Published in final edited form as:

J Proteomics. 2017 August 08; 165: 69–74. doi:10.1016/j.jprot.2017.06.013.

Highly reproducible improved label-free quantitative analysis of cellular phosphoproteome by optimization of LC-MS/MS gradient and analytical column construction

Nagib Ahsan^{1,2,†}, Judson Belmont^{3,†}, Zhuo Chen^{4,†}, James G. Clifton⁵, and Arthur R. Salomon^{2,3,4,*}

¹Division of Biology and Medicine, Brown University, Providence, RI 02903, USA

²Center for Cancer Research Development, Proteomics Core Facility, Rhode Island Hospital, Providence, RI 02903, USA

³Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, RI 02912, USA

⁴Department of Chemistry, Brown University, Providence, RI 02912, USA

⁵Department of Molecular Pharmacology, Physiology and Biotechnology, Brown, University, Providence, RI 02912, USA

Abstract

Expanding the sequencing depth of the peptides with a statistically significant quantitative change derived from a biological stimulation is critical. Here we demonstrate that optimization of LC gradient and analytical column construction can reveal over 30,000 unique peptides and 23,000 phosphopeptides at high confidence. The quantitative reproducibility of different analytical workflows was evaluated by comparing the phosphoproteome of CD3/4 stimulated and unstimulated T-cells as a model system. A fritless, 50 cm-long column packed with 1.9 μ m particles operated with a standard pressure HPLC significantly improved the sequencing depth 51% and decreased the selected ion chromatogram peak spreading. Most importantly, under the optimal workflow we observed an improvement of over 300% in detection of significantly changed phosphopeptides in the stimulated cells compared with the other workflows. The discovery power of the optimized column configuration was illustrated by identification of significantly altered phosphopeptides harboring novel sites from proteins previously established as important in T cell signaling including A-Raf, B-Raf, c-Myc, CARMA1, Fyn, ITK, LAT, NFAT1/2/3, PKC α , PLC γ 1/2, RAF1, and SOS1. Taken together, our results reveal the analytical power of optimized chromatography using sub 2 μ m particles for the analysis of the T cell

*Corresponding Author: Arthur R. Salomon, PhD, Associate Professor, Brown University, 70 Ship Street, Providence, RI 02903, USA, art@drsalomon.com, Fax: +1-401-863-6087.

[†]Authors contributed equally

Conflict of Interest: The authors declare no conflict of interest

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

phosphoproteome to reveal a vast landscape of significantly altered phosphorylation changes in response to T cell receptor stimulation.

Keywords

Sub 2 μ m C18 particles; Label free quantification; LC-MS/MS; Quantitative Phosphoproteomics; T Cell signaling

Mass spectrometry (MS)-based proteomics has played an indispensable role in wide-scale analysis of complex protein mixtures. The technology has been employed in the wide-scale characterization of post-translational modifications (PTMs) and protein interactions [1, 2]. A major challenge in proteomic identification and quantitation is the large dynamic range of protein abundance contained in cellular lysates: four orders of magnitude in yeast and at least seven orders of magnitude in human cells [3,4]. Improvements in peptide separation technology are crucial for complex proteome characterization. To analyze the proteome with high complexity, two-dimensional separation based on gels or liquid chromatography has been used [5-8]. With these methods, LC-MS/MS is preceded by an additional mode of fractionation, such as strong cation exchange (SCX), SDS-PAGE, isoelectric focusing (IEF), and high pH RPLC. Although these methods can reduce sample complexity during LC-MS/MS and thereby increase sequencing depth, they do require more starting material (up to mg level) and each additional separation step decreases the quantitative reproducibility of replicate analyses, frustrating the extraction of biologically relevant insights.

Post translational modifications (PTMs) play a critical role in regulating many signaling pathways. Therefore, identification and quantification of post translationally modified peptides, particularly phosphorylated peptides, is highly desirable. However, identification of phosphopeptides by mass spectrometry is complicated by the high dynamic range of in vivo phosphorylation and low stoichiometry in biological systems [9]. Therefore, to date many methods have been developed to enrich phosphopeptides using a variety of approaches including ion exchange chromatography, metal oxide surfaces, immobilized metal ion affinity chromatography and enrichment by phosphospecific antibodies [10-17].

Quantitation of the phosphopeptides is also very critical as variable phosphorylation alters many biological pathways and can correlate with disease states [18]. Optimization of quantitative reproducibility with each technique while maintaining the highest possible sequencing depth will maximize the number of biologically relevant insights delivered from a proteomics experiment.

