Phosphopeptide enrichment methods for mass spectrometry analysis

Introduction

Gaining a better understanding of normal cellular signaling and its modulation in disease is a critical goal of mass spectrometry-based proteomic analyses. In particular, measurement of changes in post-translational modifications (PTMs), such as phosphorylation across samples, is a key metric for activity of signaling pathways and changes in activity post-treatment. Many successful studies have utilized the power of mass spectrometry to provide insights into these changes including projects that have (1) determined novel sites of protein phosphorylation, ubiquitination, acylation, methylation, or protease cleavage, (2) identified and validated drug targets, (3) discovered biomarkers, (4) explored the mechanism of action of drugs/chemical modulators, and (5) elucidated off-target drug effects. In all of these cases, the enrichment of the PTM of interest was the key step to allow these discoveries to be made. Without enrichment, the signal of PTM peptides is lost in the background of all other, unmodified peptides in a cell line, tissue, or biofluid sample. There are various published methods for investigation of the phosphoproteome of cells and tissues, with three main categories emerging, as shown in **Figure 1** and **Table 1**.

Enrichments

Metal Affinity Enrichment

This method involves the use of coordinated metal ions (Immobilized Metal Affinity Chromatography, IMAC) or metal oxides (Metal Oxide Affinity Chromatography, MOAC) on beads to pull down any phosphopeptide based on interaction of the positively charged metal group with the negatively charged phosphate group(s) on peptides (1). **Figure 1A** shows the structure of an Fe-NTA IMAC bead binding a phosphopeptide.

Motif-based Enrichment (PTMScan® Discovery)

This method involves the use of specialized motif or PTM antibodies to immunoaffinity-enrich for specific classes of phosphopeptides based on the modification itself and/or sequence characteristics flanking the phosphosite (2, 3). **Figure 1B** shows the general strategy for standard and motif antibody development.

Pathway-based Enrichment (PTMScan Pathways)

This method involves the use of validated site-specific antibodies multiplexed into one reagent that targets critical signaling nodes from various signaling pathways or protein types such as kinases (4). **Figure 1C** shows pathways targeted and coverage of one pathway (T cell receptor signaling) using the PTMScan® Multi-Pathway Enrichment Reagent.

Each of these methods has its advantages and disadvantages. Metal affinity enrichments such as Fe-IMAC provide the largest datasets from the lowest sample amounts. These enrichments, however, are unfocused with respect to the phosphopeptides identified and quantified, sometimes causing important, lower abundance proteins and sites to be missed.

PTMScan® Discovery experiments are more focused on subpopulations within the phosphoproteome that match particular consensus motifs

Figure 1.

