Published in final edited form as:

Electrophoresis. 2014 December ; 35(24): 3430–3440. doi:10.1002/elps.201400153.

Analytical challenges translating mass spectrometry-based phosphoproteomics from discovery to clinical applications

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Abstract

Phosphoproteomics is the systematic study of one of the most common protein modifications in high throughput with the aim of providing detailed information of the control, response, and communication of biological systems in health and disease. Advances in analytical technologies and strategies, in particular the contributions of high-resolution mass spectrometers, efficient enrichments of phosphopeptides, and fast data acquisition and annotation, have catalyzed dramatic expansion of signaling landscapes in multiple systems during the past decade. While phosphoproteomics is an essential inquiry to map high-resolution signaling networks and to find relevant events among the apparently ubiquitous and widespread modifications of proteome, it presents tremendous challenges in separation sciences to translate it from discovery to clinical practice. In this mini-review, we summarize the analytical tools currently utilized for phosphoproteomic analysis (with focus on MS), progresses made on deciphering clinically relevant kinase-substrate networks, MS uses for biomarker discovery and validation, and the potential of phosphoproteomics for disease diagnostics and personalized medicine.

Keywords

Kinase; MS; Phosphoproteomics

1 Introduction

Deciphering information flow through different signaling pathways of a biological network and understanding how these signals can be manipulated is both challenging and rewarding. While one can carry out numerous large-scale analyses on genomic or metabolite levels, a more comprehensive examination of signaling proteins and their modifications is necessary in order to truly understand signaling at the molecular level [1, 2]. Such examinations would allow for comparison of temporal cellular activity, differentiation within cell types, dynamic

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The authors have declared no conflict of interest.

feedback mechanisms, network cross-talk, modifications during disease formation and progression, and response of a biological system to drug treatments. Protein phosphorylation, as one major posttranslational modification, is deeply involved in all of these phenomena [3,4]. Through its numerous roles, protein phosphorylation/dephosphorylation can act as a molecular switch for enzyme activity regulation [5–7] and a beacon for protein translocation, synthesis, and degradation [8, 9]. It is also a critical step in energy generation and biological reactions [10], and an important component in many protein–protein interactions [11, 12], among other functions. Better understanding of the roles of protein phosphorylation is the cornerstone of our growing knowledge about health and disease onset and progression [13–18].

Radioisotope ^{32}P played a vital role in the discovery of protein phosphorylation and subsequent important characterizations of phosphoproteins [19–25]. Today ^{32}P radioisotope labeling has been mainly used for in vitro kinase assays, where kinase activity within a biological sample is measured in vitro by incubating a purified kinase with an exogenous substrate in the presence of ATP- γ - ^{32}P . The use of the radioisotope is efficient with this method, but it is a safety concern and requires strict regulation. Therefore, the development of phosphorylation-dependent antibodies was a welcome event for researchers. Phospho-specific antibodies are typically developed by immunizing rabbits or mice with synthetic phosphopeptides representing the amino acid sequence surrounding the phosphorylation site of the target protein [26]. The availability of phospho-specific antibodies has opened the door for the improvement of traditional methods, as well as the development of new immunoassay techniques. The main caveat in utilizing phospho-specific antibodies is that successful detection is dependent on the specificity, availability, and affinity of the antibody for the phosphoprotein of interest. Comparison studies found that only general phosphotyrosine antibodies are of sufficient quality for confident detection of universal tyrosine phosphorylation [27,28], while the quality of site-specific anti-phospho antibodies varies greatly. Nevertheless, antibodies have remained the major tool to verify protein phosphorylation in most biological studies.

Notwithstanding many years of successful use and numerous studies, the traditional methods for phosphorylation analysis (including Western Blotting and single-target ELISA assays) are still quite low-throughput (see Fig. 1 for relative throughput comparison). The development of antibody and protein microarrays for high-throughput screening has become the standard in multiplexed functional protein analysis [29, 30]. Most often, for phosphorylation, a phospho-specific antibody is used for a particular site of interest that has been carefully matched with the capture antibody [31]. This type of protein microarrays has been used as a powerful tool for detailed and comprehensive analysis of signaling networks, allowing researchers to quantitatively screen signaling targets of interest throughout various alterations in parallel. The relatively new development of reverse phase protein microarrays (RPPMA) has become particularly appealing due to high reproducibility and robustness of the approach, and the need for only small amounts of clinical samples—a usual bottleneck in such studies [32–34]. Protein microarrays, however, have a number of clear disadvantages [35]. These include difficulties in developing a good antibody match (particularly phospho-antibodies) and detection of single modification sites, especially since many relevant protein targets have multiple highly important phosphorylation sites, as is the case with Erk, Bim,

Raf, MEK, mTOR, and so on. In some cases, phosphorylation on different sites of the same protein has been shown to produce opposite effects [36, 37]. Therefore, arrays cannot be used in discovery studies to profile proteins with underdeveloped and lesser known phosphorylation sites, and are restricted to small-scale studies that profile a limited number of proteins with known phosphorylation sites.

MS has become the method of choice for multiplexed large-scale phosphoproteomic analyses (Fig. 1), enabling unbiased, simultaneous detection, and comparative quantification of phosphorylation events in discovery mode. Recent advances in instrumentation, chemical separation, and bioinformatics have facilitated global and comprehensive phosphoproteomic analyses in multiple biological systems [14,38–43]. Quantitative phosphoproteomics has become an unparalleled source of new biological information obtained in a discovery-based manner and is the workhorse for many R&D signal transduction studies. Since there have been a number of excellent reviews in the field [35,44–48], we highlight here only a few examples in sample preparation, quantitation, and data analyses that are relevant to the readership of the journal and briefly discuss future perspectives of the field.

2 Current options and advances in phosphopeptide isolation

In spite of the tremendous improvements to the new MS instruments, analysis of phosphorylation events and phosphoproteins has remained challenging for multiple reasons [49,50]. First, in general the stoichiometry of phosphorylation is relatively low and transient in nature—only a fraction of the available intracellular pool of a protein is phosphorylated at any given time as a result of a stimulus. Second, the phosphorylated sites on proteins might vary, implying that any given phosphoprotein is heterogeneous (i.e. it exists in several different phosphorylated forms). Third, many of the signaling molecules are present at low abundance within cells. Fourth, most analytical techniques, including MS, used for studying protein phosphorylation have a limited dynamic range, which means that although major phosphorylation sites may be located easily, minor sites might be difficult to identify. Finally, phosphatases could dephosphorylate residues unless precautions are taken to inhibit their activity during preparation and purification steps *in vitro*.

Therefore, to carry out an effective phosphoproteome analysis, special sample preparation procedures need to be considered. While protein extraction methods, denaturing conditions, and enzyme selection are all important, the single most critical step in successful phosphoproteome analysis is the initial isolation of the phosphorylated peptides—enrichment of phosphorylated sample from the nonphosphorylated background. In rare cases, the phospho-isolation can be done on the protein stage (as with immunoprecipitation), although most commonly it is carried out at the peptide level in order to identify phosphorylation sites in following steps (a simplified example of common procedural variations is shown in Fig. 2).

There have been a number of approaches, used alone or in combination, to achieve enrichment with varying success rates, including ion exchange chromatography [51–53], phosphoamino acid antibodies [54–56], covalent manipulation of the phosphate groups [57–60], IMAC- and MOAC-based phosphopeptide enrichment [61–64], and fractionation

techniques [65–67]. Each is effective in its own way and can be used for specific sample types and objectives in mind (more complete recent reviews of phosphopeptide enrichment methods can be found in [44–47, 68]). Currently, the most common techniques are based on metal oxide chelation to the phosphate groups. These include immobilized metal ion chromatography (IMAC) and metal oxide beads, such as TiO_2 , ZrO_2 , Al_2O_3 , SnO_2 , etc. [61–64, 69–73].

