Journal of proteome research

Article

pubs.acs.org/jpr

Rapid Analyses of Proteomes and Interactomes Using an Integrated Solid-Phase Extraction−Liquid Chromatography−MS/MS System

Nadine A. Binai,[†,‡] Fabio Marino,[†,‡] Peter Soendergaard,[§] Nicolai Bache,[§] Shabaz Mohammed,*[,†,‡,∥,⊥] and Albert J. R. Heck*[,†,‡]

[†]Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, [‡]Netherlands Proteomics Centre, University of Utrecht, Padualaan 8, 3584 CH, Utrecht, The Netherlands

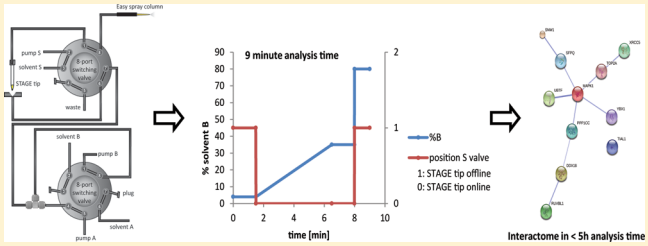
[§]Thermo Fisher Scientific, Edisonsvej 4, DK-5000, Odense C, Denmark

[∥]Chemistry Research Laboratory, Department of Chemistry, University of Oxford, Mansfield Road, OX1 3TA, Oxford, United Kingdom

[⊥]Department of Biochemistry, University of Oxford, South Parks Road, OX1 3QU, Oxford, United Kingdom

Supporting Information

ABSTRACT: Here, we explore applications of a LC system using disposable solid-phase extraction (SPE) cartridges and very short LC−MS/MS gradients that allows for rapid analyses in less than 10 min analysis time. The setup consists of an autosampler harboring two sets of 96 STAGE tips that function as precolumns and a short RP analytical column running 6.5 min gradients. This system combines efficiently with several proteomics workflows such as offline prefractionation methods, including 1D gel electrophoresis and strong-cation exchange chromatography. It also enables the analysis of interactomes obtained by affinity purification with an analysis time of approximately 1 h. In a typical shotgun proteomics experiment involving 36 SCX fractions of an AspN digested cell lysate, we detected over 3600 protein groups with an analysis time of less than 5.5 h. This innovative fast LC system reduces proteome analysis time while maintaining sufficient proteomic detail. This has particular relevance for the use of proteomics within a clinical environment, where large sample numbers and fast turnover times are essential.



KEYWORDS: *nanoliquid chromatography, SPE−LC, solid-phase extraction, STAGE tips, mass spectrometry, protein analysis, proteomes and interactomes*

INTRODUCTION

Over the previous decade, bottom-up proteomics methods have been under continuous improvement and are now able to reveal proteomes with extensive depth.[1−4] Most recently, these improvements have allowed the first drafts of the human proteome to be completed.[5,6] These achievements are possible, in part, due to advances in ultra-high-pressure liquid chromatography (UHPLC) and the increasing speed and sensitivity of mass spectrometers.[7−9] For the chromatographic component, the focus has been predominantly on column peak capacity and proteome depth rather than chromatographic speed. However, a large number of proteome experiments generate relatively simple mixtures that would benefit from rapid LC methods in order to increase sample throughput. Such experiments include affinity purifications,[10,11] chemical proteomics,[12,13] and a number of methods based on prefractionation.[14,15] However, the need for rapid analysis not been completely ignored. For example, Shen and colleagues successfully used a submicron-particle-packed RP column to achieve an 8 min gradient, which was applied for the identification of proteins from bacterial digests.[16] Ding et al. demonstrated a fast approach with a 2D method of two short reverse-phase HPLC runs, aiming to obtain a proteome within a day.[17] Akeroyd and colleagues, on the other hand, used high flow rates to realize very short gradient times (3.5 min) for the screening of microbial libraries.[18] Besides the adjustments in the liquid chromatography part, Baker et al. utilized 15 min gradients combined with the additional separation of ions within the mass spectrometer, using an ion mobility cell for gas-phase separation.[19] Electrophoretic methods have also been used for rapid peptide separation. This was most recently demonstrated by Moini and Martinez, who coupled strong field capillary electrophoresis to MS detecting peptides from single protein digests in 1 min.[20]

Here, we describe and evaluate an LC setup composed of a semiautomated solid-phase extraction (SPE) step for sample concentration and cleanup and an ultrashort reverse-phase (RP) column that is capable of rapid gradient chromatography, a substantial improvement to the setup described earlier by Hørning et al.[21] A more detailed description of this system and initial test results are given in an article by Falkenby et al.[22]

Received: September 29, 2014
Published: November 25, 2014

Briefly, the LC incorporates single-use SPE cartridges or, as they are more commonly called, stop and go extraction (STAGE) tips.[23,24] The use of disposable C18 SPE cartridges as precolumns in this setup results in reduced carryover and increased robustness, which makes this ideal for problematic samples. Furthermore, the immediate use of the STAGE tip as a precolumn shortens sample handling and avoids sample losses between clean up and analysis. While in the setup of Hørning and colleagues[21] isocratic elution was used, this new system generates a gradient via two syringe pumps, providing improved separation of the peptides. Flow rates of 1.5 μL/min guarantee speed and robustness over nanoflow setups. The automated sample loading setup and short cycle times make this system appropriate for high throughput of similar samples, such as in the case of clinical samples that require testing large patient cohorts. Here, we show that this system, coupled to a fast mass spectrometer, allows for very fast throughput with satisfactory identification rates, ultimately putting less demand on MS instrument time.

MATERIALS AND METHODS

Cell Culture and Stimulation

Human T-lymphoma (Jurkat) and cervix carcinoma (HeLa) cells were cultured in RPMI and DMEM, respectively. Each medium was supplemented with 10% FCS, 10 mM L-glutamine, and 5% penicillin/streptomycin (all from Lonza, Braine-l'Alleud, Belgium). Jurkat cells were serum-starved for 1 h before stimulation with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Zwijndrecht, The Netherlands) or DMSO (Sigma-Aldrich) for 20 min. The cells were harvested and immediately washed with ice-cold PBS. The cell pellet was stored at −80 °C until lysis.

Sample Preparation

Cells were lysed in lysis buffer containing 8 M urea (Sigma-Aldrich), 50 mM ammonium bicarbonate (AMBIC; Sigma-Aldrich), and protease inhibitor tablets (Roche, Basel, Switzerland), pH 8. Lysates were cleared by centrifugation for 10 min at 20 000*g* and 4 °C. Supernatant was transferred into a new tube, and the protein amount was determined using a Bradford protein assay (Bio-Rad, Hercules, CA, USA). Next, proteins were digested utilizing the filter-assisted sample preparation (FASP) protocol with minor alterations.[25] Briefly, the reduced and alkylated sample was first treated with Lys-C (Sigma-Aldrich) in an enzyme/substrate ratio of 1:50 (w/w) for 4 h, and then trypsin (Promega, Madison, WI, USA) was applied o/n at 37 °C in an enzyme/substrate ratio of 1:100 (w/w). AspN digests were obtained by incubating the sample first with AspN (Roche) at an enzyme/substrate ratio of 1:100 for 4 h at RT. Subsequently, 100 μL of digestion buffer (1 M urea in 50 mM AMBIC) containing AspN in an enzyme/substrate ratio of 1:200 was added and incubated o/n at RT. Resulting peptide mixtures were desalted using Seppak 1 cm^3 columns (Waters Corporation, Milford, MA, USA).

