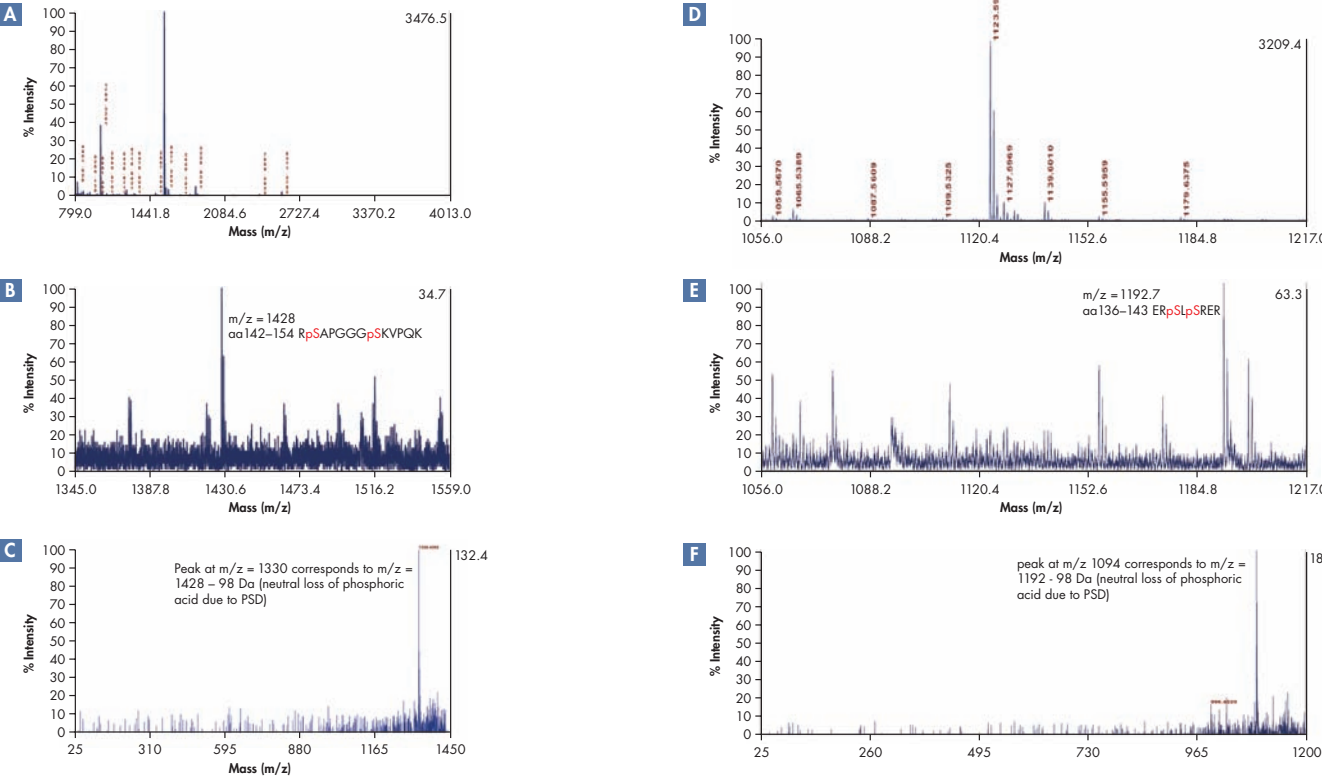
Coomassie-stained 2D gels of total cell lysate (upper panel) and phosphoprotein fraction (lower panel). Prominent spots (arrows) are not present in the phosphoprotein fraction, indicating their efficient removal during the phosphoprotein purification process. Red circles indicate landmarks for correct spot matching of the two images.

Identification of phosphoproteins after separation by 2D-PAGE



2D gels of **A** unprocessed cell lysate and **B** phosphoprotein fraction eluted from the PhosphoProtein Purification Kit column. Phosphoproteins were visualized by staining with ProQ Diamond and subsequent scanning on a Fuji FLA 3000 fluorescence scanner. Identified proteins are annotated according to their NCBI database entry.

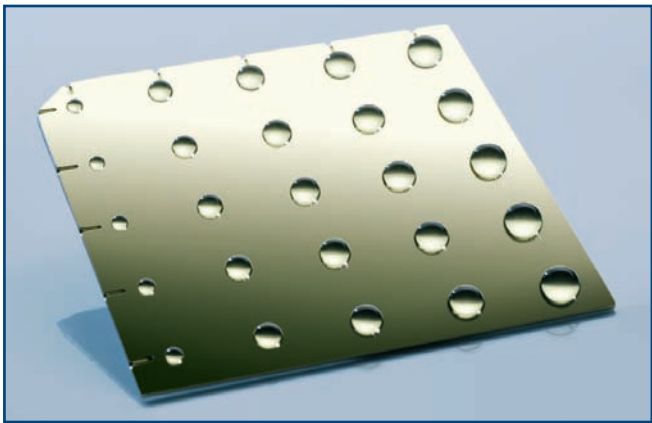
Phosphopeptide validation on Mass-Spec-Focus IMAC Chips



A MALDI spectrum of a crude nucleophosmin digest on a stainless steel target. **B** The same crude digest was processed using a Mass-Spec-Focus IMAC Chip. **C** MS/MS spectrum of the phosphopeptide peak ($m/z = 1428$) shown in **B**. **D** MALDI spectrum of a crude pre-mRNA splicing factor SRP20 digest on a stainless steel target. **E** The same crude digest was processed using a Mass-Spec-Focus IMAC Chip. **F** MS/MS spectrum of the phosphopeptide peak ($m/z = 1192$) shown in **E**.

Summary

- Phosphoproteins could be efficiently separated using the PhosphoProtein Purification Kit
- Purified phosphoproteins were separated by 2D-PAGE and detected using a phosphoprotein-specific stain
- Mass-Spec-Focus IMAC Chips enabled enrichment of phosphopeptides and subsequent validation of candidate phosphoproteins by MALDI-MS
- We are currently working on identifying phosphorylation sites in the isolated phosphoproteins, in order to produce a preliminary 'phosphoproteome map' for HeLa cells



Mass-Spec-Focus Chip

Acknowledgement: We would like to thank the central bioanalytics department of the Cologne Center of Molecular Medicine at the University of Cologne for access to their spot-picking robot and helpful assistance.