IMAC is based on the affinity of cationic metal ions such as Fe^{3+} and Ga^{3+} toward negatively charged phosphate groups. A typical procedure calls for loading of the peptides onto IMAC beads/column under acidic pH, washing away of the unbound nonphosphopeptides, and subsequent elution of the phosphopeptides under basic pH or a competing phosphate salt. Since originally introduced by Andersson and Porath in 1986 [74], IMAC has become a staple in phosphoproteomic analyses. Despite good success, due to acidic peptides also showing affinity toward metal ions, IMAC has typically shown relatively poor selectivity in complex samples. There have been a number of attempts to alleviate this drawback, including methyl esterification of acidic groups, addition of organic solvents, and adjustments in pH [75–77]. These alterations, however, either complicate the MS analysis, as in the case of methylation, or only marginally improve selectivity.

The introduction of metal oxide affinity chromatography (MOAC) method, most commonly TiO_2 and ZrO_2 , addressed the issue of relatively poor selectivity of IMAC. Compared with IMAC, MOAC chelation has large adsorption capacity, strong-binding affinity, high mechanical, and chemical stability even at very low pH, and unique ion-exchange properties [45]. It is these advantages that permit the use of very low pH and additional selectivity “enhancers,” mainly α -hydroxy acids such as DHB, glycolic acid, and lactic acid, which compete against acidic peptides to achieve high selectivity. There have been multiple studies comparing various metal ions and oxides [78–80], different selectivity enhancers [81–83], other enrichment conditions [81,84,85], and even combining MOAC with IMAC in sequential manner (SIMAC [86]), providing a wide range of procedural choices available to any researcher. However, the problem of poor reproducibility among different laboratories and even within the same laboratory when using the identical procedure remains to be resolved. Besides the lack of speed of mass spectrometer that might introduce biases in sampling during data acquisition, we and many others believe that the lack of reproducibility and consistency in the results are partially due to the heterogeneous conditions of the enrichment reactions. These lower the binding effectiveness as a result of nonlinear kinetic behavior, problems with solvation, unequal distribution of the reactive groups, and poor access to the binding molecules. To address this need, our group introduced a homogenous step for phosphopeptide enrichment based on a water-soluble nanosize dendrimer that is functionalized to selectively target phosphate groups on peptides [87]. Besides excellent solubility, the advantages of using dendrimers as a support include high structural and chemical homogeneity, compact spherical shape, high branching, and controlled surface functionalities [88]. Termed polymer-based metal ion affinity capture (PolyMAC), the technique uses high density titanium ions on the dendrimer surface to capture phosphopeptides in a homogeneous aqueous environment, eliminating the heterogeneous-binding conditions that limit the reproducibility of solid-phase isolation techniques [87, 89, 90]. In the second step, the PolyMAC-bound phosphopeptides are isolated out of solution

via another functional group (“handle”) on the dendrimer that covalently binds to capture beads (general principle is depicted in Fig. 3). A high concentration ratio of the reactive groups to the “handle” groups facilitates the complete recovery of the bound phosphopeptides while eliminating the need for extra steps to remove excess reagents.

Despite the effectiveness of recently introduced and modified enrichment methods, including the improved IMAC, MOAC, and PolyMAC approaches, phosphopeptide enrichment alone is far from sufficient for large-scale phosphoproteomics studies. The limitations of LC-MS sensitivity, high sample complexity, and restricted-binding capacity demand that an additional fractionation step is utilized, typically prior to enrichment. Some of the most successful comprehensive phosphoproteomic studies have combined phosphopeptide isolation with chromatographic methods such as strong cation and anion exchange (SCX [65,91], SAX [92,93]), HILIC [66], and electrostatic repulsion–hydrophilic interaction chromatography (ERLIC) [67, 94]. Each fractionation technique has its own benefits and drawbacks [95]. SCX and SAX take advantage of the inherent negative charge of the phosphorylated peptides, thus further separating them from other peptides. However, the elution buffer is usually not compatible with subsequent enrichment or LC-MS steps, and the resulting peptides need to be desalted. HILIC offers a very clean fractionation option because only volatile aqueous and organic solvents are utilized, although it also suffers from poor peptide separation. ERLIC addresses the issue by combining the advantages of ion exchange and hydrophilic interaction and has since gained traction in the phosphoproteomic community. Finally, recent advances in the chromatographic materials have enabled the development of RPLC as another outstanding separation method [96]. Here, the fractionation is carried out at higher than normal pH (usually 8–10), thus ensuring its orthogonality with acidic RPLC during LC-MS analysis. Nowadays, the combination of appropriate fractionation with enrichment methods frequently generates thousands or even tens of thousands of unique phosphopeptides in a single set of experiments [10, 38, 85, 97, 98].

3 Quantitative measurement of protein phosphorylation

Advances in instrumentation and analytical techniques have made it possible to identify thousands of phosphorylation sites in a single LC-MS analysis, but quantitation of phosphorylation events is necessary for the interpretation of changes in complex biological systems. Current quantitation methods generally follow one of two strategies. The first is to use a tag to label proteins or peptides *in vivo* or *in vitro* and compare ion intensities to determine relative abundance ratios in a single MS analysis. The second strategy, typically called the label-free method, does not employ a label and instead relies upon the ion intensities or LC-MS peak areas to determine the relative or absolute abundances of analytes in two or more analyses.

SILAC, stable isotope labeling by amino acids in cell culture, is the most popular technique in proteomic studies to label proteins *in vivo* [99], which is also the case for phosphoproteomic studies. SILAC has been successfully applied to several large-scale studies of the phosphoproteome, including analyses of hepatocytes [100] and the yeast pheromone response [91]. However, SILAC depends on the metabolic processes of the cell

for incorporation of isotope-labeled amino acids during protein synthesis, which has traditionally limited SILAC quantitation to cell culture. As a result, recent efforts have focused on adapting the SILAC procedure to primary cells, tissue samples, and even whole organisms [101–103]. Mice labeled by SILAC have been used to generate standard peptides for analysis of phosphoproteome changes during chemically induced skin carcinogenesis in a mouse model [104].

Metabolic labeling of proteins in vivo followed by MS analysis permits quantitation of protein phosphorylation in a highly reproducible manner, but it can be cost prohibitive, and suitable cell lines or organisms may not be readily available. In these cases, chemically tagging proteins or peptides in vitro is an attractive alternative. Chemical tagging is carried out typically after protein extraction, so it is applicable to virtually all sample types. Additionally, experiments using chemical tags generally require less time than methods that rely on protein turnover within an organism. Dimethyl labeling represents a cost- and time-effective method for protein quantitation [105]. Adding an isotopic label through an enzymatic reaction is another alternative for nonspecific in vitro protein labeling. If the digestion of proteins using trypsin is done in ^{18}O -labeled water, a 4 Da shift is produced, which is sufficient for differentiating peptides in high-resolution MS analyses [106].