Immune Precipitation (IP) and In-Gel Digestion

Cells were lysed in IP lysis buffer consisting of 150 mM NaCl (Merck, Darmstadt, Germany), 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 10% glycerol (all Sigma-Aldrich), phosStop phosphatase inhibitor tablet, and protease inhibitor tablet (both Roche) in 20 mM Tris, pH 7.5. To homogenize the samples, a douncer with pestle B was used three times for 30 s on ice. Lysates were cleared by centrifugation for 10 min at 20 000*g* and 4 °C. Supernatant was transferred into a new tube, and the protein amount was determined by Bradford protein assay (Bio-Rad). For each immune precipitation, 600 μg of lysate was mixed with 30 μL of phospho-p44/42 MAPK XP rabbit mAb sepharose bead conjugate (Cell Signaling Technology, Danvers, MA, USA) and incubated o/n at 4 °C on a rotator. After five washing steps with lysis buffer, proteins were denatured by boiling for 5 min in XT loading buffer (Bio-Rad). The denatured sample was then separated on a criterion precast 4−12% bis-tris gel (Bio-Rad). Each gel lane was cut into 11 pieces, and a standard in-gel digestion with trypsin (Promega) was performed as described previously.[26]

Western Blot

Fifty micrograms of protein lysate was separated on a Novex precast 4−12% bis-tris gel (Life Technology, The Netherlands) and subsequently transferred onto nitrocellulose membrane. To detect phosphorylated proteins, western blot analysis was performed using anti-phospho-p44/42 MAPK XP antibody (Cell Signaling Technology). Equal loading was confirmed by incubating the membrane with α-tubulin antibody (Pierce, Rockford, IL, USA).

Strong-Cation Exchange Chromatography (SCX)

Strong-cation exchange chromatography was used as described earlier.[27] We applied an improved SCX system to fractionate the samples. Briefly, an Agilent 1100 HPLC system (Aglilent, Santa Clara, CA, USA) was equipped with an Opti-Lynx (Optimized Technologies, Portland, OR, USA) trapping cartridge and a Zorbax Bio-SCX II column (0.8 mm i.d. × 50 mm length, 3.5 μm; Agilent). Solvent A consisted of 0.05% formic acid in 20% acetonitrile, and solvent B contained 0.05% formic acid and 0.5 M NaCl in 20% acetonitrile. Six hundred micrograms of labeled peptide mixture was loaded, and a total of 50 fractions (1 min each, 50 μL elution volume) were collected and dried down in a vacuum concentrator. The salt gradient used for elution of the peptides was as follows: 0−0.01 min (0−2% B), 0.01−8.01 min (2−3% B), 8.01−14.01 min (3−8% B), 14.01−28 min (8−20% B), 28−38 min (20−40% B), 38−48 min (40−90% B), 48−54 min (90% B), and 54−60 min (0% B).

Liquid Chromatography (LC)−Mass Spectrometry (MS)

The Easy SPE−LC (Thermo, in development) was operated with an easy spray column (Reprosil C18, 1.9 μm particles packed in 200 μm i.d. capillary of 7 cm length with an 15 μm i.d. polished emitter). The sample was loaded onto C18 STAGE tips, which were then placed in the autosampler. While the analytical column is equilibrated with solvent A (0.1% formic acid), a robotic arm moves the STAGE tip to a connector in the flow path. The 5 min gradient from 4 to 35% solvent B (99.9% acetonitrile, 0.1% formic acid) is then applied through the STAGE tip, eluting the peptides to the analytical column, where they are further separated and elute into the mass spectrometer via an Easy-ESI interface (Thermo). The

Table 1. Run Schedule of the Easy SPE−LC System

activity	time
equilibration/placing STAGE tip	0−1.5 min
gradient 4−35% B	1.5−6.5 min
elution 35% B	6.5−8 min
column wash 80% B/removing STAGE tip	8−9 min
total analysis time per sample	**9 min**

dx.doi.org/10.1021/pr501011z | J. Proteome Res. 2015, 14, 977−985

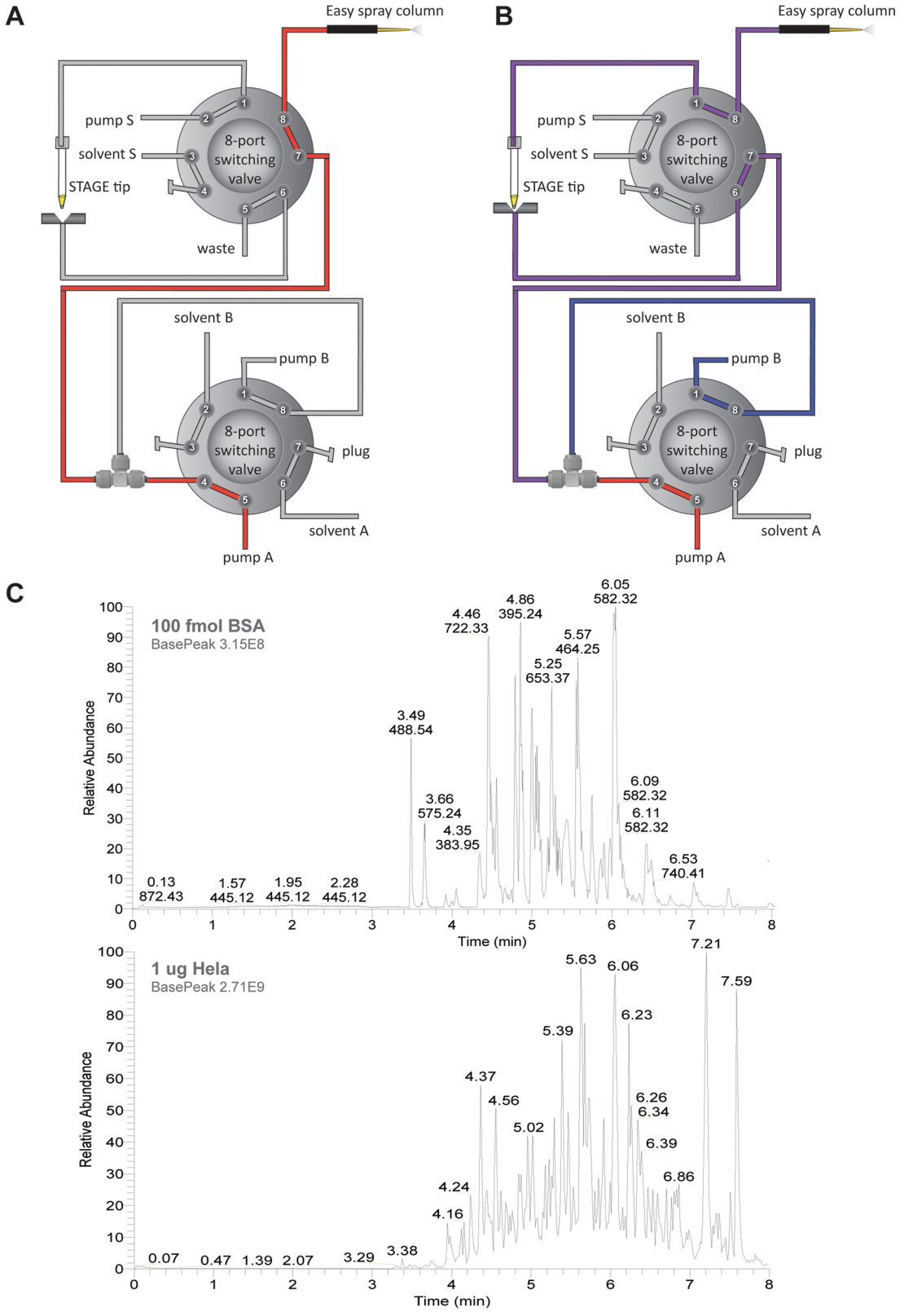


Figure 1. Experimental setup of the Easy SPE–LC system. (A) The sample is immobilized on the STAGE tip, which is moved from the autosampler into the flow line. At the same time, the analytical column is equilibrated with solvent A. (B) The gradient is generated by pumps A and B, and the solvents flow through the STAGE tip to the analytical column, where peptides are separated by hydrophobicity and sprayed into the mass spectrometer. (C) Typical base peak ion chromatograms of BSA and HeLa digests. Note that the acquisition starts 1 min after the beginning of the method, leading to a total analysis time of 9 min.

valves and pumps are controlled through preconfigured software (Table 1).

