Phospho-iTRAQ: Assessing Isobaric Labels for the Large-Scale Study Of Phosphopeptide Stoichiometry

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Supporting Information

ABSTRACT: The ability to distinguish between phosphopeptides of high and low stoichiometry is essential to discover the true extent of protein phosphorylation. We here extend the strategy whereby a peptide sample is briefly split in two identical parts and differentially labeled preceding the phosphatase treatment of one part. Our use of isobaric tags for relative and absolute quantitation (iTRAQ) marks the first time that isobaric tags have been applied for the large-scale analysis of phosphopeptides. Our Phospho-iTRAQ method focuses on the unmodified counterparts of phosphorylated peptides, which thus circumvents the ionization, fragmentation, and phospho-enrichment difficulties that hamper quantitation of stoichiometry in most common phosphoproteomics methods. Since iTRAQ enables multiplexing, simultaneous (phospho)proteome comparison between internal replicates and multiple samples is possible. The technique was validated on multiple instrument platforms by adding internal standards of high stoichiometry to a complex lysate of control and EGF-stimulated HeLa cells. To demonstrate the flexibility of Phospho-iTRAQ with regards to the experimental setup, the proteome coverage was extended through gel fractionation, while an internal replicate measurement created more stringent data analysis opportunities. The latest developments in MS instrumentation promise to further increase the resolution of the stoichiometric measurement of Phospho-iTRAQ in the future. The data have been deposited to the ProteomeXchange with identifier PXD001574.

KEYWORDS: phosphoproteome, stoichiometry, iTRAQ, dephosphorylation, threshold calculation, in-gel Phospho-iTRAQ

INTRODUCTION

One of the most extensively studied protein modifications is phosphorylation, a reversible posttranslational modification (PTM) with a central role in a broad range of cellular processes such as cell metabolism, homeostasis, transcription, and apoptosis. Because of their transient character, phosphorylations initiate and propagate signal transduction pathways, which make these fundamental to inter- and intracellular communication.1,2 Over 2% of the genome encodes for kinases, the family of enzymes that catalyze the transfer of a phosphate group to serine, threonine, or tyrosine residues. It is estimated that 30% of all proteins are phosphorylated, which illustrates the high prevalence of this PTM.3 Detection of phosphorylation is nevertheless challenging considering the dynamic regulation, low stoichiometry, and heterogeneous character of the phosphosites.4 Numerous dedicated phosphoproteomics workflows have therefore been developed to unravel phosphorylation networks and the activity of their modulating enzymes (as extensively reviewed in refs 2 and 5).

In these workflows, phosphopeptides are typically enriched prior to mass spectrometry analysis to remove the strong background interference caused by the bulk of nonphosphorylated peptides.4 Apart from requiring thorough optimization and a corresponding high level of expertise of the analyst, enrichment is also responsible for sample loss and limited quantitation accuracy.5 Subsequent mass spectrometry analysis is furthermore confounded by low ionization efficiency and inferior fragmentation of phosphopeptides. Conventional MS/MS analysis with collision-induced dissociation predominantly results in the neutral loss of the phosphate group, which leads to poor sequence coverage due to reduced backbone fragmentation. An additional stage of fragmentation in MS3 or the use of alternative fragmentation technologies, such as higher energy C-trap dissociation or electron transfer dissociation, can partially facilitate the detection and identification of phosphopeptides.5,6 While these methodologies are not yet routinely applied in most laboratories, they have allowed multiple specialized studies to report a grand total of over 100 000 phosphorylation sites.2

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To draw accurate biological conclusions, it is mandatory to obtain quantitative information on the phosphomodifications. Most quantitative approaches aim to elucidate the relative phosphorylation changes between samples by employing direct label-free comparisons of phosphoenriched fractions or metabolic or chemical labeling methods such as stable isotope labeling by amino acids in cell culture (SILAC), use of isobaric tags for relative and absolute quantitation (iTRAQ), or tandem mass tag (TMT). Such methods, however, forego the investigation of phosphorylation stoichiometry, also known as occupancy, since such analysis requires the simultaneous monitoring of the unphosphorylated counterpart. However, keeping this complete picture is crucial, for example, for in vitro studies of specific kinases where artificially high enzyme concentrations or prolonged incubation times often result in the annotation of low-level spurious phosphosites that are biologically irrelevant. The stoichiometry measurement of a single phosphosite is possible by spiking specific isotopically labeled (AQUA) peptides of that particular phosphorylated peptide and its unmodified complement, but this strategy is inconvenient for large-scale applications and requires prior knowledge of the phosphorylation site. Additionally, for comparative studies, the bias of the sample preparation steps cannot be excluded with AQUA, and specific knowledge of the relative protein concentrations is not available as with the former mentioned labeling approaches.