Isobaric tags for relative and absolute quantification (iTRAQ) [107] and tandem mass tags (TMT) [108] take a different approach. Isotopic labeling with SILAC, ^{18}O proteolysis, dimethyl labeling, and others produce mass shifts at the MS level that can be used for quantitation while MS/MS is used to gain peptide sequence information. On the other hand, iTRAQ and TMT labels have the same nominal mass and cannot be distinguished in precursor scans. Fragmentation of the tags during MS/MS produces distinct low-mass reporter ions that are used to calculate relative peptide abundances. Peptides that have been labeled with different versions of an isobaric tag will have identical ionization efficiencies and chromatographic behavior. This enables more accurate quantitation but also necessitates MS/MS scans for the generation of both quantitative and sequence data. Isobaric tags are popular in phosphoproteomics and have been applied in the identification and quantification of phosphorylation events in a variety of systems, including mammalian stem cells [109] and plants [110]. However, there have been concerns that coisolation of near-isobaric peaks, particularly in higher plexed experiments, may skew reporter ion ratios and lead to compression of detected differences in protein abundance [111, 112]. Evaluations of the accuracy and consistency of 4-plex iTRAQ in comparison to 8-plex iTRAQ and 6-plex TMT have reached conflicting conclusions [113, 114], but ratio distortion may be eliminated through an additional isolation and fragmentation event [115].

Because of its greater compatibility with different sample and instrument types, label-free quantitation has become an increasingly attractive alternative to isotope labeling. A popular way to achieve label-free quantitation is to use the number of times a peptide precursor ion is selected for MS/MS as an indication of the abundance of that peptide in the original sample [116]. This method, termed spectral counting, is fast and intuitive and has been successfully applied to phosphoproteomic studies [117]. However, the protein abundance indexes created from spectral count data are biased toward high abundance proteins, so quantitation of low-abundant proteins lacks reproducibility. Label-free quantitation can also

be accomplished through a comparison of peptide precursor ion intensities across multiple analyses. Label-free quantitation based upon extracted ion chromatograms or total ion intensities has a higher dynamic range than that of labeling strategies, but a major pitfall is variation in LC-MS performance from run to run [118]. Ideally, error due to variations in LC-MS performance can be corrected through spiked-in standards. For instance, isotope-labeled synthetic peptides are employed as internal standards in absolute quantification (AQUA) [119]. This technique is easily applied to the study of a few proteins but may be difficult to use in global level studies because a vast number of reference peptides are needed. To omit isotope labeling altogether, some have proposed utilizing peptides from an endogenous house-keeping protein as a standard [120]. This approach eliminates the need for a spiked-in standard, but it may be challenging to find a protein that is consistently expressed across all samples, especially when phosphorylation is being examined. Therefore, many label-free methods still rely upon a spiked-in standard to improve normalization.

We recently introduced a label-free method called Library-Assisted eXtracted Ion Chromatogram (LAXIC) quantitation [121]. The LAXIC strategy employs a spiked-in synthetic peptide library that has been designed to consecutively elute across the entire chromatogram. Localized normalization of phosphopeptides by library peptides that coelute within a small time frame accounts for fluctuating ion suppression effects and allows more accurate quantitation even when LC-MS performance varies.

4 Mapping kinase-substrate networks

There are well over 500 kinases that are responsible for phosphorylation; the genes encoding this enzyme class account for nearly 2% of the human genome [122]. When the slightly smaller number of phosphatases is added to that of the kinases, it becomes clear that a sizeable portion of the human proteome is dedicated to the regulation of phosphorylation. In fact, kinase activity is typically under tight control both temporally and spatially [123], and loss of regulation can lead to a disease state formation. Thus, mapping high-resolution kinase-substrate networks can elucidate signaling pathways, point to the molecular mechanisms of many diseases, and assist drug development for targeted therapies.

Besides the traditional genetic methods, there are a multitude of high-throughput strategies to identify protein kinase substrates. In general, these methods can be divided into three major categories: in vitro kinase assays, in vivo phosphoproteomics, and kinase-substrate association arrays. In vitro kinase assays involve the incubation of the kinase of interest with putative substrates, often cell extracts, in the presence of ATP. Phosphorylation of the substrates is then detected by radiometric [124], fluorometric [125], or other means. These assays are widely accessible, but they require a purified kinase and often suffer from promiscuous kinase activity due to a high concentration of kinase without the chaperones and binding partners that would be present in vivo. Kinase assays can also be performed with peptide libraries [126], phage expression libraries [127], and protein/peptide arrays [100, 128–130] for higher throughput, but again, these methods can produce high false-positive rates. On the other hand, global profiling of phosphoproteomes among different cellular states can provide information about the dynamic changes that occur during

signaling events [38, 75], but measuring global phosphorylation changes alone will not illustrate which substrates are directly associated with the kinase of interest. The third major category of detecting kinase-substrate relationships is based on their associations. Yeast-two-hybrid systems [131, 132] and affinity purification methods [123] have been used to identify kinase substrates, but, in general, the transience of kinase-substrate interactions limits the efficacy of these methods.

While still challenging, significant progress has been made in the effort to dissect kinase-substrate relationships, particularly through quantitative MS. For example, work has been done to identify the substrates of Aurora and Polo-like kinases in mitotically arrested cells by quantitative phosphoproteomics [133]. However, kinase assays using cell extracts suffer from high background due to endogenous kinase activity. To combat this problem, Shokat and coworkers developed the analog-sensitive kinase allele approach that employs kinases engineered to exclusively accept an ATP analog [134, 135]. The phosphorylation due to the engineered kinase can then be easily differentiated from that of the endogenous kinases and quantified by mass spectrometry. Others have demonstrated that a heating step before the kinase reaction reduces endogenous kinase activity [136]. We recently introduced a strategy for the identification of kinase substrates, termed kinase assay linked with phosphoproteomics (KALIP), which integrates in vitro and in vivo approaches for detection of direct kinase substrates [41,137]. First, phosphorylated proteins or peptides are extracted from cell lysate and dephosphorylated. Endogenous kinase activity is inhibited by covalent modification with a small molecule before the kinase of interest is added to the mixture. The proteins phosphorylated in the kinase reaction are then identified and quantified by mass spectrometry. Next, endogenously phosphorylated proteins are extracted and quantified from cells with and without kinase inhibitor treatment. Overlap of in vitro kinase assay identifications with the in vivo kinase-dependent phosphoproteome produces confident identification of bona fide kinase substrates [41,137,138]. Future work in the realm of kinase-substrate mapping will focus on translating highly specific methods for the identification of true kinase substrates to high-throughput platforms for use in signaling network analysis and drug discovery.

5 Phosphoproteome data validation and clinical potential

As more phosphoproteomic data are being published every month and we gain new understanding of its comprehensive roles in cellular life, the need to study phosphorylation mis-regulation in clinical setting is quickly becoming apparent. Unlike genomic profiling, phosphoproteome analysis provides critical information about normal and improper signaling events in a more immediate and time-relevant manner. This is especially critical in cancer, where phosphorylation abnormalities are often the main cause of disease progression and can act as a marker for its onset. Currently, more than 30% of drugs in development are targeting lipid and protein kinases [139]. As a result, kinases and phosphatases are well on their way to be the most druggable protein targets in cancer, immune diseases, neurological, and other ailments. There are already quite a few examples of small kinase inhibitors being successfully used for treatment of breast cancer, chronic myelogenous leukemia, acute myeloid leukemia, and nonsmall cell lung cancer. With these early successes, profiling

protein phosphorylation and related enzyme activities will likely emerge as a routine and critical element in therapy selection.

Numerous phosphoproteomic studies are available detailing the signaling pathways and potential treatment outcomes for a number of phosphorylation-dependent cancers, including those linked to misregulation of EGFR, RAF, HER, ALK, ROS, ABL, and many others [140–142]. Besides examining cancer-related pathways and drug perturbation, these studies can shed light on the underlying mechanisms of drug sensitivity and resistance, activation of feedback loops, and predictions of the most effective multi-therapy treatment regimen [143–146]. The current translational success rate from first-in-human studies to drug registration is only 11–15% [147] and has not significantly improved in over a decade [148], making the drug discovery process highly inefficient. While the hypothesis still remains to be confirmed, phosphoproteomic analyses, in combination with pharmacogenomic screenings, hold promise for significantly improving this process due to the proximity of phosphorylation events to disease-causing signaling networks and mechanisms [35].