Certainly, improvements in instrument sampling rates such as with the Orbitrap mass spectrometer and improved chromatographic peptide separations have led many researchers to explore the utility of complex proteome characterization without peptide pre-fractionation [3, 19, 20]. In addition, analytical column length has been shown previously to improve sequencing depth [3]. However, compared to traditional 3 μ m or 5 μ m reversed-phase C18 particles, sub-2 μ m C18 particles decrease the diffusion distances of solutes while minimizing the band spreading, improving column efficiency of reversed-phase separations. Another strategy is to operate a long column (50 cm or even longer), which provides more theoretical plates, with a long LC gradient (several hours or more) [21]. A major technical

difficulty in using sub-2 μm particles and long columns is the high column backpressure, which has been previously overcome by ultra-high pressure liquid chromatography (UHPLC) [22-24] or a column heater [21]. A true wide-scale understanding of a biological system requires statistical analysis of biological replicates to correctly understand biological variation. Mass spectrometry-based peptide quantification methods can be either label-free or incorporate a stable isotope label such as stable isotope labels with amino acids in cell culture (SILAC) [25], or isobaric tags for relative and absolute quantitation (iTRAQ) [26].

In this report, the analysis of the T cell phosphoproteome using an optimized chromatographic configuration that reduces backpressure using a fritless column packed with sub-2 μm particles is explored. Although many recent reports have impressively expanded the depth of proteomic sequencing, far fewer reports have examined the relationship between sequencing depth and reproducibility of proteomic and phosphoproteomic quantitation through replicate analyses. Pre-fractionation of proteomic samples prior to LC-MS can increase the yield of peptide identifications, however, this strategy increases the variability of the quantitative data analysis and increases the overall cost and instrument time required. For analyses without prefractionation, minimization of the necessity for specialized equipment not available in many proteomic labs can provide higher accessibility of sensitive proteomic capabilities to more labs and greatly simplify the analysis.

The Jurkat T cell clone E6-1 was obtained from American Tissue Culture Collection (Manassas, VA) and cells were cultured and maintained according to standard protocols. For the CD3/4 stimulation, after harvest, Jurkat cells were treated with anti-CD3 and anti-CD4 antibody in PBS (clone OKT3 and OKT4; eBioscience, San Diego, CA) for 3 min at 37°C. Cell lysis, protein extraction and trypsin digestion followed by Sep-Pack C18 column purification was performed as previously described [27], with the exception that TFA was used instead of acetic acid. Eluents containing peptides were lyophilized for 48 hours to dryness. Phosphopeptides were enriched with Titansphere Phos-TiO tips (GL Sciences, Tokyo Japan) following the manufacturer's protocol.

Analytical columns 50 and/or 15 cm long were packed in-house with ReproSil-Pur C18-AQ 1.9 μm particles (Dr. Maisch GmbH, Ammerbuch, Germany) or 3 μm Monitor particles. Detailed protocols for column packing are provided in the supplementary methods section. Tryptic peptides were analyzed by a fully automated phosphoproteomic technology platform [28, 29]. The nanoLC-MS/MS experiments were performed with an Agilent 1200 Series Quaternary HPLC system (Agilent Technologies, Santa Clara, CA) connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA) (see Supplementary Material and Methods for details). Peptide spectrum matching of MS/MS spectra from whole cell lysate Jurkat tryptic digest samples was performed against a human-specific database (UniProt; downloaded 2/1/2013) using MASCOT v. 2.4 (Matrix Science, Ltd, London W1U 7GB UK). A concatenated database containing 144,156 “target” and “decoy reversed” sequences was employed to estimate the false discovery rate (FDR) [30]. Peptide assignments from the database search were filtered down to 1% false discovery rate (FDR) by a logistic spectral score, as previously described [30, 31]. To validate the position of the

phosphorylation sites, the Ascore algorithm [32] was applied to all data, and the reported phosphorylation site position reflected the top Ascore prediction.

Relative quantification of phosphopeptide abundance was performed via calculation of selected ion chromatograms (SIC) peak areas. Retention time alignment of individual replicate analyses was performed as previously described [33]. A minimum SIC peak area equivalent to the typical spectral noise level of 1000 was required of all data reported for label-free quantitation. Individual SIC peak areas were normalized to the peak area of the exogenously spiked phosphopeptide FQpSEEQQQTEDELQDK added prior to phosphopeptide enrichment and reversed-phase elution into the mass spectrometer. Quantitative analysis was applied to five biological replicate experiments from separate batches of cells. To select the phosphopeptides that show a statistically significant change in abundance between CD3/4 stimulated and unstimulated cells, q-values for multiple hypothesis tests were calculated based on p-values from two-tailed unpaired Student's t tests using the R package QVALUE as previously described [34, 35]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD005650.