One interesting labeling strategy that is specifically designed to quantify occupancy circumvents the common difficulties of standard phosphopeptide detection by focusing on the nonphosphorylated counterparts of the phosphopeptides. Herein, a peptide sample is briefly split in two identical parts, which are differentially labeled preceding a phosphatase treatment of one part and mock-treatment of the other. Immediately afterward, the two parts are recombined for the subsequent LC–MS/MS analysis. Dephosphorylation of a phosphopeptide will induce a 79.979 mass shift resulting in a skewed label ratio in the unphosphorylated peptide’s reporter region, which now specifies the phosphorylation stoichiometry. Although the principle has been described previously, only one phosphatase-based workflow was adapted specifically for the large-scale study of a complete lysate whereby known phosphopeptides present in a database were quantified by isotopic labels. Although this report is extensively referenced, the method is not yet routinely applied for discovery purposes. As an alternative approach, each half of a peptide mixture can be labeled with isobaric tags prior to the phosphatase treatment of one-half. By virtue of the complete multiplexing until the fragmentation stage and subsequent quantitation at the MS2 level, isobaric labels have the capacity to analyze up to ten samples simultaneously. Previous reports have already prototyped this strategy with TMT and iTRAQ labels on relatively simple protein samples but have concentrated mainly on the precision of the stoichiometry measurement.

Here we extend for the first time the isobaric tag equivalent of this approach for the large-scale quantitative phosphoanalysis of complex mixtures. The technique was validated on three different instrument platforms on epidermal growth factor (EGF)-stimulated HeLa cells. We discuss a novel data analysis approach for the discovery of high stoichiometry phosphopeptides in complex peptide mixtures and present a way to interpret the sizable data set without prior matching of the peptide identifications to a phospho-database. Because all peptides and not only the phosphorylated fraction of the proteome is measured in this approach, instruments with high-speed acquisition capabilities and adequate resolution for accurate reporter quantitation are indispensable. Still, because of the minimal technical variation that is introduced throughout the workflow (as for Wu et. al.20), implementation of extensive fractionation and purification steps, such as protein gel-electrophoresis and 2DLC at the peptide level, allows for more in-depth coverage of the (phospho) proteome. What makes this approach even more attractive is that the iTRAQ multiplexing allows for a replicate measurement within one experiment, which creates a new data analysis workflow that meets the stringent requirements of peptide quantitation. All three instrument panels were able to annotate a large set of known phosphoproteins. Extending the latter sample fractionation and using novel methodologies to avoid dilution of reporter ratios in MS/MS will allow for the annotation of increasing numbers of phosphopeptide with increasingly low stoichiometries in the future.

### MATERIALS AND METHODS

#### Chemicals and Materials

Cells were obtained from ATCC. The growth medium, EGF, media supplements, phosphate buffered saline (PBS), and the PeppermintStick internal protein standard were from Invitrogen (Carlsbad, CA, USA). Readyprep sequential extraction kit, tributylphosphine (TBP), 4x Laemmli sample buffer, 2-mercaptoethanol (Invitrogen), Tris-HCl precast polyacrylamide gels, and the Precision Plus Protein Marker were obtained from BioRad (Hercules, CA, USA). Triethylammonium bicarbonate (TEAB), sodium dodecyl sulfate (SDS), N-cyclohexyl-3-aminopropanesulfonic acid (CAPS), calcium chloride (CaCl₂), and tween-20 were from Millipore (Billerica, MA, USA). Modified trypsin was acquired from Promega (Fitchburg, WI, USA), and the iTRAQ reagents were from ABSciex (Framingham, MA, USA). Acetonitrile and formic acid were from Biosolve, LCMS-grade (Dieuze, France). Alkaline phosphatase was obtained from different manufactures: calf intestinal phosphatase was from New England Biolabs (Ipswich, MA, USA) and Sigma-Aldrich (St. Louis, MO, USA), thermosensitive alkaline phosphatase was from Promega, and Escherichia coli alkaline phosphatase was from Sigma-Aldrich. All other reagents were purchased from Sigma-Aldrich unless stated otherwise.

#### Cell Culture and Lysis

HeLa cells were cultured at 37 °C in Dulbecco’s Modified Eagle Medium (5% CO₂) supplemented with 1% (w/v) l-glutamine, 10% (w/v) FBS, and 50 IU/mL penicillin/streptomycin. Cells were starved in FBS-free medium for 17 h before harvesting. For the EGF stimulation, the medium was removed, enriched with 150 ng/mL EGF, and immediately readed to the cells for 10 min. Next, the EGF-stimulated and control cells were incubated for 15 min at 37 °C with PBS-based dissociation buffer (Invitrogen) and detached by cell scraping. Cells were washed twice by precooled PBS and centrifuged at 4 °C. All subsequent steps were also performed at 4 °C unless declared otherwise. Cell lysis was performed by suspending the cell pellet in the R1-buffer from the Readyprep sequential extraction kit at a density of 1 mL/10⁶ cells. R1 comprises 40 mM Tris-buffer, which we supplemented with 2% (v/v) phosphatase inhibitor cocktail 2&3 (P5726 and P0044, Sigma-Aldrich), 2% (v/v) TBP, 0.3% (v/v) benzozase...
(E1014, Sigma-Aldrich), and 1 tablet/10 mL R1 complete mini EDTA-free protease inhibitor cocktail (11836170001, Roche, Penzberg, Germany). After sonication in an ice-bath for 10 min (Branson 2510 sonication bath), the protein extracts were mixed with 25:1 volumes of precooled acetone for overnight incubation at −20 °C. The next day, after a centrifugation step at maximum speed (14 000 rpm) for 15 min, the acetone was removed, and the pellet was resuspended in 0.5 M TEABC. After an additional spin, the pellet was removed, and the supernatant was withheld as the “R1 extract.” A two-step lysis method was performed to the EGF-treated cells before gel fractionation, which thereby extended the “Phospho-iTRAQ” method to increase the protein coverage. The protein extract of the cells in the R1-buffer was centrifuged at a maximum speed.