Drug discovery to alleviate negative effects from abnormal kinase activity is currently the major objective in phosphoproteomics. However, an equally important area is biomarker discovery. Clinically useful protein biomarkers can provide earlier and better diagnosis for more effective treatments, improved prognosis, and preventative screening that can identify patients at highest risk and subsequently offer intervention [149–152]. Measurement of specific phospho-signatures is increasingly appealing as a predictive tool for drug response and patient selection. Numerous studies have applied MS-based proteomic strategies to discover candidate protein biomarkers for a wide range of diseases. To date, however, few protein biomarkers identified using proteomics, especially phosphoproteomics, have been introduced in clinical practice [153–156]. Even with significant advances in instrumentation, multidimensional fractionation, enrichment strategies, and high-abundant protein depletion methods, detection of low-abundant disease-relevant phosphoproteins from complex matrices—especially biofluids—continues to be difficult. More importantly, verification and validation of biomarkers has been the most challenging task.

After identification of potential biomarkers, the most commonly used approaches for their validation are ELISA, Western Blotting, and antibody/aptamer arrays [157]. These assays are predominantly based on antibodies. For phosphoproteins as potential targets, even in rare cases when high-quality phospho-specific antibodies can be generated, the development process is very long, costly and often does not offer sufficient multiplexing capabilities. With a positive outcome of true biomarker verification being far from certain, such a strategy is difficult to justify. Similar to the discovery phase of the experiment, MS-based analytical platform would still appear to be a desirable strategy for biomarker validation due to its medium-throughput capabilities and requirement for relatively lower upfront preparative cost when compared to the antibody-based assays.

Selected reaction monitoring (SRM, also referred to as multiple reaction monitoring (MRM)) is one of the MS-based approaches that can offer specificity, robustness, reproducibility, and quantitative capabilities of an antibody without the upfront development costs [155,158,159]. It offers the ability to reproducibly and accurately distinguish and

quantify peptides/phosphopeptides from multiple samples with high sensitivity. While in some cases enrichment (e.g. SISCAPA [160, 161] or high-abundant protein depletion [162–164]) are necessary to achieve a lower limit of quantitation for SRM, deficiency in low-abundant analyte quantitation is a common challenge and is a trade-off for other powerful benefits. The SRM technique has already demonstrated the ability to quickly evaluate potential protein/phosphoprotein biomarker candidates from dozens of proteins in each run [165–169]. This multiplexing ability significantly increases the value of the method. With further procedural and instrumentation improvements, the ability to examine and verify close to a hundred biomarker candidates per analysis is now available. These advances greatly increase the rate of success and allow more efficient generation of a panel of candidates to serve as potential markers [155, 170]. Multiple studies in the recent years have examined SRM limits of detection and quantitation, accuracy, precision, and variability between experiments and even different labs [171–173]. As a result, the method has been established as compatible with rigorous requirements for genuine biomarker verification, thus further confirming its promise in translational clinical research [174]. The application of targeted MS circumvents the drawbacks of antibodies and other affinity-based agents, and provides a powerful alternative route for biomarker validation.

With improvements in clinical sample handling, direct disease phosphorylation analysis and biomarker verification streamlining, profiling phosphorylation signaling can become an integral part of clinical research, preventative diagnostics, and personalized medicine. Phosphoproteomics will enable classification of individual cancer subtypes on the molecular level, identification and validation of disease biomarkers, and discovery of novel treatment options. It will help reveal the mechanisms of drug resistance and better elucidate and predict the tumor response to therapies [175]. While at this stage a vast majority of phosphoproteomic analyses are still employed in a laboratory R&D setting, further advances of the field will soon bring this powerful discovery and profiling strategy into clinical settings.

6 Conclusion

We are now living in a postgenomic era, as it is becoming apparent that DNA sequence alone is not sufficient to predict disease dynamics. Drastic advancements in phosphoproteomics technologies, such as instrumentation, sample preparation, quantitation methods, and bioinformatics, have provided us with abundance of information regarding biochemical signaling networks and disease progression pathways. Research efforts have been increasingly refocused away from experiments of large-scale phosphoproteome profiling toward targeted functional phosphorylation analyses that provide more manageable and relevant datasets. We are currently witnessing a strong push to utilize and integrate comprehensive phosphoproteomic strategies into targeted systems biology approaches for clinical research. While comprehensive analytical separation tools have been utilized to study model systems, they cannot be directly applied to relevant clinical sources [13, 101]. The development of novel analytical strategies and techniques will be tremendously valuable for target validation, disease subclassification, targeted therapy formulation, drug resistance analysis, and prediction of treatment outcomes [176, 177]. As a result, the outcomes of targeted phosphoproteomic analyses (Fig. 4) on the molecular and

physiological scale, though incredibly challenging, could have important medical implications and may become a new frontier in drug discovery and personalized medicine.

Acknowledgments

This project has been funded by National Institutes of Health grants GM088317 and GM088226.

Abbreviations

IMAC	immobilized metal ion chromatography
iTRAQ	isobaric tags for relative and absolute quantitation
MOAC	metal oxide affinity chromatography
SILAC	stable isotope labeling by amino acids in cell culture
SRM	selected reaction monitoring
TMT	tandem mass tags

References

1. Berrade L, Garcia AE, Camarero JA. *Pharm Res.* 2011; 28:1480–1499. [PubMed: 21116694]
2. Hunter T. *Cell.* 2000; 100:113–127. [PubMed: 10647936]
3. Cohen P. *Nat Cell Biol.* 2002; 4:E127–E130. [PubMed: 11988757]
4. Pawson T, Scott JD. *Trends Biochem Sci.* 2005; 30:286–290. [PubMed: 15950870]
5. Case N, Thomas J, Sen B, Styner M, Xie Z, Galior K, Rubin J. *J Biol Chem.* 2011; 286:39450–39456. [PubMed: 21956113]
6. Cole PA, Shen K, Qiao Y, Wang D. *Curr Opin Chem Biol.* 2003; 7:580–585. [PubMed: 14580561]
7. Yu S, Huang H, Iliuk A, Wang WH, Jayasundera KB, Tao WA, Post CB, Geahlen RL. *J Biol Chem.* 2013; 288:10870–10881. [PubMed: 23447535]
8. Kuwahara H, Nishizaki M, Kanazawa H. *J Biochem.* 2008; 143:349–358. [PubMed: 18084041]
9. Hong JJ, Yankee TM, Harrison ML, Geahlen RL. *J Biol Chem.* 2002; 277:31703–31714. [PubMed: 12077122]
10. Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalarski CE, Elledge SJ, Gygi SP. *Proc Natl Acad Sci USA.* 2008; 105:10762–10767. [PubMed: 18669648]
11. Mayya V, Lundgren DH, Hwang SI, Rezaul K, Wu L, Eng JK, Rodionov V, Han DK. *Sci Signal.* 2009; 2:ra46. [PubMed: 19690332]
12. Yamada M, Suzuki K, Mizutani M, Asada A, Matozaki T, Ikeuchi T, Koizumi S, Hatanaka H. *J Biochem.* 2001; 130:157–165. [PubMed: 11432792]
13. Hanahan D, Weinberg RA. *Cell.* 2000; 100:57–70. [PubMed: 10647931]
14. Tan CS, Bodenmiller B, Pasculescu A, Jovanovic M, Hengartner MO, Jorgensen C, Bader GD, Aebersold R, Pawson T, Linding R. *Sci Signal.* 2009; 2:ra39. [PubMed: 19638616]
15. Harsha HC, Pandey A. *Mol Oncol.* 2010; 4:482–495. [PubMed: 20937571]
16. Van Eyk JE. *Circ Res.* 2011; 108:490–498. [PubMed: 21335431]
17. Gaestel M, Kotlyarov A, Kracht M. *Nat Rev Drug Discov.* 2009; 8:480–499. [PubMed: 19483709]
18. Iwai LK, Benoist C, Mathis D, White FM. *J Proteome Res.* 2010; 9:3135–3145. [PubMed: 20438120]
19. Summers TA, Creutz CE. *J Biol Chem.* 1985; 260:2437–2443. [PubMed: 3156130]
20. Li H, Liu JP, Smith R, Robinson PJ. *Mol Cell Endocrinol.* 1996; 122:159–171. [PubMed: 8902846]
21. Guy GR, Philip R, Tan YH. *Electrophoresis.* 1994; 15:417–440. [PubMed: 8055870]