We first compared the performance of a fritless, 50 cm analytical column with 1.9 μm particles with an integrated electrospray tip that was self-pulled or from a commercial source (New Objective, FS360-75-8-N-5-C70, Woburn, MA). Fritless columns were first employed in the capillary electrochromatography (CEC) field [36, 37, 38]. Five different LC gradients (2 hr, 3 hr, 4.5 hr, 6 hr, 8 hr) were used to characterize equal portions of Jurkat T cell whole cell lysate. Over 23,000 unique peptides corresponding to 4,000 unique proteins were identified using either the 6 or 8 hr LC gradients at a 1% FDR estimated by decoy database approach (Fig. S1). The in-house pulled 50 cm-long, 1.9 μm C18 analytical column performed nearly identically to the commercially pulled column in terms of PSM yield (Fig. S1). Reversed-phase separations were performed with a 50 cm-long, 1.9 μm C18 analytical column using an Agilent 1200 quaternary pump operated at less than 300 bar (Fig. S2). To verify the stability of this fritless 50 cm, 1.9 μm C18 column, the HPLC pressure was cycled from 0 to ~260 bar a total of 100 times. The length of the packing bed and flow rate was measured after every 10 cycles (Table S1). The results clearly showed that both the packing bed length and flow rate at the electrospray tip were unaltered through 100 cycles.

The optimal reversed-phase gradient length of this newly constructed 50 cm-long, 1.9 μm column was examined using five different LC gradients (2 hr, 3 hr, 4.5 hr, 6 hr, 8 hr) (Table S2 and S3). Among the five gradients, the 6 hr gradient yielded the highest number of PSMs (30,403) which corresponded to 4679 protein groups (Fig. S3). However, the 3 hr linear gradient also provided the best balance between sequencing depth and acquisition time yielding 88% of the maximal PSMs in half the time. Other groups have also demonstrated that over 90% of protein or peptide PSMs can be identified using a 3 hr gradient with a 50 cm column [3]. In addition, use of a 50 cm column with sub-2 μm particles produced peak capacities up to 500 within a 120 min gradient [39]. Other reports demonstrated that sequencing depth also increased with increasing peak capacity while peak capacity increased with increasing gradient length and decreasing particle diameter [39-41]. Based on these

earlier studies and our preliminary data, the subsequent phosphopeptide quantitative analysis was performed using a 3 hr gradient.

Quantitative reproducibility is essential to maximize extraction of biologically relevant insights from proteomic experiments. To compare the quantitative reproducibility of analytical columns with either 1.9 or 3 μm particles, Jurkat T cells stimulated for 3 min with CD3/4 antibody crosslink were compared to unstimulated cells (control) after phosphopeptide enrichment by the TiO_2 method from whole cell lysates. A 50 cm column packed with 1.9 μm particles was compared to 15 and 50 cm columns constructed with 3 μm particles. The 15 cm, 3 μm column was included because this configuration represents a commonly used column employed in proteomic labs. For each specific column configuration, intensity-based label-free quantitative analysis with retention time alignment [29, 33] was applied to quantitate the five biological replicate analyses. All selected ion chromatogram (SIC) peak areas from each LCMS run were normalized to the signal intensity of an exogenously spiked phosphopeptide (FQpSEEQQTEDELQDK) standard that was added to every sample at equal levels and co-purified with the T cell phosphopeptides. This standard peptide was quantified reproducibly in all the replicate LC-MS experiments with less than 20% CV across all replicates, highlighting the reproducibility of the quantitation in this study (Table S4). A comprehensive list of all phosphorylated peptides with Mowse score >20 including reversed database hits from every replicate in the CD3/4 stimulated LC-MS analysis is provided in Table S5. All the detailed quantitative proteomic data is provided in Table S6.

Although both 50 cm column configurations were operated at a 60-100 nl/min flow rate due to the backpressure generated from the length of the column and use of a standard HPLC pump compared to 200-300nl/min for the 15 cm configuration, the FWHM medians were smaller for the 50 cm configurations (Fig. S4). Although the bulk majority of peaks quantified in the study relied on greater than 10 MS scans across their selected ion chromatogram (Fig. S4), there was also no correlation at all between FWHM and q-value for the three column configurations tested (Fig. S5). In addition, the column configurations using 60-100 nl/min also showed a high degree of reproducibility in the five biological replicates analyzed which resulted in pairwise replicate peak area R^2 correlation coefficients of ~0.9 (Fig. S6A-B) compared to 0.8 (Fig. S6C) for the 15 cm configuration. Overall, the difference in flow rate did not greatly impact the reproducibility of quantitation between 15 and 50 cm configurations. A histogram analysis of retention times and the selected ion chromatograms obtained from the 50cm 1.9 μm column showed a true distribution of peptides quantified in this experiment ranged from 50-200 min (Fig. S7). Furthermore, compared to the 3 μm particles, the 1.9 μm particles decreased the SIC peak spreading (Fig. S8). In addition, MA plot around the 0 line, especially for the 50 cm, 1.9 μm column configuration also indicates the absence of any bias in the fold changes across a range of peak areas, supporting the quality of the quantitative data (Fig. S9). The distribution of q-values was significantly altered for the 1.9 μm column compared to the 3 μm columns, suggesting an increase in the reproducibility of replicate analyses with this column configuration (Fig. S10). When examining the distribution of the calculated phosphopeptide SIC peak areas, more than 30% of the identified significantly changed phosphopeptides