UHPLC runs on HeLa digests were performed using an Easy nano-LC (Thermo, Bremen, Germany) equipped with an easy

dx.doi.org/10.1021/pr501011z | J. Proteome Res. 2015, 14, 977–985

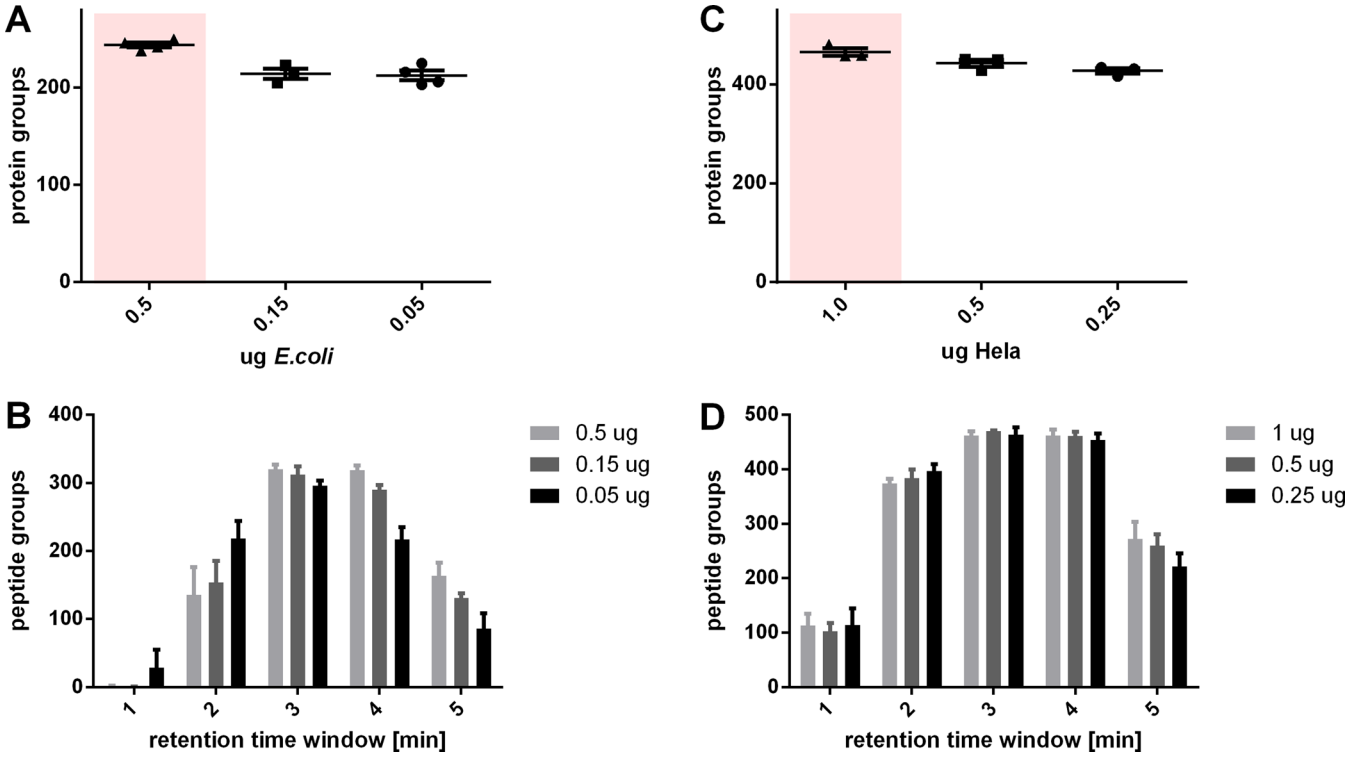


Figure 2. Benchmarking the Easy SPE–LC system. Decreasing amounts (0.5, 0.15, and 0.05 μg for *E. coli* and 1, 0.5, and 0.25 μg for HeLa) of standard tryptic digests were analyzed in technical replicates with the Easy SPE–LC system. The resulting numbers of identified protein groups from digests from *E. coli* (A) and human HeLa cells (C) are plotted, including the mean of replicate runs and SEM. The identified peptides of (B) *E. coli* and (D) HeLa are plotted in 1 min retention time bins.

spray column (Pepmap RSLC, C18, 100 Å, 2 μm particles packed in 75 μm i.d. capillary of 50 cm length, Thermo).[4] The sample was picked up from the autosampler vial plate and loaded into the loop using the Pump S at 20 μL/min with solvent A (0.1% formic acid). During the sample pick up, 5 and 1 μL of solvent A were used to equilibrate the precolumn and the analytical column, respectively, at a controlled back pressure of 700 bar. The sample was loaded from the loop on the back-flushed trap column (Thermo, PepMap RSLC, C18, 100 Å, 5 μm particles packed in 5 mm trap column with 300 μm i.d.), and the trap was connected to waste for the time needed to wash with 20 μL of solvent A. After the loading/washing step, the back-flushed trap column was then switched online with the analytical column, and a gradient of solvent A and B (99.9% acetonitrile, 0.1% formic acid) was started. The gradient for the separation ranged from 7 to 30% of solvent B in 22, 37, 67, 97, and 157 min, depending on method length. After each gradient, the column was washed for 2 min by increasing the buffer B concentration to 100% followed by conditioning the system with 93% buffer A for at least 15 min (Supporting Information Table 1). The applied flow rate was 150 nL/min.

Both LC setups were interfaced with a QExactive mass spectrometer (Thermo). The peptides were eluted from the reverse-phase column and directly sprayed into the mass spectrometer. The instrument was operated in data-dependent acquisition mode using the following settings: ESI voltage, 1.9 kV; inlet capillary temperature, 275 °C; scan range, 350–1500 m/z; full scan automatic gain control target, 3×10^6 ions at 35 000 resolution; Orbitrap full scan maximum injection time, 250 ms; up to 10 precursor ions were picked for fragmentation; ms2 scan AGC target was 5×10^4 ions at 17 500 resolution; maximum injection time, 120 ms; normalized collision energy, 25; dynamic exclusion time, 10 s; and isolation window, 1.5 m/z. Some settings were modified for the SPE–LC runs as indicated in Supporting Information Figure 1.