To include more proteins, the pellet was resuspended in the R3-buffer (containing urea, thiourea, detergents, and ampholytes) from the Readyprep sequential extraction kit and combined with the initial R1 lysate, which together formed the “R1+R3 extract”. The protein content of all the extracts was determined by the 2D Quant Kit (GE Healthcare, Little Chalfont, United Kingdom).

**Gel Fractionation and Digest**

Twenty microgram samples of the “R1 extract” from both the EGF-stimulated and control cells were digested analogous to the iTRAQ protocol (ABSciex). A 500 μg sample of the “R1+R3 extract” derived from the EGF-stimulated cells was diluted 3:1 in 4x Laemmli sample buffer and reduced with 10% (v/v) BME for 10 min at 95 °C. Next, 1.5% (w/w) of the Peppermint Stick internal protein standard (IS1) was added, and the sample was loaded in the central well of the 10% Criterion Tris−HCl Gel, 11 cm IPG + 1 well. Electrophoresis of the gel was performed for 30 min at 150 V and 60 min at 200 V in a Criterion Cell (Biorad) and monitored by the Precision Plus Protein Marker in the outer well. After a fixation and washing step, the gel was cut into four equal pieces comprising different molecular weight fractions, which were transferred to glass tubes. The proteins were in-gel digested according to optimized conditions.24 All the obtained peptides were brought to dryness by the SpeedVac and stored at −20 °C until further use.

**Labeling**

Peptides of the R1 extracts were redissolved in 40 μL of 50 mM TEABC, while peptides from the molecular weight fractions of

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**Figure 1.** The Phospho-iTRAQ approach workflow. (A) Experimental workflow: in the “in-solution” approach (left panel), the soluble protein extracts (R1) of control and EGF-stimulated HeLa cells were spiked with an internal peptide standard (IS2) of heavy phosphopeptides and compared by Phospho-iTRAQ. When only one sample is mined (EGF), the flexibility of the protocol allows for gel purification and fractionation and thus for complementing the soluble protein extract (R1) with the hydrophobic fraction diluted in strongly denaturing buffer (R3) to increase the number of annotated phosphopeptides (“in-gel” approach, right panel). The EGF-stimulated cells were spiked with a phosphoprotein internal standard (IS3) before fractionation on a 1D PAGE into four molecular weight fractions followed by Phospho-iTRAQ (Supporting Information). (B) Phospho-iTRAQ protocol: a peptide sample is briefly split in two identical parts and differentially labeled preceding the phosphatase treatment of one part. Afterward, samples are immediately recombined and split into three parts for the LC–MS/MS analysis on three different instruments. (C) Data Analysis: raw data was processed by the respective vendor’s software, and MGF-files were searched against the SwissProt Human database using Mascot. Exported DAT-files were imported into Rover for ranking of the iTRAQ ratios and were further analyzed in Excel. (D) Data: initially phosphorylated peptides have skewed iTRAQ ratios and arise out of the center of the log-normal distribution of the whole precursor population. The mean of the log-normal ratio distribution is located around zero since the vast majority of the peptides in the data have equal 114/115 or 116/117 reporters.
the R1+R3 extract were redissolved in 80 μL of 50 mM TEABC. As a second internal standard (IS²), ± 7 pmol of each phosphopeptide from the heavy MS phosphomix 1, 2, and 3 (MSP1H, MSP2H, MSP3H: Sigma-Aldrich. Composition, see Supporting Information) was spiked into each sample. Subsequently, R1 extracts were equally divided in two parts (Figure 1), and the fraction of the R1+R3 extract was divided in four parts of 20 μL. Before the iTRAQ labeling, each reporter was reconstituted in 250 μL of ethanol and combined with the related label of a second 4-plex set. For the R1 extracts, the two halves of the control sample were labeled with 50 μL of the 114 and 115 reporter, the two halves of the EGF-stimulated sample with the 116 and 117 reporter, and each quarter of the four molecular weight fractions of the R1+R3 extract with 50 μL of a different 4-plex label (see Supporting Information). The labeling was performed for 2 h at room temperature by continuous shaking before samples were dried out.