detected in the 50 cm-long, 1.9 μ m C18 column were below 1E7, while this percentage was less than 14% with the 3 μ m C18 column configurations (Table S7 and Fig S11).

Phosphoproteomic analysis revealed significant differences in phosphopeptide identification yields among the columns tested (Table 1). For instance, the 50 cm, 1.9 μ m particle column yielded over 23,000 unique phosphopeptide PSMs when combining both the stimulated and unstimulated samples. In contrast, a total of 15,173 and 14,494 phosphopeptide PSMs were detected using the 3 μ m particle, 50 cm and 15 cm columns, respectively (Table 1). At a strict cutoff of q -value < 0.01 for the replicate analyses, a total of 7900, 2117 and 1391 unique phosphopeptides were significantly changed in abundance >2 -fold in CD3/4 stimulated T cell compared with unstimulated cells when analyzed by 50 cm 1.9 μ m, 50 cm 3 μ m, and 15 cm 3 μ m particle columns, respectively (Table 1). These results revealed that the 1.9 μ m C18 column reproducibly detected 373% more phosphopeptides with greater than 2 fold significant change and 299% more phosphopeptides with greater than 10 fold significant change compared to the 3 μ m C18 column. Statistical analysis with the more conservative Benjamini-Hochberg procedure also yielded similar results (see details in the supplementary method section, Table S8). Interestingly, despite the anticipated decrease in numbers of PSMs with $q < 0.01$ in all configurations with this more stringent statistical test, we observed over 300% increase in PSMs with $q < 0.01$ in the optimal configuration (50 cm 1.9 μ m) compared to the 50 cm 3 μ m and 15 cm 3 μ m configurations with or without fold-change thresholding for both algorithms (Table S8).

The distribution of fold changes calculated between the stimulated and unstimulated cells was compared for the 1.9 μ m and 3 μ m column configurations (Fig. S12). This analysis revealed that the distribution of fold changes for the 1.9 μ m configuration was broader than with the 3 μ m configurations, indicating an elevated dynamic range. Overall, the 50 cm, 3 μ m column missed 73% and the 15 cm, 3 μ m column missed 76% of the unique phosphorylation sites residing on significantly changed phosphopeptides compared to the the optimized 50 cm, 1.9 μ m column (Fig. 1A). A similar trend was observed when only unique phosphotyrosine sites were compared (Fig. 1B). A volcano plot analysis showed that 74%, 68% and 30% of phosphopeptides were not significantly changed in response to CD3/4 stimulation in T cells when analyzed by 3.0 μ m, 15 cm, 3.0 μ m, 50 cm and 1.9 μ m, 50 cm column, respectively (Fig 1C-E). However, the percentage of elevated peptides (>2 fold, $q < 0.01$) was very similar among the columns. Interestingly, the percentage of peptides with significantly decreased abundance (<2 fold, $q < 0.01$) were gradually increased with increasing of column length and decreasing particle size (Fig 1C-E).

Although kinases play a prominent role in the pathway activated by crosslinking the T cell receptor, phosphatases and negative feedback pathways are also very important in these networks to regulate autoimmune disease and to control the duration of activation of T cells. Recent studies from our lab have revealed previously uncharacterized negative feedback pathways from Vav1, Zap-70, SLP76, and PLC γ 1 to receptor proximal signaling molecules in T cells [27, 42-46]. At this point, the vast majority of the phosphorylation sites on phosphopeptides with reduced abundance are not yet biologically characterized. Future studies will determine the role, if any, of these newly discovered sites in feedback regulation of the signaling network. A comparative heatmap analysis of the KEGG T cell pathway

annotated proteins observed in this study showed many novel phosphorylation sites that are significantly changed in response to T cell stimulation (Fig. 2). The 1.9 μ m, 50 cm column configuration significantly outperformed the other configurations. This configuration revealed both the expected elevation of phosphopeptide abundance on canonical phosphorylation sites downstream of the T cell receptor as well as novel sites on previously established T cell signaling proteins.