Data Analysis

Raw data were initially processed with Proteome Discoverer 1.4 (Thermo). The created peak lists were searched with Mascot (Matrix Science, Version 2.4) using the human UniProt database (20 496 entries, Sep 2012) and the following parameters: 50 ppm precursor mass tolerance and 0.05 Da fragment ion tolerance. Trypsin was specified as enzyme, the fragment ion type as ESI-QUAD-TOF up to two missed cleavages were accepted, oxidation of methionine was set up as variable modification, and cysteine carbamidomethylation as fixed modification. Percolator calculated the target FDR with a strict cutoff of 0.01.[28] After identification and quantification, all results were combined and filtered with the following criteria: Mascot ion score of at least 20 on peptides and proteins, maximum peptide rank 1, high peptide confidence, maximum search engine rank 1.

IP data were loaded into CRAPome,[29] which was run with default settings. Normalized spectral counting was used for quantification. The normalization is done by CRAPome and based on the length of the peptide sequence. Results were filtered for known interactions with an iRef score of 1 and for true interactions with a rather stringent SAINT score > 0.8.

Pathway enrichment analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov).[30] The heatmap was created using the [R] package ggplot2 (R Core Team).

dx.doi.org/10.1021/pr501011z | J. Proteome Res. 2015, 14, 977–985

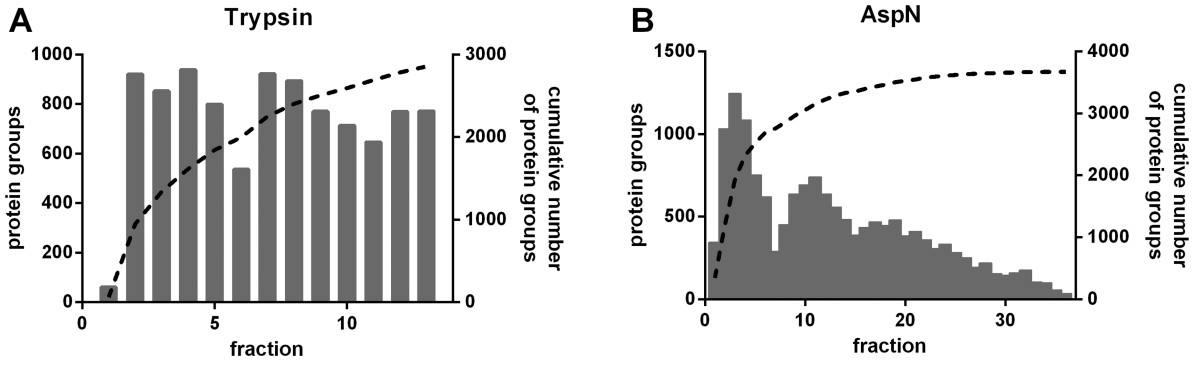


Figure 3. SCX prefractionation of a HeLa digest substantially increased the number of protein identifications. (A) Thirteen fractions of an SCX from 400 μg of tryptic HeLa digest were analyzed with the Easy SPE–LC system. (B) Thirty six fractions from 300 μg of AspN digest were measured. The gray bars show the number of identified protein groups per run. The dashed line depicts the cumulative number of protein groups.

RESULTS AND DISCUSSION

Identification of 500 Protein Groups Using a 5 min Gradient

The Easy SPE–LC system provides a unique design (Figure 1) where the sample is applied via an SPE step followed by a very short RP analytical column separation (see ref 22 for a more detailed description). A flow rate of 1.5 μL/min ensures high sample throughput and increased robustness.[22] To aid throughput and reduce sample carryover, the sample is initially loaded off-line onto a single-use STAGE tip. The autosampler of the system can handle 2 × 96 STAGE tips. After equilibration of the analytical column, the STAGE tip is placed in-line with the analytical column, after which a gradient is applied. As depicted in Table 1, the total analysis time of one run is 9 min. The gradient time is 6.5 min and is composed of the 5 min ramping from 4 to 35% solvent B plus the 1.5 min elution at 35%. During this step, the peptides are displaced from the STAGE tip onto the analytical column, where peptides are resolved prior to mass spectrometric analysis. Given the short gradient time, it is beneficial to couple this setup to a fast mass spectrometer; in our case, a QExactive mass analyzer was utilized.[31] The resolving power of this system is demonstrated with a 100 fmol BSA digest, showing baseline resolution of the peptide peaks in the ion chromatogram (Figure 1C). With a calculated average peak width at half-maximum of 1.54 s, the peak capacity (13.4%) was 147. These results are in line with the results from Falkenby et al.,[22] demonstrating interlaboratory reproducibility of the system. The lag time of 3 min is due to the void volume between the STAGE tip and the analytical column. We initially characterized the sensitivity and comprehensiveness of the setup on complex samples by analyzing aliquots of standard cell digests. For *Escherichia coli*, maximal results were achieved with 500 ng of digest; some 200 protein groups were identified with the 5 min gradient. Interestingly, only a small reduction in performance was observed by decreasing this amount 10-fold, i.e., 50 ng (Figure 2A). Investigating the number of identified peptides in 1 min retention time windows revealed no obvious differences between analyses of differing amounts of material (Figure 2B). Since there is a pronounced increase in complexity between bacterial and mammalian proteomes, we also evaluated the performance of the system on more complex human cell samples. HeLa cells are widely used in proteomics studies to benchmark new technologies.[3,32] Here, maximal results were achieved with 1 μg of HeLa digest, leading to the identification of over 450 protein groups (Figure 2C). Similar to the *E. coli* experiment, reduction of the injected amount (in this case, 4 times less) resulted in a marginal decrease (ca. 400 identified protein groups). These results are also reflected at the peptide level (Figure 2D). Furthermore, we evaluated the most efficient number of MS2 scans per full scan. Optimal results were achieved using a top-10 method (10 MS2 scans per full scan). As described in the work of Kelstrup et al., optimization of settings, such as AGC targets and max injection times (IT), can improve peptide identifications.[33] Further optimization of the settings led to the identification of around 500 protein groups per run (Supporting Information Figure 1). The Jensen group managed similar results using only slightly different AGC target settings and ion injection times,[22] demonstrating excellent consistency in performance between laboratories. Translating this result into protein identifications per minute, we obtained a remarkable 76 protein groups per minute of gradient time. Therefore, we hypothesized that the current ultrafast system would be particularly beneficial for the analysis of proteome samples of intermediate complexity, of which we describe several varieties further below.

Rapid Analysis of Prefractionated Samples: Ion-Exchange Chromatography and Tryptic Peptides

Because 2D setups have proven to be a successful strategy for mining in-depth proteomes,[14,34,35] we used the SPE–LC to analyze SCX fractions, a common method of prefractionation for complex samples.[36,37] The same HeLa digest as that used above was subjected to offline SCX fractionation,[27] where a portion of each fraction was subsequently analyzed with the Easy SPE–LC. When analyzing the 13 most appropriate SCX fractions, a total of 2833 protein groups could be identified in only 84.5 min of accumulated (6.5 min per SCX fraction) gradient time (117 min of total analysis time) (Figure 3A). These identification rates are competitive with current 1D (UHPLC) strategies both in analysis time and in number of identified proteins (Supporting Information Figure 2).