22. Burnett G, Kennedy EP. J Biol Chem. 1954; 211:969–980. [PubMed: 13221602]
23. Krebs EG, Fischer EH. J Biol Chem. 1955; 216:113–120. [PubMed: 13252011]
24. Fischer EH, Krebs EG. J Biol Chem. 1955; 216:121–132. [PubMed: 13252012]
25. Hunter T. Philos Trans R Soc Lond B Biol Sci. 1998; 353:583–605. [PubMed: 9602534]
26. Blaydes JP, Vojtesek B, Bloomberg GB, Hupp TR. Methods Mol Biol. 2000; 99:177–189. [PubMed: 10909085]
27. Panse S, Dong L, Burian A, Carus R, Schutkowski M, Reimer U, Schneider-Mergener J. Mol Divers. 2004; 8:291–299. [PubMed: 15384422]
28. Zerweck J, Masch A, Schutkowski M. Methods Mol Biol. 2009; 524:169–180. [PubMed: 19377944]
29. Hause RJ, Kim HD, Leung KK, Jones RB. Expert Rev Proteomics. 2011; 8:565–575. [PubMed: 21999828]
30. Perlee L, Christiansen J, Dondero R, Grimwade B, Lejnine S, Mullenix M, Shao W, Sorette M, Tchernev V, Patel D, Kingsmore S. Prot Sci. 2004; 2:9.
31. Gembitsky DS, Lawlor K, Jacovina A, Yaneva M, Tempst P. Mol Cell Prot. 2004; 3:1102–1118.
32. Mueller C, Liotta LA, Espina V. Mol Oncol. 2010; 4:461–481. [PubMed: 20974554]
33. Malinowsky K, Wolff C, Ergin B, Berg D, Becker KF. J Cell Physiol. 2010; 225:364–370. [PubMed: 20625992]
34. Wulfkühle JD, Speer R, Pierobon M, Laird J, Espina V, Deng J, Mammano E, Yang SX, Swain SM, Nitti D, Esserman LJ, Belluco C, Liotta LA, Petricoin EF 3rd. J Proteome Res. 2008; 7:1508–1517. [PubMed: 18257519]
35. Morris MK, Chi A, Melas IN, Alexopoulos LG. Drug Discov Today. 2013; 19:425–432. [PubMed: 24141136]
36. Steelman LS, Abrams SL, Whelan J, Bertrand FE, Ludwig DE, Basecke J, Libra M, Stivala F, Milella M, Tafuri A, Lunghi P, Bonati A, Martelli AM, McCubrey JA. Leukemia. 2008; 22:686–707. [PubMed: 18337767]
37. Steelman LS, Chappell WH, Abrams SL, Kempf RC, Long J, Laidler P, Mijatovic S, Maksimovic-Ivanic D, Stivala F, Mazzarino MC, Donia M, Fagone P, Malaponte G, Nicoletti F, Libra M, Milella M, Tafuri A, Bonati A, Basecke J, Cocco L, Evangelisti C, Martelli AM, Montalto G, Cervello M, McCubrey JA. Aging (Albany NYx). 2011; 3:192–222.
38. Olsen JV, Blagoev B, Gnäd F, Macek B, Kumar C, Mortensen P, Mann M. Cell. 2006; 127:635–648. [PubMed: 17081983]
39. Pan C, Olsen JV, Daub H, Mann M. Mol Cell Prot. 2009; 8:2796–2808.
40. Rubbi L, Titz B, Brown L, Galvan E, Komisopoulou E, Chen SS, Low T, Tahmasian M, Skaggs B, Muschen M, Pellegrini M, Graeber TG. Sci Signal. 2011; 4:ra18. [PubMed: 21447799]
41. Xue L, Wang WH, Iliuk A, Hu L, Galan JA, Yu S, Hans M, Geahlen RL, Tao WA. Proc Natl Acad Sci USA. 2012; 109:5615–5620. [PubMed: 22451900]
42. Andersen JN, Sathyanarayanan S, Di Bacco A, Chi A, Zhang T, Chen AH, Dolinski B, Kraus M, Roberts B, Arthur W, Klinghoffer RA, Gargano D, Li L, Feldman I, Lynch B, Rush J, Hendrickson RC, Blume-Jensen P, Paweletz CP. Sci Transl Med. 2010; 2:43ra55.
43. Alcolea MP, Casado P, Rodriguez-Prados JC, Van-haesebroeck B, Cutillas PR. Mol Cell Prot. 2012; 11:453–466.
44. Engholm-Keller K, Larsen MR. Proteomics. 2013; 13:910–931. [PubMed: 23404676]
45. Leitner A, Sturm M, Lindner W. Anal Chim Acta. 2011; 703:19–30. [PubMed: 21843671]
46. Kanshin E, Michnick S, Thibault P. Semin Cell Dev Biol. 2012; 23:843–853. [PubMed: 22683502]
47. Imamura H, Wakabayashi M, Ishihama Y. Semin Cell Dev Biol. 2012; 23:836–842. [PubMed: 22683501]
48. Roux PP, Thibault P. Mol Cell Prot. 2013; 12:3453–3464.
49. Nita-Lazar A, Saito-Benz H, White FM. Proteomics. 2008; 8:4433–4443. [PubMed: 18846511]
50. Steen H, Jeblanathirajah JA, Rush J, Morrice N, Kirschner MW. Mol Cell Prot. 2006; 5:172–181.
51. Hung CW, Kubler D, Lehmann WD. Electrophoresis. 2007; 28:2044–2052. [PubMed: 17523138]