Our quantitative data is completely consistent with previously described phosphorylation site changes on canonical T cell signaling proteins. For example, our study revealed TCR stimulation-induced elevated phosphorylation on immunoreceptor tyrosine activation motif phosphorylation sites on receptor subunits TCR ζ Y⁶⁴, Y⁷², Y⁸³, Y¹¹¹, Y¹⁴², CD3 ϵ Y¹⁸⁸, Y¹⁹⁹, CD3 δ Y¹⁴⁹, Y¹⁶⁰. Elevated ITAM phosphorylation is a hallmark of T cell activation [47]. Additionally, elevated phosphorylation was also observed on downstream canonical T cell activation sites on ITK Y⁵¹², ERK1 T²⁰²Y²⁰⁴, Erk2 T¹⁸⁵Y¹⁸⁷, p38 Y¹⁸², ZAP70 Y²⁹² and Y^{492,493}, MEK1 S²¹⁸, S²²² as previously described [27, 42-49]. Although decreased phosphorylation is much less characterized within the TCR signaling pathway, this study revealed decreased phosphorylation on the Calcineurin phosphatase substrate phosphorylation sites on NFAT1 at T³²⁵ and S³²⁶. Calcineurin-mediated dephosphorylation of NFAT is required for its import from the cytosol into the nucleus where it regulates key transcriptional targets [50]. The canonical sites observed uniquely to be significantly elevated in abundance with the 1.9 μ m, 50 cm column include CD3 δ Tyr¹⁶⁰, TCR ζ Tyr^{83,111}, and PLC γ 1 Tyr¹²⁵³. The discovery power of the optimized column configuration was illustrated by a plethora of novel sites seen only with this column, significantly changed 3 min after T cell receptor stimulation on proteins previously established to play important roles in the signaling pathway including A-Raf, B-Raf, c-Myc, CARMA1, Fyn, ITK, LAT, NFAT1/2/3, PKC α , PLC γ 1/2, RAF1, and SOS1 (Fig. 2).

Recently described phosphoproteomic approaches generally employ a targeted enrichment prior to LC-MS/MS analysis coupled with automated workflows. One recent study identified over 10,000 phosphopeptides and was able to decrease the variability among the samples [10, 13, 15]. Our results are in good agreement with earlier reports that demonstrated increasing column length and gradient time increased PSM yield [3]. Biological researchers are primarily concerned with detection of significant changes in the proteome or phosphoproteome following a biological stimulation or perturbation of cells through the analysis of biological replicates. Our optimized analytical column configuration significantly improved quantitative reproducibility, enabling detection of changes in the phosphoproteome through biological replicates. The improved ability of the 1.9 μ m C18 configuration to detect large fold changes in phosphopeptide abundance may be attributed to this column's improved ability to reproducibly quantitate low abundance peptides.

In conclusion, the ideal column configuration reported here represents an inexpensive and highly accessible approach to analyze the phosphoproteome of cells. The use of a fritless 50 cm column packed with 1.9 μ m particles provides dramatic improvements in detection of significantly altered phosphopeptides without the need for specialized UHPLC or column heating apparatus. This analysis emphasizes the importance of consideration of the

quantitative reproducibility of proteomic methods in lieu of a monolithic focus on sequencing depth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to acknowledge financial support from NIH grant R01 AI083636. In addition, this research is based in part upon work conducted using the Rhode Island NSF/EPSCoR Proteomics Share Resource Facility, which is supported in part by the National Science Foundation EPSCoR Grant No. 1004057, National Institutes of Health Grant No. 1S10RR027027, a Rhode Island Science and Technology Advisory Council grant, and the Division of Biology and Medicine, Brown University.