**Dephosphorylation**

The labeled peptides in the 20 vials (both R1 extracts were split in two, and each gel fraction of the R1+R3 extract was split in four) were redissolved in 12 μL of calf intestinal phosphatase (CIP) buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT) and incubated for 45 min at room temperature to remove the residual free labels. Next, the 114–116 and 115–117 labeled peptides were combined, which resulted in 10 vials (114–116 and 115–117 for the "R1 extracts" as well as the four molecular weight fractions of the "R1+R3 extract"). To the 115–117 samples, 30 μL of the dephosphorylating enzyme cocktail (6.25% 0.25U/μL *Escherichia coli* alkaline phosphatase (P4069, Sigma-Aldrich)), 31.25% 1U/μL CIP 1 (M2920, New England Biolabs), 31.25% 10U/μL CIP 2 (P4978, Sigma-Aldrich), and 31.25% 1U/μL thermosensitive alkaline phosphatase (M9910, Promega) was added. For the 114–116 control samples, 30 μL of CIP buffer was supplemented before all samples were incubated at 37 °C for the overnight dephosphorylation. Phosphatases were inactivated the next day by heating the samples for 30 min at 85 °C in the presence of EDTA (50 mM). The 114–116 and 115–117 units were merged, and the five samples were cleaned by C18 tips (Sigma-Aldrich) to desalt and to remove free labels and excess of phosphatases. The peptide samples that were eluted with 80% acetonitrile (ACN), 20% water, and 0.1% formic acid (FA) were split into three equal parts and were dried out immediately.

**LC–MS/MS Analysis**

Peptides were resolved in 0.1% FA, and approximately 0.5–1 μg was brought on column each run. The nanoLC–MS/MS analysis of the three parts was performed on different ESI mass spectrometry platforms: the first part on a TripleToF 5600 (ABSciex), the second part on a QExactive (Thermo Fisher), and the third part on a SYNAPT G2-Si (Water Cooperation).

(1) For the analysis on the TripleToF 5600, the chromatography was done on an Eksigent ekspert nanoLC 400 System on NanoLC column, 3 μm, ChromXP C18 CL, with a 120 min gradient going from 5–90% ACN, 0.1% FA, and a 300 nL/min flow. During acquisition, each survey scan accumulated precursors in the range of 400–1250 m/z for 250 ms, from which the top 20 were fragmented for MS/MS every cycle at a ratio of 180 ms/precursor. Resolution was >15 k in MSMS; dynamic exclusion 10s.

(2) The QExactive was coupled to a Dionex Ultimate 3000 R5 nanoLC system. After trapping on a precolumn, peptides were separated on the Acclaim PepMap100, 75 μm × 50 cm by a 90 min gradient, whereby the amount of ACN 0.1% FA increased from 5–70%, also at a constant flow rate of 300 nL/min. In a scan range of 380–2000 m/z, a full MS and data-dependent MS/MS scan was performed on the top 10 by a normalized collision energy (NCE) 30 stepped + collision energy 10% in all cases. Resolution was >25 k in MSMS; dynamic exclusion 25 s.

For the measurements on the SYNAPT G2-Si, a NanoACQUITY system separated the samples with a Waters BEH C18, 75 μm × 150 mm analytical column after a trapping step of 8 min. Through changing the amount of ACN, 0.1% FA changed from 1–40% for 90 min at a constant flow rate of 300 nL/min. The range of 400–1600 m/z was inspected during the 0.1 s survey scan, from which the top ten of the precursors were fragmented by ramping the collision energy in the "iTRAQ" mode for a 0.1 s/MS/MS scan if the threshold of 10 k counts/spectrum was reached. Resolution was >20 k in MSMS; dynamic exclusion 30 s.

**Data Analysis**

Data acquisition, recording, and preprocessing were carried out with software of the respective mass spectrometer manufacturers. Equally, the raw data were processed by specialized software packages of the vendors: ProteinPilot 4.0.8085 for the TripleToF 5600 of ABSciex, Proteome Discoverer 1.4 for the Thermo Scientific QExactive, and PLGS 3.0.1 for the SYNAPT G2-Si of Waters Cooperation. The same software packages were used to export the created peak lists in the MGF format. The database searches of these MGF files were performed with Mascot 2.4.0. (Matrix Science) against the SwissProt Human database (59 084 sequences, supplemented with the sequences of the internal standards). Initially, the prevalence of the most common modifications was explored by an error-tolerant database search.55 Details and search parameters can be found in the Supporting Information. The result files of these searches were exported as Mascot-DAT file formats (Peptide e-value <0.05), and each run was then imported separately in the Rover software for individual processing (https://code.google.com/p/compomics-rover/). After obtaining Rover statistics on the 114/115 and 116/117 ratios, the data were filtered in order to export the log2 ratios and z-scores of unique or "razor" peptides for consecutive data validation. Data validation and frequency plot distribution of z-scores (at a bin size of 0.1) were done in Excel, whereby the z-scores of the four molecular weight fractions of the R1+R3 extracts were combined. Additional database searches were performed by changing the variable modifications in order to annotate the "heavy" internal standard phosphopeptides (see Supporting Information).

