

# Quantitative Phosphoproteome Analysis using Stable Isotope Labeling and Affinity Purification

Sun-Il Hwang, Deborah Lundgren, Sung Hee Park, Viveka Mayya, Karim Rezaul, and David K. Han

Department of Cell Biology, Center for Vascular Biology, University of Connecticut  
School of Medicine, 263 Farmington Avenue, Farmington, CT 06030

## Introduction

Most of the signal transduction in eukaryotic cells is mediated by reversible phosphorylation on specific protein motifs. However, phosphoproteome analysis by mass spectrometry is difficult due to the low abundance of phosphoproteins and poor fragmentation pattern of phosphopeptides. To observe the dynamic changes of phosphorylation, we utilized the Stable Isotope Labeling by Amino acids in Cell culture (SILAC) methodology and affinity purification of phosphoproteins (1-6). Here we investigated stromal cell-derived factors 1-alpha (SDF-1 $\alpha$ ) mediated signaling, a known factor for cell proliferation and migration (7).

## Methods

Jurkat T cells were labeled in RPMI 1640 medium with 10% dialyzed fetal bovine serum and antibiotics containing light L-leucine (Leu-0,  $^{12}\text{C}_6$ ) and L-lysine (Lys-0,  $^{12}\text{C}_6, ^{14}\text{N}_2$ ) or heavy L-leucine (Leu-6,  $^{13}\text{C}_6$ ) and L-lysine (Lys-8,  $^{13}\text{C}_6, ^{15}\text{N}_2$ ) for at least five passages. The light isotope-labeled Jurkat T cells were treated with SDF-1 $\alpha$  for 5 minutes at 37°C. After stimulation, phosphoproteins were purified with affinity purification kit according to manufacturer's instruction (QIAGEN). The eluates were mixed 1:1 and run on 1-D PAGE gel (Figure 1). The gel bands were cut into small pieces, trypsinized and analyzed with LTQ LC-MS/MS (Thermo Finnigan).

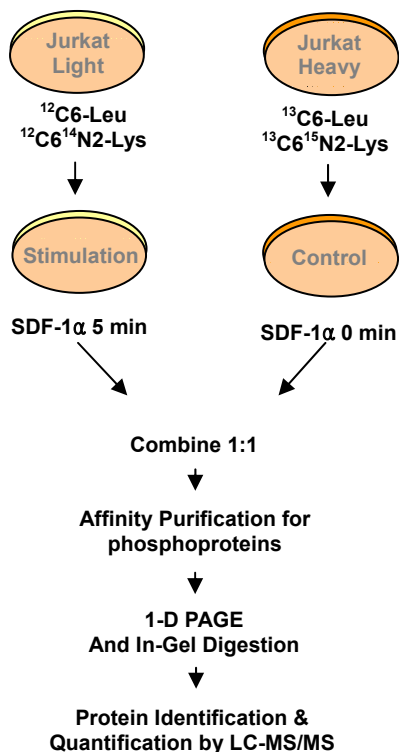


Figure 1. Schematic procedure of phosphoprotein purification and quantification

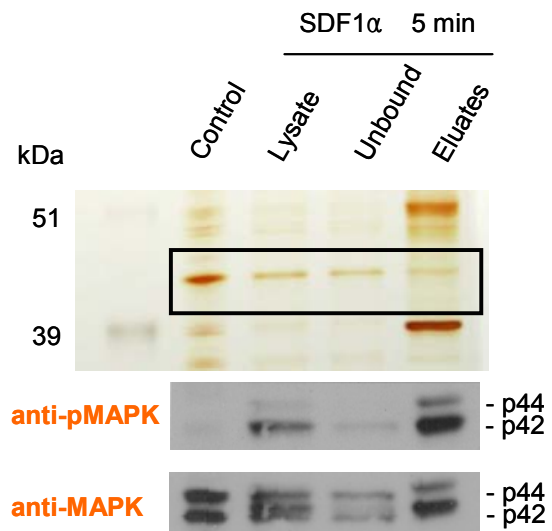


Figure 2. Validation of purification for the phosphoprotein by Western blotting. The gel was visualized with silver staining. The pMAPK (phospho-mitogen activated protein kinase) and MAPK were used for Western blotting.

## Results

A total of 183,857 MS/MS spectra were acquired from 24 gel bands. These spectra were interpreted by automated data processing using the SEQUEST algorithm for protein identification. The protein database from Universal Protein Resource (UniProt) was used to search the un-interpreted MS/MS spectra with differential modification of +6 for heavy leucine-labeled peptide and +8 for heavy lysine-labeled peptide. XPRESS software isolates the light- and heavy-isotope peptide elution profiles, determines the area of each peptide peak, and calculates the abundance ratio based on these areas in an automated fashion. These quantification ratios were confirmed by manual validation and correction. With high stringent criteria of SEQUEST score, we identified over 1,000 proteins (include single hits), and quantified over 800 proteins. Among these, we identified over 500 unique phosphorylation sites from 400 proteins. Next, we tested the efficiency of purification by Western blotting using an antibody against mitogen-activated protein kinase (MAPK) (Figure 2). We found that phospho-MAPK was efficiently purified by this technology. Consistent with this test, we also found a dual specificity mitogen-activated protein kinase kinase 1 (MPK1\_HUMAN) was up-regulated ~1.94 fold. These results indicate that this methodology is useful to purify phosphoprotein during SDF-1 $\alpha$  signaling.

## References

1. Ong SE, Blagojev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics*. 1(5): 376-86.
2. Oda Y, Nagasu T, Chait BT. (2001) Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat Biotechnol*. 19(4):379-82.
3. Ficarro SB, McClelland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM. (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat Biotechnol*. 20(3):301-5.
4. Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. (2004) Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc Natl Acad Sci U S A*. 101(33):12130-5.
5. Metodiev MV, Timanova A, Stone DE. (2004) Differential phosphoproteome profiling by affinity capture and tandem matrix-assisted laser desorption/ionization mass spectrometry. *Proteomics*. 4(5):1433-8.
6. Ueda K, Kosako H, Fukui Y, Hattori S. (2004) Proteomic identification of Bcl2-associated athanogene 2 as a novel MAPK-activated protein kinase 2 substrate. *J Biol Chem*. 279(40):41815-21.
7. Ganju RK, Brubaker SA, Meyer J, Dutt P, Yang Y, Qin S, Newman W, Gropman JE. (1998) The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. *J Biol Chem*. 273(36):23169-75.