References

1. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003; 422(6928):198–207. [PubMed: 12634793]
2. Choudhary C, Mann M. Decoding signalling networks by mass spectrometry-based proteomics. *Nat Rev Mol Cell Biol*. 2010; 11(6):427–39. [PubMed: 20461098]
3. Pirmoradian M, Budamgunta H, Chingin K, Zhang B, Astorga-Wells J, Zubarev RA. Rapid and deep human proteome analysis by single-dimension shotgun proteomics. *Mol Cell Proteomics*. 2013; 12(11):3330–8. [PubMed: 23878402]
4. Zubarev RA. The challenge of the proteome dynamic range and its implications for in-depth proteomics. *Proteomics*. 2013; 13(5):723–6. [PubMed: 23307342]
5. Gautier V, Mouton-Barbosa E, Bouyssie D, Delcourt N, Beau M, Girard JP, Cayrol C, Burlet-Schiltz O, Monsarrat B, Gonzalez de Peredo A. Label-free quantification and shotgun analysis of complex proteomes by one-dimensional SDS-PAGE/NanoLC-MS: evaluation for the large scale analysis of inflammatory human endothelial cells. *Mol Cell Proteomics*. 2012; 11(8):527–39. [PubMed: 22518033]
6. Gilar M, Olivova P, Daly AE, Gebler JC. Orthogonality of separation in two-dimensional liquid chromatography. *Anal Chem*. 2005; 77(19):6426–34. [PubMed: 16194109]
7. Gilar M, Olivova P, Daly AE, Gebler JC. Two-dimensional separation of peptides using RP-RP-HPLC system with different pH in first and second separation dimensions. *J Sep Sci*. 2005; 28(14):1694–703. [PubMed: 16224963]
8. Zhou F, Sikorski TW, Ficarro SB, Webber JT, Marto JA. Online nanoflow reversed phase-strong anion exchange-reversed phase liquid chromatography-tandem mass spectrometry platform for efficient and in-depth proteome sequence analysis of complex organisms. *Anal Chem*. 2011; 83(18):6996–7005. [PubMed: 21851055]
9. Mann M, Ong SE, Gronborg M, Steen H, Jensen ON, Pandey A. Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *Trends Biotechnol*. 2002; 20(6):261–8. [PubMed: 12007495]
10. Abelin JG, Patel J, Lu X, Feeney CM, Fagbami L, Creech AL, Hu R, Lam D, Davison D, Pino L, Qiao JW, Kuhn E, Officer A, Li J, Abbatiello S, Subramanian A, Sidman R, Snyder E, Carr SA, Jaffe JD. Reduced-representation Phosphosignatures Measured by Quantitative Targeted MS Capture Cellular States and Enable Large-scale Comparison of Drug-induced Phenotypes. *Mol Cell Proteomics*. 2016; 15(5):1622–41. [PubMed: 26912667]
11. Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc Natl Acad Sci U S A*. 2004; 101(33):12130–5. [PubMed: 15302935]
12. Giansanti P, Stokes MP, Silva JC, Scholten A, Heck AJ. Interrogating cAMP-dependent kinase signaling in Jurkat T cells via a protein kinase A targeted immune-precipitation phosphoproteomics approach. *Mol Cell Proteomics*. 2013; 12(11):3350–9. [PubMed: 23882029]

13. Humphrey SJ, Azimifar SB, Mann M. High-throughput phosphoproteomics reveals in vivo insulin signaling dynamics. *Nat Biotechnol.* 2015; 33(9):990–5. [PubMed: 26280412]
14. Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jorgensen TJ. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol Cell Proteomics.* 2005; 4(7):873–86. [PubMed: 15858219]
15. R P, Post H, Fitzpatrick MA, Garrigues LB, Wu W, MacGillavry HD, Hoogenraad CC, Heck AJ, Altelaar AF. Robust, Sensitive, and Automated Phosphopeptide Enrichment Optimized for Low Sample Amounts Applied to Primary Hippocampal Neurons. *J Proteome Res.* 2016
16. Sugiyama N, Masuda T, Shinoda K, Nakamura A, Tomita M, Ishihama Y. Phosphopeptide enrichment by aliphatic hydroxy acid-modified metal oxide chromatography for nano-LC-MS/MS in proteomics applications. *Mol Cell Proteomics.* 2007; 6(6):1103–9. [PubMed: 17322306]
17. Zhou H, Ye M, Dong J, Corradini E, Cristobal A, Heck AJ, Zou H, Mohammed S. Robust phosphoproteome enrichment using monodisperse microsphere-based immobilized titanium (IV) ion affinity chromatography. *Nat Protoc.* 2013; 8(3):461–80. [PubMed: 23391890]
18. Pagel O, Loroch S, Sickmann A, Zahedi RP. Current strategies and findings in clinically relevant post-translational modification-specific proteomics. *Expert Rev Proteomics.* 2015; 12(3):235–53. [PubMed: 25955281]
19. Cristobal A, Hennrich ML, Giansanti P, Goerdayal SS, Heck AJ, Mohammed S. In-house construction of a UHPLC system enabling the identification of over 4000 protein groups in a single analysis. *Analyst.* 2012; 137(15):3541–8. [PubMed: 22728655]
20. Nagaraj N, Kulak NA, Cox J, Neuhauser N, Mayr K, Hoerning O, Vorm O, Mann M. System-wide perturbation analysis with nearly complete coverage of the yeast proteome by single-shot ultra HPLC runs on a bench top Orbitrap. *Mol Cell Proteomics.* 2012; 11(3) M111 013722.
21. Thakur SS, Geiger T, Chatterjee B, Bandilla P, Frohlich F, Cox J, Mann M. Deep and highly sensitive proteome coverage by LC-MS/MS without prefractionation. *Mol Cell Proteomics.* 2011; 10(8) M110 003699.
22. MacNair JE, Lewis KC, Jorgenson JW. Ultrahigh-pressure reversed-phase liquid chromatography in packed capillary columns. *Anal Chem.* 1997; 69(6):983–9. [PubMed: 9075400]
23. MacNair JE, Patel KD, Jorgenson JW. Ultrahigh-pressure reversed-phase capillary liquid chromatography: isocratic and gradient elution using columns packed with 1.0-micron particles. *Anal Chem.* 1999; 71(3):700–8. [PubMed: 9989386]
24. Yin X, Liu X, Zhang Y, Yan G, Wang F, Lu H, Shen H, Yang P. Rapid and sensitive profiling and quantification of the human cell line proteome by LC-MS/MS without prefractionation. *Proteomics.* 2014; 14(17-18):2008–16. [PubMed: 25044409]
25. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics.* 2002; 1(5):376–86. [PubMed: 12118079]
26. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlett-Jones M, He F, Jacobson A, Pappin DJ. Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics.* 2004; 3(12):1154–69. [PubMed: 15385600]
27. Nguyen V, Cao L, Lin JT, Hung N, Ritz A, Yu K, Jianu R, Ulin SP, Raphael BJ, Laidlaw DH, Brossay L, Salomon AR. A new approach for quantitative phosphoproteomic dissection of signaling pathways applied to T cell receptor activation. *Mol Cell Proteomics.* 2009; 8(11):2418–31. [PubMed: 19605366]
28. Yu K, Salomon AR. PeptideDepot: flexible relational database for visual analysis of quantitative proteomic data and integration of existing protein information. *Proteomics.* 2009; 9(23):5350–8. [PubMed: 19834895]
29. Yu K, Salomon AR. HTAPP: high-throughput autonomous proteomic pipeline. *Proteomics.* 2010; 10(11):2113–22. [PubMed: 20336676]
30. Elias JE, Gygi SP. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat Methods.* 2007; 4(3):207–14. [PubMed: 17327847]