Rapid Analysis of SCX-Fractionated Nontryptic Digests

The use of multiple proteases has proven to be beneficial for extending proteome coverage.[37−39] However, the use of alternative enzymes for protein digestion creates peptides with properties that can be substantially different, compared to the very well-characterized analysis of tryptic digests. Hence, the question arises as to which types of peptides are most useful for mass spectrometric sequencing. We hypothesize that a screening with the SPE–LC system can provide rapid and

dx.doi.org/10.1021/pr501011z | J. Proteome Res. 2015, 14, 977−985

useful insight. Therefore, we tested the performance and screening capabilities of the LC with nontryptic digests. A HeLa cell lysate was digested with the endoproteinase AspN, and an aliquot was subsequently fractionated with SCX. Where trypsin produces predominantly 2+ and 3+ peptides, because it cleaves peptides at the two major basic residues, AspN generates peptides with a larger range of basic residues (and therefore higher charge states). This is reflected by the higher abundances of peptides being detected in the later SCX fractions (Supporting Information Figure 3). To determine whether these highly charged peptides provide a worthy addition for protein identification, we screened every fraction on the Easy SPE–LC system. The latter 20 fractions of the SCX run contained salt at concentration levels that can compromise standard nano-LC systems. We argue that our system could therefore be beneficial because the SPE setup does not require a prior desalting step, as this occurs on the STAGE tip itself. The total gradient time for the 36 analyzed fractions was 234 min (analysis time 324 min). In this experiment, 3671 protein groups could be identified. However, the cumulative number of protein groups leveled off after the first 15 fractions (Figure 3B). Only 275 protein groups resulted from the latter 20 fraction containing >4+ charge states. The current generation of instruments still cannot overcome the limitations of CID for highly charged peptides.[40] For longer/higher charged peptides, other fragmentation techniques such as ETD[41] or EThcD[42] may be more suitable. Strikingly, we were able to do this entire experiment in 5.4 h, compared to the several days that one would need with a regular LC–MS system, thus demonstrating the screening potential of the system. Such screens take a fraction of a day.

Rapid Analysis of Peptides Following In-Gel Digestion

Another frequently used fractionation method for protein samples before LC–MS is separation by SDS-PAGE.[43] It is a popular method since it removes detergents and other low molecular weight impurities.[44] To mimic a typical experiment, 50 μg of HeLa protein lysate was separated, and each gel lane was divided in to 11 sections (in triplicate). After digestion, each section was analyzed using the Easy SPE–LC system. In 71.5 min cumulative gradient time (99 min total analysis time), approximately 1800 protein groups could be detected. An overlap of 75% between lanes was observed, demonstrating excellent reproducibility of the system (Figure 4). Furthermore, this setup is very robust, with issues observed with only 6 out of 986 samples run to date, equating to an error rate of just 0.6%. Runs that failed were predominantly due to a blockage of the tip. Our findings correspond well with the results of Falkenby et al.,[22] who showed only minor performance loss after 192 consecutive injections of partially digested *E. coli* lysate.

Rapid Analysis of Interactomes Acquired by Affinity Purification

Since pull-down experiments result in medium-complexity samples as far as the number of proteins present is concerned, we hypothesized that such experiments would make an ideal partnership with analysis by the Easy SPE–LC system. We tested the use of the system under these circumstances by studying the interaction partners of a central signaling molecule, extracellular signal regulated kinase (ERK), with human Jurkat T cells. Previously, our laboratory extensively studied protein kinase A (PKA) signaling in this cell type.[10,45] However, protein kinase C (PKC), another member of the AGC kinase family, plays an important role in the activation of

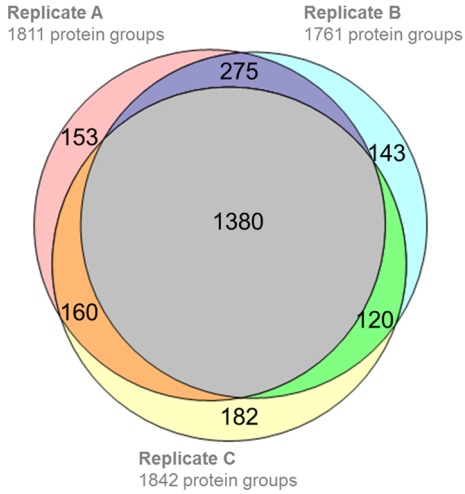


Figure 4. Analysis of gel-LC (SDS-PAGE) samples: in-gel digestion (11 sections) of an SDS-PAGE separated HeLa sample was performed in triplicate. The Venn diagram shows the overlap between the experiments.

T cells upon T-cell receptor engagement, a crucial function of the immune system. Here, we utilize the PKC activator phorbol 12-myristate 13-acetate (PMA) to trigger downstream signaling, resulting in the activation of the mitogen activated protein kinases (MAPK) ERK1/2 through phosphorylation, as validated by western blot analysis (Figure 5A). By using an antibody specific to the activated form of ERK1/2 for a pull-down experiment, interactions depending upon phosphorylation could be detected. Next to the phosphospecific ERK antibody, we used an isotype control antibody coupled to the same type of beads as a negative control. In total, 1490 protein groups were identified by IP using the antibody against the activated form of ERK1/2; however, 897 were also found in the negative control, suggesting a large number of nonspecific interactions. Several software algorithms have been developed to evaluate putative protein–protein interactions that often use the background data that is generated by the negative control.[46−48] We used a recently developed software tool, the SAINT express function in CRAPome, to discriminate between true and contaminant interactions,[29,49] resulting in a list of 177 potential interactors (Supporting Information Table 2). Alignment with the iRef database further confirmed the identification of 11 known interactors of phospho-ERK1 within our data set (Table 2). The top hit was upstream-binding factor 1 (UBTF), which acts as part of the transcription machinery to activate ribosomal gene transcription upon phosphorylation by ERK.[50] ERK (MAPK1) itself was also detected in our data. As expected, significantly higher spectral counts for MAPK1 were identified in the stimulated cells compared to that in the untreated cells (Table 2), which fits with the increased phosphorylation of ERK observed in the western blot (Figure 5A). Next, we searched for pathways of these new putative interacting proteins, and we formed three groups: (i) detected only without the stimulus, (ii) only upon stimulation, and (iii) interacting under both conditions. For all three groups, the enriched pathways were extracted using the reactome pathways function in DAVID. Several pathways were detected in more than one of the groups. By clustering the *p*-values of the enrichment, we were able to define several functional clusters. Pathways upon stimulation with PMA included amino acid metabolism,[51] proteasomal degradation, and cell cycle regu-