52. Xu Y, Sprung R, Kwon SW, Kim SC, Zhao Y. *J Proteome Res.* 2007; 6:1153–1157. [PubMed: 17330948]
53. Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. *Proc Natl Acad Sci USA.* 2004; 101:12130–12135. [PubMed: 15302935]
54. Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, Zhang H, Zha XM, Polakiewicz RD, Comb MJ. *Nat Biotechnol.* 2005; 23:94–101. [PubMed: 15592455]
55. Ross AH, Baltimore D, Eisen HN. *Nature.* 1981; 294:654–656. [PubMed: 6171737]
56. Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, Nardone J, Lee K, Reeves C, Li Y, Hu Y, Tan Z, Stokes M, Sullivan L, Mitchell J, Wetzel R, Macneill J, Ren JM, Yuan J, Bakalarski CE, Villen J, Kornhauser JM, Smith B, Li D, Zhou X, Gygi SP, Gu TL, Polakiewicz RD, Rush J, Comb MJ. *Cell.* 2007; 131:1190–1203. [PubMed: 18083107]
57. Oda Y, Nagasu T, Chait BT. *Nat Biotechnol.* 2001; 19:379–382. [PubMed: 11283599]
58. McLachlin DT, Chait BT. *Anal Chem.* 2003; 75:6826–6836. [PubMed: 14670042]
59. Tao WA, Wollscheid B, O'Brien R, Eng JK, Li XJ, Bodenmiller B, Watts JD, Hood L, Aebersold R. *Nat Methods.* 2005; 2:591–598. [PubMed: 16094384]
60. Bodenmiller B, Mueller LN, Mueller M, Domon B, Aebersold R. *Nat Methods.* 2007; 4:231–237. [PubMed: 17293869]
61. Porath J. *J Chromatogr.* 1988; 443:3–11. [PubMed: 2844839]
62. Pinkse MW, Uitto PM, Hilhorst MJ, Ooms B, Heck AJ. *Anal Chem.* 2004; 76:3935–3943. [PubMed: 15253627]
63. Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jorgensen TJ. *Mol Cell Prot.* 2005; 4:873–886.
64. Kweon HK, Hakansson K. *Anal Chem.* 2006; 78:1743–1749. [PubMed: 16536406]
65. Ballif BA, Villen J, Beausoleil SA, Schwartz D, Gygi SP. *Mol Cell Prot.* 2004; 3:1093–1101.
66. McNulty DE, Annan RS. *Mol Cell Prot.* 2008; 7:971–980.
67. Alpert AJ. *Anal Chem.* 2008; 80:62–76. [PubMed: 18027909]
68. Beltran L, Cutillas PR. *Amino Acids.* 2012; 43:1009–1024. [PubMed: 22821267]
69. Ficarro SB, Parikh JR, Blank NC, Marto JA. *Anal Chem.* 2008; 80:4606–4613. [PubMed: 18491922]
70. Neville DC, Rozanas CR, Price EM, Gruis DB, Verkman AS, Townsend RR. *Protein Sci.* 1997; 6:2436–2445. [PubMed: 9385646]
71. Leitner A, Sturm M, Hudecz O, Mazanek M, Smatt JH, Linden M, Lindner W, Mechtler K. *Anal Chem.* 82:2726–2733. [PubMed: 20201521]
72. Leitner A, Sturm M, Smatt JH, Jarn M, Linden M, Mechtler K, Lindner W. *Anal Chim Acta.* 2009; 638:51–57. [PubMed: 19298879]
73. Wolschin F, Wienkoop S, Weckwerth W. *Proteomics.* 2005; 5:4389–4397. [PubMed: 16222723]
74. Andersson L, Porath J. *Anal Biochem.* 1986; 154:250–254. [PubMed: 3085541]
75. Ficarro SB, McClell ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM. *Nat Biotechnol.* 2002; 20:301–305. [PubMed: 11875433]
76. Tsai CF, Wang YT, Chen YR, Lai CY, Lin PY, Pan KT, Chen JY, Khoo KH, Chen YJ. *J Proteome Res.* 2008; 7:4058–4069. [PubMed: 18707149]
77. Ndassa YM, Orsi C, Marto JA, Chen S, Ross MM. *J Proteome Res.* 2006; 5:2789–2799. [PubMed: 17022650]
78. Vilasi A, Fiume I, Pace P, Rossi M, Pocsfalvi G. *J Mass Spectrom.* 2013; 48:1188–1198. [PubMed: 24259207]
79. Lai AC, Tsai CF, Hsu CC, Sun YN, Chen YJ. *Rapid Commun Mass Spectrom.* 2012; 26:2186–2194. [PubMed: 22886815]
80. Negroni L, Claverol S, Rosenbaum J, Chevet E, Bonneau M, Schmitter JM. *J Chromatogr B.* 2012; 891–892:109–112.
81. Jensen SS, Larsen MR. *Rapid Commun Mass Spectrom.* 2007; 21:3635–3645. [PubMed: 17939157]

82. Sugiyama N, Masuda T, Shinoda K, Nakamura A, Tomita M, Ishihama Y. *Mol Cell Prot.* 2007; 6:1103–1109.
83. Aryal UK, Ross AR. *Rapid Commun Mass Spectrom.* 2010; 24:219–231. [PubMed: 20014058]
84. Lemeer S, Pinkse MW, Mohammed S, van Breukelen B, den Hertog J, Slijper M, Heck AJ. *J Proteome Res.* 2008; 7:1555–1564. [PubMed: 18307296]
85. Zhou H, Low TY, Hennrich ML, van der Toorn H, Schwend T, Zou H, Mohammed S, Heck AJ. *Mol Cell Prot.* 2011; 10 M110 006452.
86. Thingholm TE, Jensen ON, Robinson PJ, Larsen MR. *Mol Cell Prot.* 2008; 7:661–671.
87. Iliuk AB, Martin VA, Alicie BM, Geahlen RL, Tao WA. *Mol Cell Prot.* 2010; 9:2162–2172.
88. Boas U, Heegaard PM. *Chem Soc Rev.* 2004; 33:43–63. [PubMed: 14737508]
89. Iliuk A, Jayasundera K, Schluttenhofer R, Tao WA. *Methods Mol Biol.* 2011; 790:277–285. [PubMed: 21948422]
90. Iliuk A, Tao WA. *Methods Mol Biol.* 2009; 527:117–129. [PubMed: 19241010]
91. Gruhler A, Olsen JV, Mohammed S, Mortensen P, Faergeman NJ, Mann M, Jensen ON. *Mol Cell Prot.* 2005; 4:310–327.
92. Han G, Ye M, Zhou H, Jiang X, Feng S, Jiang X, Tian R, Wan D, Zou H, Gu J. *Proteomics.* 2008; 8:1346–1361. [PubMed: 18318008]
93. Dai J, Wang LS, Wu YB, Sheng QH, Wu JR, Shieh CH, Zeng R. *J Proteome Res.* 2009; 8:133–141. [PubMed: 19053533]
94. Zarei M, Sprenger A, Gretzmeier C, Dengjel J. *J Proteome Res.* 2013; 12:5989–5995. [PubMed: 24144214]
95. Zarei M, Sprenger A, Metzger F, Gretzmeier C, Dengjel J. *J Proteome Res.* 2011; 10:3474–3483. [PubMed: 21682340]
96. Gilar M, Olivova P, Daly AE, Gebler JC. *J Sep Sci.* 2005; 28:1694–1703. [PubMed: 16224963]
97. Di Palma S, Boersema PJ, Heck AJ, Mohammed S. *Anal Chem.* 2011; 83:3440–3447. [PubMed: 21443167]
98. Villen J, Beausoleil SA, Gerber SA, Gygi SP. *Proc Natl Acad Sci USA.* 2007; 104:1488–1493. [PubMed: 17242355]
99. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. *Mol Cell Proteomics.* 2002; 1:376–386. [PubMed: 12118079]
100. Amanchy R, Zhong J, Molina H, Chaerkady R, Iwahori A, Kalume DE, Gronborg M, Joore J, Cope L, Pandey A. *J Proteome Res.* 2008; 7:3900–3910. [PubMed: 18698806]
101. Pan C, Kumar C, Bohl S, Klingmueller U, Mann M. *Mol Cell Proteomics.* 2009; 8:443–450. [PubMed: 18952599]
102. Geiger T, Cox J, Ostasiewicz P, Wisniewski JR, Mann M. *Nat Methods.* 2010; 7:383–385. [PubMed: 20364148]
103. Kruger M, Moser M, Ussar S, Thievensen I, Lubner CA, Forner F, Schmidt S, Zanivan S, Fassler R, Mann M. *Cell.* 2008; 134:353–364. [PubMed: 18662549]
104. Zanivan S, Meves A, Behrendt K, Schoof EM, Neilson LJ, Cox J, Tang HR, Kalna G, van Ree JH, van Deursen JM, Trempus CS, Machesky LM, Lindling R, Wickstrom SA, Fassler R, Mann M. *Cell Rep.* 2013; 3:552–566. [PubMed: 23375375]
105. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ. *Nat Protoc.* 2009; 4:484–494. [PubMed: 19300442]
106. Yao X, Freas A, Ramirez J, Demirev PA, Fenselau C. *Anal Chem.* 2001; 73:2836–2842. [PubMed: 11467524]
107. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlett-Jones M, He F, Jacobson A, Pappin DJ. *Mol Cell Proteomics.* 2004; 3:1154–1169. [PubMed: 15385600]
108. Thompson A, Schafer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Johnstone R, Mohammed AK, Hamon C. *Anal Chem.* 2003; 75:1895–1904. [PubMed: 12713048]