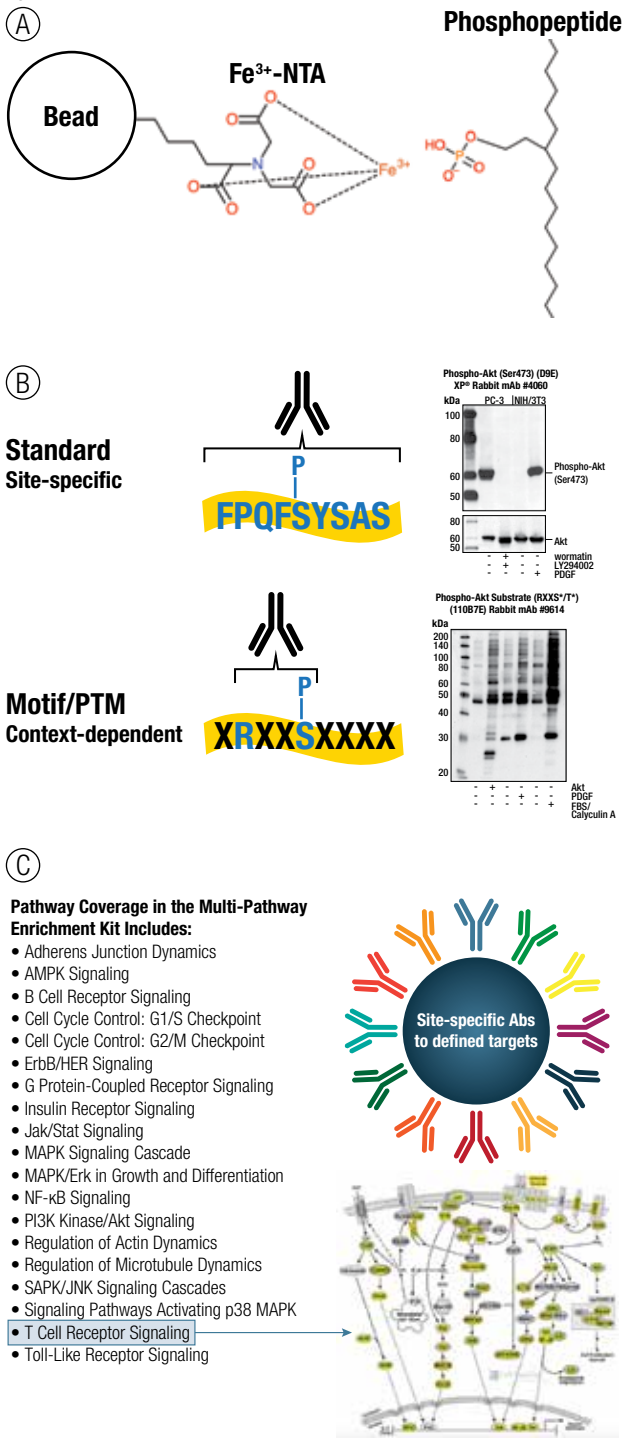

Figure 1: Methods for phosphopeptide enrichment. **1A:** Fe-IMAC enrichment. **1B:** PTMScan® Discovery antibody-based enrichment. **1C:** PTMScan® Multi-Pathway Enrichment.

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Table 1

Product #	PTMScan® Kit Description	Motif
Phospho-Tyr		
#5636	Phospho-Tyrosine Mouse mAb (P-Tyr-100) Kit	y
#8803	Phospho-Tyrosine Rabbit mAb (P-Tyr-1000) Kit	y
Phospho-Ser/Thr		
#5561	Phospho-Akt Substrate Motif mAb 1 Kit	RXX(s/t)
#5563	Phospho-Akt Substrate Motif mAb 2 Kit	RXRXX(s/t)
#5564	Phospho-AMPK Substrate Motif Kit	LXRXX(s/t)
#12267	Phospho-ATM/ATR Substrate Motif Kit	(s/t)Q
#12170	Phospho-CK2 Substrate Motif Kit	(s/t)(D/E)X(D/E)
#14990	Phospho-MAPK Substrate Motif Kit	PXtP
#4652	Phospho-MAPK/CDK Substrate Motif Kit	PXsP, sPX(K/R)
#10821	Phospho-PDK1 Docking Motif Kit	(F/Y)(s/t)(F/Y)
#5565	Phospho-PKA Substrate Motif Kit	(K/R)(K/R)X(s/t)
#71652	Phospho-PKC Substrate Motif Kit	(K/R)(K/R)X(s/t)X(K/R)
#14989	Phospho-Ser-Pro-Pro Motif Kit	sPP
#5566	Phospho-ST*P Motif XP® Rabbit mAb Kit	StP
#5567	Phospho-T*PP Motif XP® Rabbit mAb Kit	tPP
#91336	Phospho-Thr-X-Arg Motif [pTR] Kit	tXR
Motif Antibody Mixes		
#32948	Basophilic Kinase Substrate Motif Kit	(R/X)(R/X)X(s/t)
#25081	Phospho-Ser/Thr Motif Kit	(s/t)
#28303	Phospho-Ser/Thr-Pro Motif Kit	(s/t)P
Other Enrichments		
#20432	Phospho-Enrichment IMAC Fe-NTA Magnetic Beads	(s/t/y)
#75676	Multi-Pathway Enrichment Kit	Signaling Pathways

Table 1: PTMScan kits to enrich phosphopeptides

or phosphorylation on particular residues, such as phosphotyrosine. This allows much deeper coverage and identification of sites on low abundance/low stoichiometry sites than a more general enrichment. These methods, however, typically require more material than metal affinity enrichments, and will only provide data for the motif(s) targeted in the enrichment.