dx.doi.org/10.1021/pr501011z | J. Proteome Res. 2015, 14, 977−985

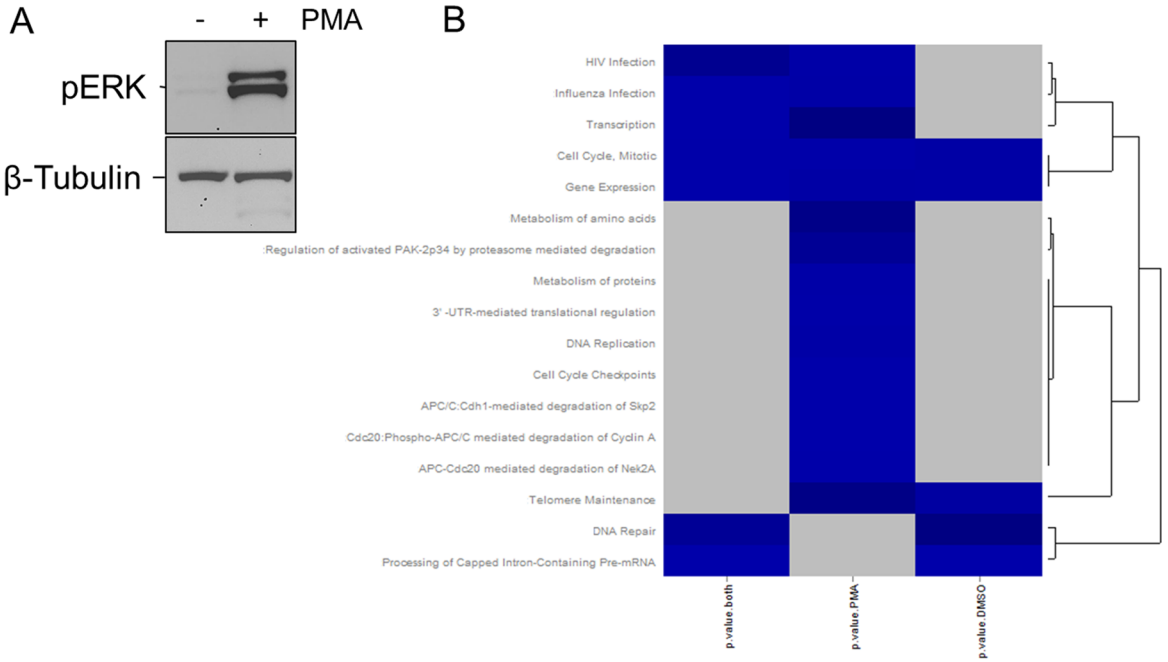


Figure 5. Fast analysis of protein interaction networks, using the interactome of phosphoERK as test-case. (A) Western blot showing the phosphorylation of ERK upon PMA stimulation. β-Tubulin was used as a loading control. (B) Heatmap of p-values of the pathway enrichment analysis from three groups of proteins: "both" contains all proteins that are found to specifically interact with pERK independent of the stimulation, "PMA" contains all proteins that specifically interact upon stimulation, and "DMSO" harbors all proteins interacting only in unstimulated cells. The darker the blue, the stronger the enrichment. Gray fields in the heatmap indicate that this pathway was not enriched under the respective conditions.

Table 2. Known Interactors of ERK[a]

		spectral counts			
gene name	protein name	IP pERK PMA	IP pERK DMSO	IP isotype	SAINT probability
UBTF	Nucleolar transcription factor 1	11	12	0	1
RUVBL1	RuvB-like 1	9	10	0	1
PPP1CC	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	6	12	0	0.97
YBX1	Nuclease-sensitive element-binding protein 1	6	5	0	0.96
SNW1	SNW domain-containing protein 1	3	4	0	0.89
TIAL1	Nucleolysin TIAR	2	5	0	0.8
DDX18	ATP-dependent RNA helicase DDX18	6	9	1	0.91
MAPK1	Mitogen-activated protein kinase 1	52	5	2	1
TOP2A	DNA topoisomerase 2-alpha	92	126	3	1
SFPQ	Splicing factor. proline- and glutamine-rich	26	54	5	0.99
XRCC5	X-ray repair cross-complementing protein 5	34	20	6	0.92

[a]SAINT/CRAPome results of the pERK pull down were filtered by SAINT probability (SP) score > 0.8, iRef = 1. Furthermore, the table contains the spectral counts for each sample.

lation[52] (Figure 5C). The identification of these known and putative interactors was performed in 214.5 min cumulative gradient time (297 min total analysis time), demonstrating the power of this system once more.

CONCLUSIONS

The Easy SPE–LC system provides a robust (error rate < 1% over 1000 runs), powerful tool for rapid and thus cost-effective proteome analysis. The unique setup with disposable STAGE tips as a precolumn reduces carry over and increases robustness. We demonstrate its versatility in the analysis of complex digests of lysates, but we believe that its real power surfaces when used in combination with either prefractionation workflows (gel-LC, SCX) or affinity purification enrichments. We envision this system to be very helpful for screening purposes, determining protein-interaction networks.

ASSOCIATED CONTENT

Supporting Information

Figure 1: Optimization of MS method for maximal protein identification. Figure 2: Comparison of short gradient 2D methods with UHPLC single runs. Figure 3: UV traces from SCX of HeLa digested with AspN or trypsin. Table 1: UHPLC gradient methods. Table 2: SAINT/CRAPome results of the pERK pull down. This material is available free of charge via the Internet at http://pubs.acs.org. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium[53] via the PRIDE partner repository with the data set identifier PXD001111.

AUTHOR INFORMATION

Corresponding Authors

*(S.M.) E-mail: shabaz.mohammed@chem.ox.ac.uk.
*(A.J.R.H.) E-mail: a.j.r.heck@uu.nl.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The Netherlands Proteomics Centre is kindly acknowledged for financial support. Part of this research was performed within the framework of the PRIME-XS project, grant no. 262067, funded by the European Union Seventh Framework Program and The Netherlands Organization for Scientific Research (NWO) supported large scale proteomics facility *Proteins@Work* (project 184.032.201) as well as the VIDI grant to S.M. (700.10.429).

■ REFERENCES

(1) Köcher, T.; Pichler, P.; Swart, R.; Mechtler, K. Analysis of protein mixtures from whole-cell extracts by single-run nanoLC−MS/MS using ultralong gradients. *Nat. Protoc.* **2012**, *7*, 882−90.

(2) Cristobal, A.; Hennrich, M. L.; Giansanti, P.; Goerdayal, S. S.; Heck, A. J.; Mohammed, S. In-house construction of a UHPLC system enabling the identification of over 4000 protein groups in a single analysis. *Analyst* **2012**, *137*, 3541−8.

(3) Nagaraj, N.; Wisniewski, J. R.; Geiger, T.; Cox, J.; Kircher, M.; Kelso, J.; Pääbo, S.; Mann, M. Deep proteome and transcriptome mapping of a human cancer cell line. *Mol. Syst. Biol.* **2011**, *7*, 548.

(4) Pirmoradian, M.; Budamgunta, H.; Chingin, K.; Zhang, B.; Astorga-Wells, J.; Zubarev, R. A. Rapid and deep human proteome analysis by single-dimension shotgun proteomics. *Mol. Cell. Proteomics* **2013**, *12*, 3330−8.

(5) Kim, M. S.; Pinto, S. M.; Getnet, D.; Nirujogi, R. S.; Manda, S. S.; Chaerkady, R.; Madugundu, A. K.; Kelkar, D. S.; Isserlin, R.; Jain, S.; Thomas, J. K.; Muthusamy, B.; Leal-Rojas, P.; Kumar, P.; Sahasrabuddhe, N. A.; Balakrishnan, L.; Advani, J.; George, B.; Renuse, S.; Selvan, L. D.; Patil, A. H.; Nanjappa, V.; Radhakrishnan, A.; Prasad, S.; Subbannayya, T.; Raju, R.; Kumar, M.; Sreenivasamurthy, S. K.; Marimuthu, A.; Sathe, G. J.; Chavan, S.; Datta, K. K.; Subbannayya, Y.; Sahu, A.; Yelamanchi, S. D.; Jayaram, S.; Rajagopalan, P.; Sharma, J.; Murthy, K. R.; Syed, N.; Goel, R.; Khan, A. A.; Ahmad, S.; Dey, G.; Mudgal, K.; Chatterjee, A.; Huang, T. C.; Zhong, J.; Wu, X.; Shaw, P. G.; Freed, D.; Zahari, M. S.; Mukherjee, K. K.; Shankar, S.; Mahadevan, A.; Lam, H.; Mitchell, C. J.; Shankar, S. K.; Satishchandra, P.; Schroeder, J. T.; Sirdeshmukh, R.; Maitra, A.; Leach, S. D.; Drake, C. G.; Halushka, M. K.; Prasad, T. S.; Hruban, R. H.; Kerr, C. L.; Bader, G. D.; Iacobuzio-Donahue, C. A.; Gowda, H.; Pandey, A. A draft map of the human proteome. *Nature* **2014**, *509*, 575−81.