109. Phanstiel DH, Brumbaugh J, Wenger CD, Tian S, Probasco MD, Bailey DJ, Swaney DL, Tervo MA, Bolin JM, Ruotti V, Stewart R, Thomson JA, Coon JJ. *Nat Methods*. 2011; 8:821–827. [PubMed: 21983960]
110. Nuhse TS, Bottrill AR, Jones AM, Peck SC. *Plant J*. 2007; 51:931–940. [PubMed: 17651370]
111. Karp NA, Huber W, Sadowski PG, Charles PD, Hester SV, Lilley KS. *Mol Cell Proteomics*. 2010; 9:1885–1897. [PubMed: 20382981]
112. Ow SY, Salim M, Noirel J, Evans C, Rehman I, Wright PC. *J Proteome Res*. 2009; 8:5347–5355. [PubMed: 19754192]
113. Pichler P, Kocher T, Holzmann J, Mazanek M, Taus T, Ammerer G, Mechtler K. *Anal Chem*. 2010; 82:6549–6558. [PubMed: 20593797]
114. Pottiez G, Wiederin J, Fox HS, Ciborowski P. *J Proteome Res*. 2012; 11:3774–3781. [PubMed: 22594965]
115. Ting L, Rad R, Gygi SP, Haas W. *Nat Methods*. 2011; 8:937–940. [PubMed: 21963607]
116. Liu H, Sadygov RG, Yates JR 3rd. *Anal Chem*. 2004; 76:4193–4201. [PubMed: 15253663]
117. Xie X, Feng S, Vuong H, Liu Y, Goodison S, Lubman DM. *Electrophoresis*. 2010; 31:1842–1852. [PubMed: 20446291]
118. Kim YJ, Zhan P, Feild B, Ruben SM, He T. *Anal Chem*. 2007; 79:5651–5658. [PubMed: 17580949]
119. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. *Proc Natl Acad Sci USA*. 2003; 100:6940–6945. [PubMed: 12771378]
120. Tabata T, Sato T, Kuromitsu J, Oda Y. *Anal Chem*. 2007; 79:8440–8445. [PubMed: 17929902]
121. Xue L, Wang P, Wang L, Renzi E, Radivojac P, Tang H, Arnold R, Zhu JK, Tao WA. *Mol Cell Prot*. 2013; 12:2354–2369.
122. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. *Science*. 2002; 298:1912–1934. [PubMed: 12471243]
123. Daub H, Olsen JV, Bairlein M, Gnad F, Oppermann FS, Korner R, Greff Z, Keri G, Stemmann O, Mann M. *Mol Cell*. 2008; 31:438–448. [PubMed: 18691976]
124. Knebel A, Morrice N, Cohen P, Embo J. 2001; 20:4360–4369. [PubMed: 11500363]
125. Martin K, Steinberg TH, Cooley LA, Gee KR, Beechem JM, Patton WF. *Proteomics*. 2003; 3:1244–1255. [PubMed: 12872225]
126. Yaffe MB, Leparc GG, Lai J, Obata T, Volinia S, Cantley LC. *Nat Biotechnol*. 2001; 19:348–353. [PubMed: 11283593]
127. Fabian MA, Biggs WH 3rd, Treiber DK, Atteridge CE, Azimioara MD, Benedetti MG, Carter TA, Ciceri P, Edeen PT, Floyd M, Ford JM, Galvin M, Gerlach JL, Grotzfeld RM, Herrgard S, Insko DE, Insko MA, Lai AG, Lelias JM, Mehta SA, Milanov ZV, Velasco AM, Wodicka LM, Patel HK, Zarrinkar PP, Lockhart DJ. *Nat Biotechnol*. 2005; 23:329–336. [PubMed: 15711537]
128. Zhu H, Snyder M. *Curr Opin Chem Biol*. 2003; 7:55–63. [PubMed: 12547427]
129. Han X, Sonoda T, Mori T, Yamanouchi G, Yamaji T, Shigaki S, Niidome T, Katayama Y. *Comb Chem High Throughput Screen*. 2010; 13:777–789. [PubMed: 20615195]
130. Lam KS, Liu R, Miyamoto S, Lehman AL, Tuscano JM. *Acc Chem Res*. 2003; 36:370–377. [PubMed: 12809522]
131. Yang X, Hubbard EJ, Carlson M. *Science*. 1992; 257:680–682. [PubMed: 1496382]
132. Staudinger J, Zhou J, Burgess R, Elledge SJ, Olson EN. *J Cell Biol*. 1995; 128:263–271. [PubMed: 7844141]
133. Kettenbach AN, Schweppe DK, Faherty BK, Pechenick D, Pletnev AA, Gerber SA. *Sci Signal*. 2011; 4:rs5. [PubMed: 21712546]
134. Shah K, Liu Y, Deirmengian C, Shokat KM. *Proc Natl Acad Sci USA*. 1997; 94:3565–3570. [PubMed: 9108016]
135. Blethrow JD, Glavy JS, Morgan DO, Shokat KM. *Proc Natl Acad Sci USA*. 2008; 105:1442–1447. [PubMed: 18234856]
136. Troiani S, Uggeri M, Moll J, Isacchi A, Kalisz HM, Rusconi L, Valsasina B. *J Proteome Res*. 2005; 4:1296–1303. [PubMed: 16083279]