Like PTMScan® Discovery, PTMScan® Pathway enrichments require more material than metal affinity, allow identification of a smaller subset of phosphopeptides, and are fixed with respect to the peptides and proteins targeted. These enrichments are directly focused on the critical regulatory sites of signaling proteins, ensuring that the key players in a given signaling network are identified and quantified. These reagents have also been designed to target both phosphopeptides as well as unmodified peptides on proteins, allowing for simultaneous monitoring of both phosphorylation status as well as changes in protein level.

This application note demonstrates the use of these three methods to profile signaling in the c-Met driven MKN-45 human cell line model of gastric carcinoma (5). MKN-45 cells were treated with DMSO or a specific inhibitor of the c-Met receptor tyrosine kinase (SU11274) and profiled with Fe-IMAC enrichment, Phospho-Tyrosine (pY) and Phospho-Serine/Threonine (pSer/pThr) PTMScan, and the PTMScan® Multi-Pathway Enrichment Reagent.

Method

MKN-45 gastric carcinoma cells were grown to 80% confluence, serum starved overnight, and treated with DMSO or 1µM SU11274 (Sigma, #S9820) for 2 hours prior to harvest. Cells were washed with PBS, scraped in Urea Lysis Buffer, sonicated, centrifuged, and total protein normalized by Bradford assay. 10mg of protein were used for antibody enrichments, 500µg for Fe-IMAC enrichment. Samples were processed according to PTMScan protocols (5), the general outline of which is shown in **Figure 2**. Protein lysates for each condition were reduced, alkylated, digested with trypsin (IMAC, pY, Multi-Pathway) or LysC (pSer/pThr), C18 reversed phase purified, and enriched with the appropriate reagent: PTMScan® Phospho-Enrichment IMAC Fe-NTA Magnetic Beads #20432 (IMAC), PTMScan® Phospho-Tyrosine Rabbit mAb (P-Tyr-1000) Kit #8803 (pY), PTMScan® Phospho-Ser/Thr Motif [pS/T] Kit #25081 (pSer/pThr), or the PTMScan® Multi-Pathway Enrichment Kit #75676 (Multi-Pathway) as shown in **Figure 2**. LysC digested peptides from the pSer/pThr enrichment were further in-solution digested with trypsin.

Enriched peptides were desalted over C18 tips (6) and run in duplicate in liquid chromatography tandem mass spectrometry (LC-MS/MS) on Thermo Scientific QExactive™ or Fusion™ Lumos™ instruments. Peptide sequences were assigned to mass spec data and score filtered using SEQUEST® and the GFY-Core platform from Harvard University (7-8), and quantification of relative peak abundance in the MS1 channel was performed using Skyline from the University of Washington (9).

Figure 2.

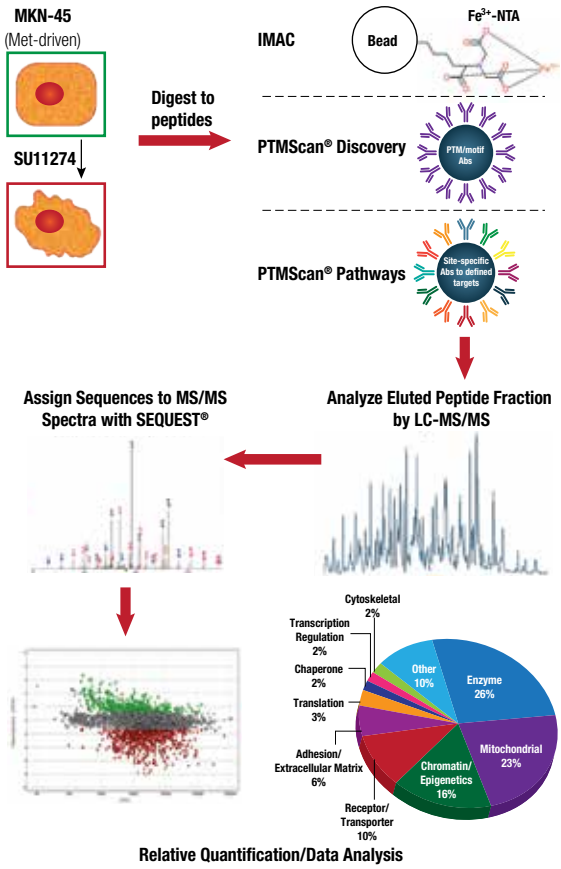

Figure 2: The PTMScan Method and experimental design.