(6) Wilhelm, M.; Schlegl, J.; Hahne, H.; Moghaddas Gholami, A.; Lieberenz, M.; Savitski, M. M.; Ziegler, E.; Butzmann, L.; Gessulat, S.; Marx, H.; Mathieson, T.; Lemeer, S.; Schnatbaum, K.; Reimer, U.; Wenschuh, H.; Mollenhauer, M.; Slotta-Huspenina, J.; Boese, J. H.; Bantscheff, M.; Gerstmair, A.; Faerber, F.; Kuster, B. Mass-spectrometry-based draft of the human proteome. *Nature* **2014**, *509*, 582−7.

(7) Zubarev, R. A.; Makarov, A. Orbitrap mass spectrometry. *Anal. Chem.* **2013**, *85*, 5288−96.

(8) Altelaar, A. F.; Munoz, J.; Heck, A. J. Next-generation proteomics: towards an integrative view of proteome dynamics. *Nat. Rev. Genet.* **2013**, *14*, 35−48.

(9) MacNair, J. E.; Patel, K. D.; Jorgenson, J. W. Ultrahigh-pressure reversed-phase capillary liquid chromatography: isocratic and gradient elution using columns packed with 1.0-micron particles. *Anal. Chem.* **1999**, *71*, 700−8.

(10) Giansanti, P.; Stokes, M. P.; Silva, J. C.; Scholten, A.; Heck, A. J. Interrogating cAMP-dependent kinase signaling in jurkat T cells via a protein kinase A targeted immune-precipitation phosphoproteomics approach. *Mol. Cell. Proteomics* **2013**, *12*, 3350−9.

(11) Lambert, J. P.; Tucholska, M.; Pawson, T.; Gingras, A. C. Incorporating DNA shearing in standard affinity purification allows simultaneous identification of both soluble and chromatin-bound interaction partners. *J. Proteomics* **2014**, *100*, 55−9.

(12) Scholten, A.; Poh, M. K.; van Veen, T. A.; van Breukelen, B.; Vos, M. A.; Heck, A. J. Analysis of the cGMP/cAMP interactome using a chemical proteomics approach in mammalian heart tissue validates sphingosine kinase type 1-interacting protein as a genuine and highly abundant AKAP. *J. Proteome Res.* **2006**, *5*, 1435−47.

(13) Ku, X.; Heinzlmeir, S.; Helm, D.; Médard, G.; Kuster, B. New affinity probe targeting VEGF receptors for kinase inhibitor selectivity profiling by chemical proteomics. *J. Proteome Res.* **2014**, *13*, 2445−52.

(14) Di Palma, S.; Hennrich, M. L.; Heck, A. J.; Mohammed, S. Recent advances in peptide separation by multidimensional liquid chromatography for proteome analysis. *J. Proteomics* **2012**, *75*, 3791−813.

(15) Motoyama, A.; Yates, J. R. Multidimensional LC separations in shotgun proteomics. *Anal. Chem.* **2008**, *80*, 7187−93.

(16) Shen, Y.; Smith, R. D.; Unger, K. K.; Kumar, D.; Lubda, D. Ultrahigh-throughput proteomics using fast RPLC separations with ESI-MS/MS. *Anal. Chem.* **2005**, *77*, 6692−701.

(17) Ding, C.; Jiang, J.; Wei, J.; Liu, W.; Zhang, W.; Liu, M.; Fu, T.; Lu, T.; Song, L.; Ying, W.; Chang, C.; Zhang, Y.; Ma, J.; Wei, L.; Malovannaya, A.; Jia, L.; Zhen, B.; Wang, Y.; He, F.; Qian, X.; Qin, J. A fast workflow for identification and quantification of proteomes. *Mol. Cell. Proteomics* **2013**, *12*, 2370−80.

(18) Akeroyd, M.; Olsthoorn, M.; Gerritsma, J.; Gutker-Vermaas, D.; Ekkelkamp, L.; van Rij, T.; Klaassen, P.; Plugge, W.; Smit, E.; Strupat, K.; Wenzel, T.; van Tilborg, M.; van der Hoeven, R. Searching for microbial protein over-expression in a complex matrix using automated high throughput MS-based proteomics tools. *J. Biotechnol.* **2013**, *164*, 112−20.

(19) Baker, E. S.; Livesay, E. A.; Orton, D. J.; Moore, R. J.; Danielson, W. F.; Prior, D. C.; Ibrahim, Y. M.; LaMarche, B. L.; Mayampurath, A. M.; Schepmoes, A. A.; Hopkins, D. F.; Tang, K.; Smith, R. D.; Belov, M. E. An LC−IMS−MS platform providing increased dynamic range for high-throughput proteomic studies. *J. Proteome Res.* **2010**, *9*, 997−1006.

(20) Moini, M.; Martinez, B. Ultrafast capillary electrophoresis/mass spectrometry with adjustable porous tip for a rapid analysis of protein digest in about a minute. *Rapid Commun. Mass Spectrom.* **2014**, *28*, 305−10.

(21) Hørning, O. B.; Kjeldsen, F.; Theodorsen, S.; Vorm, O.; Jensen, O. N. Isocratic solid phase extraction-liquid chromatography (SPE-LC) interfaced to high-performance tandem mass spectrometry for rapid protein identification. *J. Proteome Res.* **2008**, *7*, 3159−67.

(22) Falkenby, L. G.; Such-Sanmartin, G.; Larsen, M. R.; Vorm, O.; Bache, N.; Jensen, O. N. Integrated solid phase extraction-capillary liquid chromatography (speLC) interfaced to ESI-MS/MS for fast characterization and quantification of proteins and proteomes. *J. Proteome Res.* **2014**, DOI: 10.1021/pr5008575.

(23) Rappsilber, J.; Ishihama, Y.; Mann, M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* **2003**, *75*, 663−70.

(24) Ishihama, Y.; Rappsilber, J.; Mann, M. Modular stop and go extraction tips with stacked disks for parallel and multidimensional Peptide fractionation in proteomics. *J. Proteome Res.* **2006**, *5*, 988−94.

(25) Wiśniewski, J. R.; Zougman, A.; Nagaraj, N.; Mann, M. Universal sample preparation method for proteome analysis. *Nat. Methods* **2009**, *6*, 359−62.

(26) Taouatas, N.; Drugan, M. M.; Heck, A. J.; Mohammed, S. , Straightforward ladder sequencing of peptides using a Lys-N metalloendopeptidase. *Nat. Methods* **2008**, *5*, 405−7.

(27) Pinkse, M. W.; Mohammed, S.; Gouw, J. W.; van Breukelen, B.; Vos, H. R.; Heck, A. J. Highly robust, automated, and sensitive online TiO_2-based phosphoproteomics applied to study endogenous phosphorylation in *Drosophila melanogaster*. *J. Proteome Res.* **2008**, *7*, 687−97.

(28) Käll, L.; Canterbury, J. D.; Weston, J.; Noble, W. S.; MacCoss, M. J. Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat. Methods* **2007**, *4*, 923−5.