137. Xue L, Geahlen RL, Tao WA. *Mol Cell Proteomics*. 2013; 12:2969–2980. [PubMed: 23793017]
138. Wang P, Xue L, Batelli G, Lee S, Hou YJ, Van Oosten MJ, Zhang H, Tao WA, Zhu JK. *Proc Natl Acad Sci USA*. 2013; 110:11205–11210. [PubMed: 23776212]
139. Fabbro D, Cowan-Jacob SW, Mobitz H, Martiny-Baron G. *Methods Mol Biol*. 2012; 795:1–34. [PubMed: 21960212]
140. Johnson SA, Hunter T. *Nat Methods*. 2005; 2:17–25. [PubMed: 15789031]
141. Manning BD, Cantley LC. *Sci STKE*. 2002; 2002:pe49. [PubMed: 12475999]
142. Hunter T. *Curr Opin Cell Biol*. 2009; 21:140–146. [PubMed: 19269802]
143. Zanivan S, Gnad F, Wickstrom SA, Geiger T, Macek B, Cox J, Fassler R, Mann M. *J Proteome Res*. 2008; 7:5314–5326. [PubMed: 19367708]
144. Guo A, Villen J, Kornhauser J, Lee KA, Stokes MP, Rikova K, Possemato A, Nardone J, Innocenti G, Wetzel R, Wang Y, MacNeill J, Mitchell J, Gygi SP, Rush J, Polakiewicz RD, Comb MJ. *Proc Natl Acad Sci USA*. 2008; 105:692–697. [PubMed: 18180459]
145. Vojvodic M, Hansford LM, Morozova O, Blakely KM, Taylor P, Fathers KE, Moffat J, Marra M, Smith KM, Moran MF, Kaplan DR. *Curr Drug Discov Technol*. 2013; 10:283–304. [PubMed: 23701117]
146. Schweppe DK, Rigas JR, Gerber SA. *J Proteomics*. 2013; 91:286–296. [PubMed: 23911959]
147. Barrenho E, Miraldo M, Smith P. *Imp Coll Business School*. 2013:1–47.
148. Kola I, Landis J. *Nat Rev Drug Discov*. 2004; 3:711–715. [PubMed: 15286737]
149. Anderson NL. *Clin Chem*. 2010; 56:177–185. [PubMed: 19884488]
150. Apweiler R, Aslanidis C, Deufel T, Gerstner A, Hansen J, Hochstrasser D, Kellner R, Kubicek M, Lottspeich F, Maser E, Mewes HW, Meyer HE, Mullner S, Mutter W, Neumaier M, Nollau P, Nothwang HG, Ponten F, Radbruch A, Reinert K, Rothe G, Stockinger H, Tarnok A, Taussig MJ, Thiel A, Thiery J, Ueffing M, Valet G, Vandekerckhove J, Verhuven W, Wagener C, Wagner O, Schmitz G. *Clin Chem Lab Med*. 2009; 47:724–744. [PubMed: 19527139]
151. Blonder J, Issaq HJ, Veenstra TD. *Electrophoresis*. 2011; 32:1541–1548. [PubMed: 21557261]
152. Schilsky RL. *Nat Rev Drug Discov*. 2010; 9:363–366. [PubMed: 20431568]
153. Liu T, Qian WJ, Strittmatter EF, Camp DG 2nd, Anderson GA, Thrall BD, Smith RD. *Anal Chem*. 2004; 76:5345–5353. [PubMed: 15362891]
154. Ma D, Li L. *Exp Rev Proteomics*. 2012; 9:267–280.
155. Picotti P, Aebersold R. *Nat Methods*. 2012; 9:555–566. [PubMed: 22669653]
156. Surinova S, Schiess R, Huttenhain R, Cerciello F, Wollscheid B, Aebersold R. *J Proteome Res*. 2011; 10:5–16. [PubMed: 21142170]
157. Anderson NL. *Mol Cell Proteomics*. 2005; 4:1441–1444. [PubMed: 16020426]
158. Holman SW, Sims PF, Eysers CE. *Bioanalysis*. 2012; 4:1763–1786. [PubMed: 22877222]
159. Kerns EH, Di L. *Curr Drug Metab*. 2006; 7:457–466. [PubMed: 16787155]
160. Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. *J Proteome Res*. 2004; 3:235–244. [PubMed: 15113099]
161. Whiteaker JR, Zhao L, Anderson L, Paulovich AG. *Mol Cell Proteomics*. 2010; 9:184–196. [PubMed: 19843560]
162. Whiteaker JR, Zhang H, Eng JK, Fang R, Piening BD, Feng LC, Lorentzen TD, Schoenherr RM, Keane JF, Holzman T, Fitzgibbon M, Lin C, Cooke K, Liu T, Camp DG 2nd, Anderson L, Watts J, Smith RD, McIntosh MW, Paulovich AG. *J Proteome Res*. 2007; 6:828–836. [PubMed: 17269739]
163. Keshishian H, Addona T, Burgess M, Kuhn E, Carr SA. *Mol Cell Proteomics*. 2007; 6:2212–2229. [PubMed: 17939991]
164. Fortin T, Salvador A, Charrier JP, Lenz C, Lacoux X, Morla A, Choquet-Kastylevsky G, Lemoine J. *Mol Cell Proteomics*. 2009; 8:1006–1015. [PubMed: 19068476]
165. Narumi R, Murakami T, Kuga T, Adachi J, Shiromizu T, Muraoka S, Kume H, Kodera Y, Matsumoto M, Nakayama K, Miyamoto Y, Ishitobi M, Inaji H, Kato K, Tomonaga T. *J Proteome Res*. 2012; 11:5311–5322. [PubMed: 22985185]

166. Kalin M, Cima I, Schiess R, Fankhauser N, Powles T, Wild P, Templeton A, Cerny T, Aebersold R, Krek W, Gillessen S. *Eur Urol.* 2011; 60:1235–1243. [PubMed: 21741162]
167. Cima I, Schiess R, Wild P, Kaelin M, Schuffler P, Lange V, Picotti P, Ossola R, Templeton A, Schubert O, Fuchs T, Leippold T, Wyler S, Zehetner J, Jochum W, Buhmann J, Cerny T, Moch H, Gillessen S, Aebersold R, Krek W. *Proc Natl Acad Sci USA.* 2011; 108:3342–3347. [PubMed: 21300890]
168. Cox DM, Zhong F, Du M, Duchoslav E, Sakuma T, McDermott JC. *J Biomol Tech.* 2005; 16:83–90. [PubMed: 16030315]
169. Unwin RD, Griffiths JR, Leverentz MK, Grallert A, Hagan IM, Whetton AD. *Mol Cell Proteomics.* 2005; 4:1134–1144. [PubMed: 15923565]
170. Edwards AM, Isserlin R, Bader GD, Frye SV, Willson TM, Yu FH. *Nature.* 2011; 470:163–165. [PubMed: 21307913]
171. Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, Bunk DM, Spiegelman CH, Zimmerman LJ, Ham AJ, Keshishian H, Hall SC, Allen S, Blackman RK, Borchers CH, Buck C, Cardasis HL, Cusack MP, Dodder NG, Gibson BW, Held JM, Hiltke T, Jackson A, Johansen EB, Kinsinger CR, Li J, Mesri M, Neubert TA, Niles RK, Pulsipher TC, Ransohoff D, Rodriguez H, Rudnick PA, Smith D, Tabb DL, Tegeler TJ, Variyath AM, Vega-Montoto LJ, Wahlander A, Waldemarson S, Wang M, Whiteaker JR, Zhao L, Anderson NL, Fisher SJ, Liebler DC, Paulovich AG, Regnier FE, Tempst P, Carr SA. *Nat Biotechnol.* 2009; 27:633–641. [PubMed: 19561596]
172. Kuzyk MA, Smith D, Yang J, Cross TJ, Jackson AM, Hardie DB, Anderson NL, Borchers CH. *Mol Cell Proteomics.* 2009; 8:1860–1877. [PubMed: 19411661]
173. Kuhn E, Whiteaker JR, Mani DR, Jackson AM, Zhao L, Pope ME, Smith D, Rivera KD, Anderson NL, Skates SJ, Pearson TW, Paulovich AG, Carr SA. *Mol Cell Proteomics.* 2012; 11 M111 013854.
174. Liu Y, Huttenhain R, Collins B, Aebersold R. *Expert Rev Mol Diagn.* 2013; 13:811–825. [PubMed: 24138574]
175. Macek B, Mann M, Olsen JV. *Annu Rev Pharmacol Toxicol.* 2009; 49:199–221. [PubMed: 18834307]
176. Tian Q, Price ND, Hood L. *J Intern Med.* 2011; 271:111–121. [PubMed: 22142401]
177. Iliuk AB, Tao WA. *Clinica Chimica Acta.* 2013; 420:23–27.

Figure 1. Qualitative comparison map of common phosphoproteomic techniques based on the throughput level (number of analyses carried out during designated time period) and amount of data generated from each analysis.

Figure 2.
A simplified depiction of typical sample preparation steps in a MS-based phosphoproteomic experiment. The figure includes commonly used methods and “shortcuts” that can be employed during a procedure.

Figure 3.
Graphical depiction of the two-step PolyMAC phosphopeptide enrichment (M = metal ion).

Figure 4.
Graphical representation of the promise and challenge for MS-based phosphoproteomic analyses of clinical samples.