Results

Qualitative Results

To evaluate the qualitative data across the four enrichments (IMAC, pTyr, pSer/pThr, and Multi-Pathway) the number of unique identified enriched peptides and overlap between enrichments were compared (**Figure 3**). The IMAC enrichment identified more enriched peptides than the antibody-based enrichments, despite using a much lower amount of input protein. Among the antibody enrichments, phosphotyrosine had the highest number of peptides identified, followed by the pSer/pThr Motif Antibody mix, then the Multi-Pathway Enrichment. Comparing the enriched peptides identified with the four methods, we saw very little overlap between IMAC and the antibody-based PTMScan enrichments, ranging from 14.7% to 2.5%. This is consistent with previous data (5,10) showing complementarity between these enrichment methods, and in particular the critical necessity of running PTMScan phosphotyrosine enrichment in order to effectively survey the tyrosine phosphoproteome (only ~250 phosphotyrosine peptides were identified in the IMAC enrichment).

Quantitative Results

Accurate quantification of enriched peptides across samples and ability to find those peptides that change with treatment are also critical factors to successful analysis of phosphorylation. **Figure 4** highlights the number of peptides quantified in each enrichment and the number that change in abundance with SU11274 treatment. Although the Fe-IMAC analysis gave the highest number of identifications (largest circle size in **Figure 3**), phosphotyrosine PTMScan quantified thousands more SU11274-induced changes than IMAC. Likewise, pSer/pThr and Multi-Pathway enrichments gave similar numbers of changes to IMAC, even though the overall size of the datasets were smaller. This clearly demonstrates the value of complementary enrichment using both metal affinity and antibody-based methods to comprehensively assess treatment-induced changes in signaling.

Critical Signaling Pathways

Beyond bulk number of enriched peptides identified from proteins in a given pathway, the ability of each enrichment to identify the critical regulatory sites on those proteins is of the utmost importance. **Table 2** highlights coverage for known regulatory sites on representative proteins from the c-Met signaling pathway identified by one of the antibody enrichments and not found in the IMAC dataset. For c-Met, a number of sites were identified only in the phosphotyrosine enrichment, including some containing the regulatory Y1003, Y1235, and Y1349 sites. Phosphotyrosine enrichment also identified regulatory sites on the Gab1 adaptor protein. Multi-Pathway is the only enrichment able to assess protein level changes by targeting unmodified sites on c-Met and uniquely identified triply phosphorylated c-Met peptides including the Y1234 and Y1235 sites. Multi-Pathway enrichment also allowed identification of unmodified Erk1/2 and Akt peptides, as well as the critical regulatory T308 and S473 sites on Akt and other regulatory sites on Gab1 and Raf1. Other important sites on Raf1 were uniquely identified in the Ser/Thr Motif Antibody enrichment. As these results demonstrate, any experimental design incorporating only IMAC or only one of these enrichments would miss certain key signaling nodes and suffer from decreased coverage of the pathway of interest.

Figure 3.

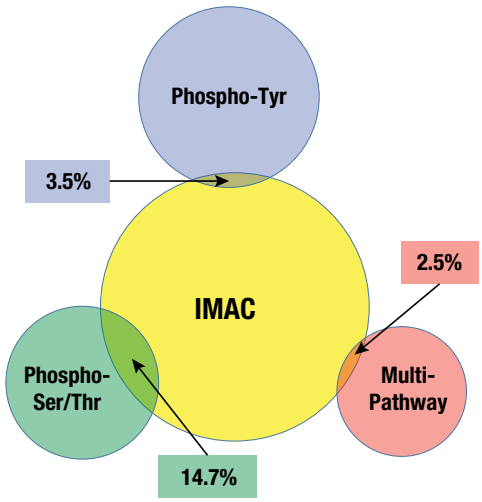

Figure 3: Venn diagram overlap of enriched peptides identified by each method.