(29) Mellacheruvu, D.; Wright, Z.; Couzens, A. L.; Lambert, J. P.; St-Denis, N. A.; Li, T.; Miteva, Y. V.; Hauri, S.; Sardiu, M. E.; Low, T. Y.; Halim, V. A.; Bagshaw, R. D.; Hubner, N. C.; Al-Hakim, A.; Bouchard, A.; Faubert, D.; Fermin, D.; Dunham, W. H.; Goudreault, M.; Lin, Z. Y.; Badillo, B. G.; Pawson, T.; Durocher, D.; Coulombe, B.; Aebersold, R.; Superti-Furga, G.; Colinge, J.; Heck, A. J.; Choi, H.; Gstaiger, M.; Mohammed, S.; Cristea, I. M.; Bennett, K. L.; Washburn, M. P.; Raught, B.; Ewing, R. M.; Gingras, A. C.; Nesvizhskii, A. I. The CRAPome: a contaminant repository for affinity purification-mass spectrometry data. *Nat. Methods* **2013**, *10*, 730−6.

(30) Huang, d. W.; Sherman, B. T.; Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **2009**, *4*, 44−57.

(31) Michalski, A.; Damoc, E.; Hauschild, J. P.; Lange, O.; Wieghaus, A.; Makarov, A.; Nagaraj, N.; Cox, J.; Mann, M.; Horning, S. Mass spectrometry-based proteomics using Q Exactive, a high-performance benchtop quadrupole Orbitrap mass spectrometer. *Mol. Cell. Proteomics* **2011**, *10*, M111.011015.

(32) Landry, J. J.; Pyl, P. T.; Rausch, T.; Zichner, T.; Tekkedil, M. M.; Stütz, A. M.; Jauch, A.; Aiyar, R. S.; Pau, G.; Delhomme, N.; Gagneur, J.; Korbel, J. O.; Huber, W.; Steinmetz, L. M. The genomic and transcriptomic landscape of a HeLa cell line. *G3: Genes, Genomes, Genet.* **2013**, *3*, 1213−24.

(33) Kelstrup, C. D.; Young, C.; Lavallee, R.; Nielsen, M. L.; Olsen, J. V. Optimized fast and sensitive acquisition methods for shotgun proteomics on a quadrupole Orbitrap mass spectrometer. *J. Proteome Res.* **2012**, *11*, 3487−97.

(34) Gilar, M.; Fridrich, J.; Schure, M. R.; Jaworski, A. Comparison of orthogonality estimation methods for the two-dimensional separations of peptides. *Anal. Chem.* **2012**, *84*, 8722−32.

(35) Gilar, M.; Olivova, P.; Daly, A. E.; Gebler, J. C. Orthogonality of separation in two-dimensional liquid chromatography. *Anal. Chem.* **2005**, *77*, 6426−34.

(36) Betancourt, L. H.; De Bock, P. J.; Staes, A.; Timmerman, E.; Perez-Riverol, Y.; Sanchez, A.; Besada, V.; Gonzalez, L. J.; Vandekerckhove, J.; Gevaert, K. SCX charge state selective separation of tryptic peptides combined with 2D-RP-HPLC allows for detailed proteome mapping. *J. Proteomics* **2013**, *91*, 164−71.

(37) Low, T. Y.; van Heesch, S.; van den Toorn, H.; Giansanti, P.; Cristobal, A.; Toonen, P.; Schafer, S.; Hubner, N.; van Breukelen, B.; Mohammed, S.; Cuppen, E.; Heck, A. J.; Guryev, V. Quantitative and qualitative proteome characteristics extracted from in-depth integrated genomics and proteomics analysis. *Cell Rep.* **2013**, *5*, 1469−78.

(38) Swaney, D. L.; Wenger, C. D.; Coon, J. J. Value of using multiple proteases for large-scale mass spectrometry-based proteomics. *J. Proteome Res.* **2010**, *9*, 1323−9.

(39) Wiśniewski, J. R.; Mann, M. Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. *Anal. Chem.* **2012**, *84*, 2631−7.

(40) Frese, C. K.; Altelaar, A. F.; Hennrich, M. L.; Nolting, D.; Zeller, M.; Griep-Raming, J.; Heck, A. J.; Mohammed, S. Improved peptide identification by targeted fragmentation using CID, HCD and ETD on an LTQ-Orbitrap Velos. *J. Proteome Res.* **2011**, *10*, 2377−88.

(41) Syka, J. E.; Coon, J. J.; Schroeder, M. J.; Shabanowitz, J.; Hunt, D. F. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9528−33.

(42) Frese, C. K.; Zhou, H.; Taus, T.; Altelaar, A. F.; Mechtler, K.; Heck, A. J.; Mohammed, S. Unambiguous phosphosite localization using electron-transfer/higher-energy collision dissociation (EThcD). *J. Proteome Res.* **2013**, *12*, 1520−5.

(43) Dzieciatkowska, M.; Hill, R.; Hansen, K. C. GeLC−MS/MS analysis of complex protein mixtures. *Methods Mol. Biol.* **2014**, *1156*, 53−66.

(44) Shevchenko, A.; Tomas, H.; Havlis, J.; Olsen, J. V.; Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **2006**, *1*, 2856−60.

(45) Oberprieler, N. G.; Lemeer, S.; Kalland, M. E.; Torgersen, K. M.; Heck, A. J.; Taskén, K. High-resolution mapping of prostaglandin E2-dependent signaling networks identifies a constitutively active PKA signaling node in $CD8^{+}CD45RO^{+}$ T cells. *Blood* **2010**, *116*, 2253−65.

(46) Nesvizhskii, A. I. Computational and informatics strategies for identification of specific protein interaction partners in affinity purification mass spectrometry experiments. *Proteomics* **2012**, *12*, 1639−55.

(47) Sowa, M. E.; Bennett, E. J.; Gygi, S. P.; Harper, J. W. Defining the human deubiquitinating enzyme interaction landscape. *Cell* **2009**, *138*, 389−403.

(48) Dazard, J. E.; Saha, S.; Ewing, R. M. ROCS: a reproducibility index and confidence score for interaction proteomics studies. *BMC Bioinf.* **2012**, *13*, 128.

(49) Teo, G.; Liu, G.; Zhang, J.; Nesvizhskii, A. I.; Gingras, A. C.; Choi, H. SAINTexpress: improvements and additional features in Significance Analysis of INTeractome software. *J. Proteomics* **2014**, *100*, 37−43.

(50) Stefanovsky, V. Y.; Pelletier, G.; Hannan, R.; Gagnon-Kugler, T.; Rothblum, L. I.; Moss, T. An immediate response of ribosomal transcription to growth factor stimulation in mammals is mediated by ERK phosphorylation of UBF. *Mol. Cell* **2001**, *8*, 1063−73.

(51) Casas-Terradellas, E.; Tato, I.; Bartrons, R.; Ventura, F.; Rosa, J. L. ERK and p38 pathways regulate amino acid signalling. *Biochim. Biophys. Acta* **2008**, *1783*, 2241−54.

(52) Lavoie, J. N.; L'Allemain, G.; Brunet, A.; Müller, R.; Pouysségur, J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J. Biol. Chem.* **1996**, *271*, 20608−16.

(53) Vizcaíno, J. A.; Côté, R. G.; Csordas, A.; Dianes, J. A.; Fabregat, A.; Foster, J. M.; Griss, J.; Alpi, E.; Birim, M.; Contell, J.; O'Kelly, G.; Schoenegger, A.; Ovelleiro, D.; Pérez-Riverol, Y.; Reisinger, F.; Ríos, D.; Wang, R.; Hermjakob, H. The PRoteomics IDEntifications (PRIDE) database and associated tools: status in 2013. *Nucleic Acids Res.* **2013**, *41*, D1063−9.