31. Yu K, Sabelli A, DeKeukelaere L, Park R, Sindi S, Gatsonis CA, Salomon A. Integrated platform for manual and high-throughput statistical validation of tandem mass spectra. *Proteomics*. 2009; 9(11):3115–25. [PubMed: 19526561]
32. Beausoleil SA, Villen J, Gerber SA, Rush J, Gygi SP. A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat Biotechnol*. 2006; 24(10):1285–92. [PubMed: 16964243]
33. Demirkan G, Yu K, Boylan JM, Salomon AR, Gruppiso PA. Phosphoproteomic profiling of in vivo signaling in liver by the mammalian target of rapamycin complex 1 (mTORC1). *PLoS One*. 2011; 6(6):e21729. [PubMed: 21738781]
34. Storey JD. The positive false discovery rate: A Bayesian interpretation and the q-value. *Annals of Statistics*. 2003; 31(6):2013–35.
35. Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A*. 2003; 100(16):9440–5. [PubMed: 12883005]
36. Lord GA, Gordon DB, Myers P, King BW. Tapers and restrictors for capillary electrochromatography and capillary electrochromatography mass spectrometry. *Journal of Chromatography A*. 1997; 768(1):9–16.
37. Mayer M, Rapp E, Marck C, Bruin GJM. Fritless capillary electrochromatography. *Electrophoresis*. 1999; 20(1):43–49. [PubMed: 10065957]
38. Gaspar A, Piyasena ME, Gomez FA. Fabrication of fritless chromatographic microchips packed with conventional reversed-phase silica particles. *Analytical Chemistry*. 2007; 79(20):7906–7909. [PubMed: 17845005]
39. Fairchild JN, Walworth MJ, Horvath K, Guiochon G. Correlation between peak capacity and protein sequence coverage in proteomics analysis by liquid chromatography-mass spectrometry/mass spectrometry. *J Chromatogr A*. 2010; 1217(29):4779–83. [PubMed: 20554284]
40. Novakova L, Vaast A, Stassen C, Broeckhoven K, De Pra M, Swart R, Desmet G, Eeltink S. High-resolution peptide separations using nano-LC at ultra-high pressure. *J Sep Sci*. 2013; 36(7):1192–9. [PubMed: 23457143]
41. Trusch M, Ehlert S, Bertsch A, Kohlbacher O, Hildebrand D, Schluter H, Tallarek U. Improved particle-packed HPLC/MS microchips for proteomic analysis. *J Sep Sci*. 2010; 33(21):3283–91. [PubMed: 21049518]
42. Cao L, Ding Y, Hung N, Yu K, Ritz A, Raphael BJ, Salomon AR. Quantitative phosphoproteomics reveals SLP-76 dependent regulation of PAG and Src family kinases in T cells. *PLoS One*. 2012; 7(10):e46725. [PubMed: 23071622]
43. Helou YA, Petrashen AP, Salomon AR. Vav1 Regulates T-Cell Activation through a Feedback Mechanism and Crosstalk between the T-Cell Receptor and CD28. *J Proteome Res*. 2015; 14(7):2963–75. [PubMed: 26043137]
44. Ji Q, Ding Y, Salomon AR. SRC homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) N-terminal tyrosine residues regulate a dynamic signaling equilibrium involving feedback of proximal T-cell receptor (TCR) signaling. *Mol Cell Proteomics*. 2015; 14(1):30–40. [PubMed: 25316710]
45. Ji Q, Salomon AR. Wide-scale quantitative phosphoproteomic analysis reveals that cold treatment of T cells closely mimics soluble antibody stimulation. *J Proteome Res*. 2015; 14(5):2082–9. [PubMed: 25839225]
46. Sjolín-Goodfellow H, Frushicheva MP, Ji Q, Cheng DA, Kadlec TA, Cantor AJ, Kuriyan J, Chakraborty AK, Salomon AR, Weiss A. The catalytic activity of the kinase ZAP-70 mediates basal signaling and negative feedback of the T cell receptor pathway. *Sci Signal*. 2015; 8(377):ra49. [PubMed: 25990959]
47. Abraham RT, Weiss A. Jurkat T cells and development of the T-cell receptor signalling paradigm. *Nat Rev Immunol*. 2004; 4(4):301–8. [PubMed: 15057788]
48. Brill LM, Salomon AR, Ficarro SB, Mukherji M, Stettler-Gill M, Peters EC. Robust phosphoproteomic profiling of tyrosine phosphorylation sites from human T cells using immobilized metal affinity chromatography and tandem mass spectrometry. *Anal Chem*. 2004; 76(10):2763–72. [PubMed: 15144186]