Phosphoprotein enrichment in the obtained protein lists was assessed by a right-sided Fisher enrichment test at a significance level of 5%. Known phosphoproteins in humans were obtained from the PhosphoSitePlus database (http://www.phosphosite.org/homeAction.do). The total number of proteins known in humans was estimated based on the described proteins in the PFAM database. Proteins involved in the EGF pathway were identified by querying the BioCarta database.

To assess the relative amount of phosphopeptides present in the lists generated by the Phospho-iTRAQ approach, these lists were compared to the known phosphopeptides present in the PhosphoSitePlus database by means of a protein blast as integrated in the NCBI blast 2.2.28 package. The procedure allowed for partial mappings between Phospho-iTRAQ peptides and the phosphopeptides. That is, stretches of at
least five amino acids in both peptide sequences had to map unambiguously to each other (irrespective of overhangs that result from, for example, tryptic miscleavages), that is, no gaps or mutations were allowed in the procedure.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD001574 and 10.6019/PXD001574.

### RESULTS AND DISCUSSION

Large-scale quantitative analysis of phospho-stoichiometry is essential to explore the true extent of phosphorylation since occupancy is associated with functionality. In this report, we therefore present for the first time the large-scale exploration of an isobaric tag-based protocol that focuses on the unphosphorylated counterparts of phosphopeptides for its analysis. Details of the optimized protocol are described in the Materials and Methods section, and a schematic overview of the workflow is given in Figure 1. For the comparative Phospho-iTRAQ analysis, HeLa cells were either treated with EGF for 10 min or left untreated before lysis. The protocol was validated on spiked-in high stoichiometry peptides and was expanded by gel fractionation of the EGF lysate, which coordinately valorized the duplication potential of 4-plex iTRAQ and its impact on data analysis.

#### Validation of the Phospho-iTRAQ Protocol through Evaluation of the Internal Phosphopeptide Standards

To validate the effectiveness of the Phospho-iTRAQ method, we added a commercially available internal phosphopeptide standard (IS2 in Figure 1) composed of 21 different heavy peptide sequences with at least one phosphorylated amino acid into the sample after digestion. A set of these spiked heavy peptides was identified in all samples (see Supporting Information). Not all peptides were annotated, however, because of the weak ionization efficiency of some of the sequences and the technical and inter-run variations characteristic of data-dependent acquisition (DDA). As observed on raw mass spectra and the processed data of the nonphosphorylated counterparts of the heavy peptides, all of the 114/115 and 116/117 iTRAQ ratios were skewed, whereby the phosphatase-treated 115 and 117 reporters substantially exceeded the 114 and 116 labels of the control parts, which hence characterized these peptides as phosphopeptides.

In Figure 2, the MS/MS spectrum and the range of the "silent" iTRAQ region of the EVQAEQPSSSSSPR peptide (m/z 519.2628, 3+) from the R1 sample are given as an example, which illustrate that the approach is able to identify phosphopeptides in complex peptide mixtures on all three instruments. Additional proof is provided if the phosphopeptides themselves are annotated (EVQAEQPSPSSPR m/z 818.3734, 2+) as these iTRAQ ratios are inverted compared to their unphosphorylated counterparts (presented in Figure 2, right upper and lower panel). In this manner, the dephosphorylation efficiency can be verified, which further allows for the accurate calculation of the stoichiometry as described elaborately in refs 12 and 19. If the native phosphorylated peptide is not annotated, the method still provides a relative simple way to identify high stoichiometry phosphopeptides on a large scale. As an alternative for the phosphatase treatment, chemical dephosphorylation by means of cerium oxide has also been suggested to ensure the sensitivity of the methodology. While the stoichiometry of the peptides is well-defined with this approach, complementary data is necessary to verify the phosphopeptide is not identified by other means.
analysis is still required to determine the exact amino acid of the identified phosphopeptide that is modified, especially in case of multiphosphorylated peptides.

Addressing the Variation between Annotated Peptides with Similar Stoichiometry

The overall average log2 Mascot ratio of the confidently annotated IS2 peptides (i.e., Mascot peptide score >25) was $-3.56$ (SD, 2.31; $N$, 42) for the control and $-2.84$ (SD, 1.46; $N$, 42) for the EGF-stimulated sample. The corresponding log2 ratios of the IS2, which was spiked after the in-gel digestion, is on average $-3.09$ (SD, 1.84; $N$, 138) for 114/115 and $-3.12$ (SD, 1.56; $N$, 138) for 116/117. Although all spiked IS2 peptides were completely phosphorylated (100% stoichiometry), generating strongly skewed “Phospho-iTRAQ ratios”, variation between the ratios of different peptides was observed as reflected by the SD. Equally, as also seen in Figure 2, the ratios of peptides that are annotated multiple times in one run or over multiple LC–MS/MS runs are not identical.

This ratio dilution is attributed to (1) underestimation of the fold change, which is a known iTRAQ bias, and (2) the “isobaric tagging elephant”, coeluting peptides with similar m/z values that are coselected for fragmentation dilute the ratios.29–32 This results in both a scattering of the ratios of nonphosphopeptides outside the center of the normal ratio distribution of the whole data set, and more importantly, contributes to the underestimation of the phosphopeptide occupancy. Most of the spectra will suffer from this dilution, as is illustrated by the fact that nearly none of the IS2 peptides (100% phosphorylated) had zero intensity at the 114 and 116 reporters, while these were not subjected to dephosphorylation. Although the isobaric tagging elephant currently still challenges the stoichiometry measurements, emerging technologies such as ion-mobility separation (SYNAPT G2-Si) and multinotch selection (Orbitrap Fusion) hold great promise to reduce this diluting effect and correspondingly increase the accuracy of isobaric labeling techniques in the near future, as was recently also suggested for the localization of organelle proteins by isotope tagging (LOPIT) technique.33–35 Furthermore, simply
improving separation of the sample prior to analysis has been shown to dramatically lower such interferences as well.32

However, because the bulk of the peptides in a data set intrinsically have 1:1 ratios, dilution will predominantly cause extreme ratios to move to the middle of the distribution. Supplemented by the notion that the fold change is generally underestimated in iTRAQ approaches, ratios are not expected to move away from the center of the distribution, and the smallest ratio of each multiply annotated sequence is arguably the most accurate one.

A Comparative (Phospho) Proteome Analysis of EGF-Stimulated and Nonstimulated HeLa Cells on Three Different Mass Spectrometry Instruments

The database searches with identical parameters resulted in the annotation of 1283 proteins out of the 31 562 spectra from the TripleTof 5600 (50% annotation rate), 1567 proteins out of the 34 629 spectra from the QExactive (42%), and 905 proteins out of the 21 822 spectra from the SYNAPT G2-Si (32%). Different LC gradients were applied, so no direct comparison between manufacturers can be made. For large-scale data mining, we applied Rover, a software specifically developed for the visualization, analysis, and validation of quantitative proteomics data. The software does not predict the phosphomodification but only performs normalization of selected ratios from mass spectrometry data sets. This allows the ranked iTRAQ ratios and corresponding z-scores of the unique and razor peptides of each run to be exported from Rover and to set cutoffs in order to select peptides with skewed label ratios (Figure 3).

The small z-scores of the IS2 peptides confirm our previous conclusions on the effectiveness of the approach to discover high stoichiometry peptides in a large data set (Figure 3). The z-scores of the spiked phosphopeptides also mark the threshold for defining phosphorylation in each data set, that is, all the annotated peptides in the data with a z-score below this value are pinpointed as highly phosphorylated. Alternatively, predefined mixtures of heavy phosphorylated and their nonphosphorylated heavy counterparts could similarly set the thresholds of different sections over the full range of occupancy states.8,20 However, even with such an alternative approach, separating the overlapping z-score ratio distribution of the phosphorylated and nonphosphorylated peptides remains extremely challenging because: (1) only a limited amount of peptides in the nonenriched data are phosphorylated in the bulk of nonphosphorylated peptides; (2) most of the phosphorylated peptides are of low stoichiometry (over 50% of the peptides have a stoichiometry <30%);20 and (3) the previously mentioned isobaric tagging elephant still leads to overlap between the normal distribution of the ratios of the nonphosphorylated peptides (1:1 ratios) and that from initially phosphorylated peptides (deviating ratios).

To assess a threshold for filtering out phosphopeptides, we first extracted the z-scores of all annotated peptides that cannot be phosphorylated: peptides with no serine, threonine, or tyrosine (No STY). On the No STY distribution, z-scores at different quantile levels (1–25% on the left site of the distribution) were chosen as a cuttoff value (Figure 3, upper panels). To select the phosphopeptides in the data, these z-score cutoffs were subsequently transposed to the ranked z-scores of the peptides that have the ability to be phosphorylated (with an STY in the sequence). Different levels, from the left percentile to the quantiles of the distribution, were applied to set the threshold on the 114/115 control sample and the 116/117 EGF-treated sample (see Table S2, Supporting Information for an example of the data analysis workflow and obtained phosphopeptide sequences). The graphs in Figure 3 (lower panel) illustrate the amount of unique phosphopeptides that are shared or only identified in one of both conditions at each selected threshold. A low cutoff only annotates high stoichiometry peptides, while a higher cutoff defines a larger set of phosphopeptides, including those with a lower stoichiometry that could be useful for discovery studies. Interestingly, on average, 35% of the identified phosphopep-
The Flexibility in Experimental Setup Provided by Phospho-iTRAQ Allows for Deeper (Phospho) Proteome Mining and Increased Reliability of Individual Phospho Events