49. Salomon AR, Ficarro SB, Brill LM, Brinker A, Phung QT, Ericson C, Sauer K, Brock A, Horn DM, Schultz PG, Peters EC. Profiling of tyrosine phosphorylation pathways in human cells using mass spectrometry. *Proc Natl Acad Sci U S A*. 2003; 100(2):443–8. [PubMed: 12522270]
50. Okamura H, Aramburu J, Garcia-Rodriguez C, Viola JP, Raghavan A, Tahiliani M, Zhang X, Qin J, Hogan PG, Rao A. Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol Cell*. 2000; 6(3):539–50. [PubMed: 11030334]

Figure 1.

Venn diagram of the number of unique phosphorylation sites residing on significantly changed phosphopeptides and volcano plot analysis of significantly changed phosphopeptides in T cells in response to CD3/4 stimulation analyzed by the three different analytical columns. A and B represent the number of unique total (serine, threonine and tyrosine) and tyrosine phosphorylation sites with significantly altered phosphopeptide abundance, respectively. Volcano plot of fold change ($\log_2$) versus q-value ($-\log_{10}$) of peak-area for 14,377, 15,114 and 22,945 phosphopeptides identified from 15 cm, 3.0 μ m Column (C), 50 cm, 3.0 μ m Column (D) and 15 cm, 1.9 μ m Column (E), respectively. Red and green circles represent the significant ($q > 0.01$) 2-fold down and up regulated peptides, respectively compared with the untreated control cells. The black circles are significant but the fold change is lower than 2. Gray circles below the blue dot horizontal line ($q = 0.01$) are non significant. Pie chart shows percentage of the peptides significantly changed compared with the untreated cells.

Table 1
Quantitative comparison of phosphopeptides derived from unstimulated and CD3/4 stimulated Jurkat cells with different analytical columns

Column length / Particle size	Total # unique phosphopeptide-spectrum matches ^a	# Unique phosphopeptide-spectrum matches with $q < 0.01$ and:		
		All	> 2-fold ^b	> 10-fold ^b
50 cm / 1.9 μ m	23131	16106	7900	660
50 cm / 3 μ m	15173	4860	2117	221
15 cm / 3 μ m	14494	3749	1391	191

^aIncludes all identified unique phosphopeptide-spectrum matches in CD3/4 stimulated and unstimulated Jurkat from five replicates.

^bIndicates number of significantly changed phosphopeptide-spectrum matches (q -value < 0.01) with 2- or 10-fold increase or decrease when comparing average peak area between CD3/4-stimulated and -unstimulated Jurkat.