It is invaluable that the approach can be extended “indefinitely” to attain higher proteome coverage without compromising quantitation. This is possible because contrary to a conventional iTRAQ, or any other quantitative, approach, samples are only briefly split for the differential dephosphorylation of one-half, and no technical variation can accumulate before or after this step (Figure 4). Only the approach of Wu et al. provides the same degree of freedom in fractionating the samples.30

Importantly, apart from fractionation, 1D PAGE also allows use of strongly denaturing buffers to solubilize the more hydrophobic proteins, which normally cannot be done in a conventional iTRAQ approach. Also, fractionation is recommended to minimize the effect of coeluted and cofragmented peptides and thus the effect of the isobaric tagging elephant.30,32 A second extension of the protocol is the use of four labels to analyze only one sample. This creates the opportunity of duplicate measurements in one experiment and generates the possibility of more stringent data analysis.

To first validate the potential impact of protein fractionation on quantitation, the EGF lysate was spiked with protein IS1 followed by gel separation into four molecular weight fractions (see Supporting Information for the validation of the molecular weight separation). The database searches of the merged MGF files confirm the expected extension of the proteome coverage: the annotation of 2070 proteins out of 105 572 spectra from the TripleTof 5600 (37% annotation rate), 2913 proteins out of 155 848 spectra from the QExactive (33%), and 1976 proteins out of 82 556 spectra from the SYNAPT G2-Si (31%).

Figure 5. Data analysis pipeline for the gel-based Phospho-iTRAQ approach (R1+R3). A replicate setup where both 114/115 and 116/117 measure the same peptides allows for more stringent data analysis. The graph demonstrates the percentages of identified phosphopeptides that are shared between the 114/115 and 116/117 replicates at different cutoff levels. We suggest to focus on those peptides that were found below the 20% cutoff (black square) in both ratios. The theoretical chance of this event happening randomly is 4% (black dotted line). The table shows the number of peptides and corresponding proteins that were isolated at this threshold on each of the platforms. The % of proteins that are known phosphoproteins (PhosphositePlus) and the respective Fisher p-value for enrichment are also given along with the proteins in this list that are known responders to EGF signaling. The Venn diagram displays the number of phosphopeptides from the left 20% percent of the distribution of either ratio that are shared between the acquisitions on the different instruments.
Additionally, of all 17 annotated peptides, only one peptide of casein β (CASB_BOVIN (I5)) was located outside the overall distribution of the 114/115: FQSEEQQQTEDELQDK, a known phosphopeptidite (Supporting Information). This illustrates that extending the protocol by additional fractionation steps at the protein level increases the proteome coverage without influencing the technique’s capability to isolate phosphopeptides.

In this gel-based Phospho-iTRAQ approach, the samples were split into four equal parts to coordinately implement an internal replicate analysis (Supporting Information). Contrary to the comparative in-solution Phospho-iTRAQ analysis (cfr. supra), 114/115 and 116/117 now independently measure the same phosphorylation event. Again, the ranked 114/115 and 116/117 z-scores of the No STY peptide population allow the setting of phosphocutoffs at different quantile levels on the STY peptide distribution. However, this time, the peptides that have a skewed ratio in both 114/115 and 116/117 can be considered to be more reliable phosphopeptides. The graph in Figure 5 illustrates how the number of such peptides greatly exceeds the amount that can be theoretically expected. Of interest, manual analysis surfaced an unexpected phenomenon that is circumvented this way: apparently an N-terminally acetylated peptide starting with an alanine creates a b1 ion of m/z 114.05, which can be mistaken during data processing to be the 114.11 reporter label. Even such unexpected phenomena, however, no longer influence the final selection of phosphopeptides when the 116/117 ratio is equally taken into account.

Taken together, we suggest to use the theoretical chance of both ratios being in the same quantile as a “false discovery cutoff” in this extended gel-based Phospho-iTRAQ strategy. Setting the threshold at 20% and requiring a duplicate measurement below this threshold (114/115 as well as 116/117) now becomes a justifiable choice, as the possibility of any peptide arriving here by chance is only 0.2 × 0.2 = 0.04, or 4%. Although a 25% overlap between the duplicate analyses at this threshold might still appear quite narrow, this is an intrinsic consequence of setting thresholds. For instance, if for one peptide the 114/115 ratio is 0.5 and the 116/117 ratio is 0.55, one ratio might very well be included at the 20% threshold for 114/115, while the other only appears at the 25% threshold for 116/117. As demonstrated in the table in Figure 5, all instruments showed a strong enrichment in phosphoproteins when the above-mentioned criteria were applied (Fisher p-value < 2.2e-16). As predicted, more proteins from the EGF pathway identified after protein fractionation, and it can thus be expected that additional fractionating at the peptide level, as was done by, for example, Wu et al. will further complete this list.

The fact that we only annotated a few known mediators in EGF signaling can be attributed to two main reasons. (1) We only sample one time point after stimulation (10 min). As shown in the landmark paper by Olsen et al., EGF-activated proteins can be divided into several temporal profiles over a 20 min range, and we thus expect to only find a portion of all the known proteins involved in EGF signaling. Some phosphorylations could thus be absent or not of high stoichiometry in our samples (supported by the notion that we start to pick them up at higher percentile thresholds). (2) Some specific peptides are not identified because they simply were not isolated for fragmentation during the DDA runs. By extending the setup and increasing the fractionation even further, as was done by Wu et al., who made a total of 20 fractions (as opposed to our four) that detected over 80 000 different peptides, should allow the digging out of more phosphorylation events from the bulk of other peptides. Note that at least on one platform, the EGF receptor was identified (phosphopeptide sequence GSTAENAEYLR), which illustrates the additional benefit of 1D PAGE as a means of including more hydrophobic proteins by the use of strongly denaturing buffers (R3). The Supporting Information shows the temporal changes in this EGF receptor pY1197 phosphorylation as detected by Olsen et. al. (http://www.phosida.com/) and illustrates that this event was detected despite the fact that it is at its decline at 10 min, after a peak at 5 min.

It is important to emphasize that the technical replicates were performed completely independently on three different instrument platforms using DDA with different LC gradients. It is thus reasonable to expect only a small overlap between these runs: (1) the above-mentioned consequence of setting a threshold is that it excludes many peptides with a lower stoichiometry near, but above, the 20% quantile; (2) a DDA run is very irreproducible, even on identical injections, and not many peptides in our analysis have been selected for fragmentation on every instrument; (3) because the data from each instrument is a merged file from four DDA runs on different MW fractions, the said variation in DDA acquisition accumulates in this data set; (4) identification of the spectra is very dependent on data quality of each individual MS/MS spectrum. Still, in the total data set, 2420 of the STY peptides from the left 20% of the distribution were present in at least one ratio of two of the three runs (see Venn diagram in Figure 5). Taken together, we argue that this considerable overlap further underlines the capabilities of the Phospho-iTRAQ approach, and we emphasize that this (gel-based) strategy can be applied on any mass spectrometry platform that has adequate speed and mass resolution.

■ CONCLUDING REMARKS

We here present and evaluate an isobaric tag based protocol that relies on phosphatase treatment of differentially labeled peptides for large-scale quantitative analysis of phosphopeptides. The Phospho-iTRAQ method focuses only on the nonphosphorylated counterparts of the phosphopeptidites and avoids specialized and relatively imprecise workflows such as phosphoenrichment. Since multiple labels are used, information on relative protein expression can still be obtained in our method.

The application of Phospho-iTRAQ on spiked-in phospho-proteins and phosphopeptides demonstrated its ability to discover high stoichiometry peptides in a large data set and this on multiple LC–MS/MS platforms. The iTRAQ data at the spectrum level allows estimation of the phosphorylation stoichiometry, while the ratio distribution of all annotated peptides allows peptides with high stoichiometry to be identified by their deviating z-scores. The dilution effect of the coeluted and coselected peptides (isobaric tagging elephant) still challenges the accuracy of the methodology but can be partially addressed by the multiplexing potential of the isobaric labels, which allows extensive fractionation and the inclusion of replicates. Furthermore, newly arising technologies, such as ion-mobility separation, hold great promise to diminish this interference and thus increase the stoichiometric resolution of the method.

Additional confirmation of the identified phosphopeptides with complementary techniques however remains necessary.
because our approach is not able to pinpoint the exact phosphorylation site. Notably, the existence of different “false localization scores” and the notion of migrating phosphate groups in collisionally activated peptides puts this shortcoming into some perspective.\textsuperscript{39,40} As with most quantitative analyses, it is up to the researcher to decide where to put the threshold in the ranked ratio distribution of large-scale data that marks the list of the phosphopeptide enriched identifications. Indeed, depending on the goal of the experiment, one can focus exclusively on the more confidently annotated high stoichiometry peptides (low cutoff), or one can rather isolate a larger but overall less reliable list of peptides for an exploratory study (high cutoff), which will thus increase the need for additional biological replicates to define the common subsets of different runs that will again augment the confidence of the phosphopeptide detection.

\section*{ASSOCIATED CONTENT}

\subsection*{Supporting Information}
Composition of the internal standards (IS\textsuperscript{1} and IS\textsuperscript{2}); scheme of the labeling of the R1+R3 extract (in-gel approach); details of the database search parameters; \%phosphoproteins identified at different thresholds in the in-solution approach; boxplot for the validation of the molecular weight separation by 1DPAGE; explanation for why extending sample fractionation does not compromise quantitative power; temporal changes in EGFR pY1197 phosphorylation as described by Olsen et al.\textsuperscript{23}; and tables showing all the annotated peptides of the IS2, an example list of the phosphopeptide enriched identifications.

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The authors declare no competing financial interest.

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