Figure 4.

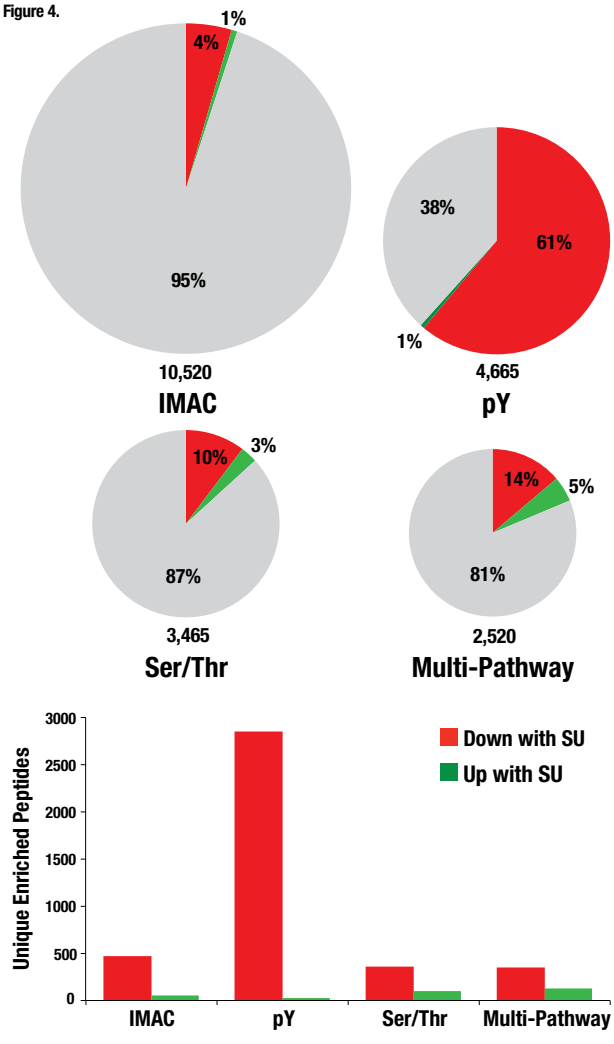

Figure 4: Quantitative overview. Number of unique enriched peptides, percentage of those peptides changing with treatment, and number of changes up/down with treatment for each method.

Table 2

Protein/Site	IMAC	pY	Ser/Thr	Multi-Pathway	SU11274:DMSO
Gab1 Y627		•			-5.3
Gab1 Y659		•			-6.6
Met T992/Y1003		•			-4.7
Met T993/Y1003		•			-49.6
Met S997/Y1003		•			-43.8
Met Y1230/Y1235		•			-102.0
Met Y1349		•			-6.7
Met Y1349/T1355		•			-6.3
Met T993/Y1003		•			-49.6
Raf1 S296			•		1.0
Raf1 S296/S301			•		1.5
Raf1 S301			•		1.0
Total Akt1				•	-1.2
Akt T308				•	-10.4
Akt1 S473				•	-2.0
Total Erk1				•	-1.2
Gab1 Y373				•	-32.5
Gab1 Y472				•	-8.3
Raf1 S339				•	1.7
Raf1Y340				•	1.7
Total Met				•	1.0
Met Y1230/Y1234/Y1235				•	-10.0
Met Y1234/Y1235/S1236				•	-62.4

Table 2: Critical regulatory sites uniquely identified in one of the antibody enrichments and not in the IMAC dataset.

Conclusion

Various methods for mass spectrometry-based phosphoproteome profiling were used to study the effects of RTK inhibitor treatment in a cell line model of human gastric carcinoma. Each of the enrichments performed has its own advantages. The metal affinity Fe-IMAC enrichment provided the largest amount of data with the lowest input sample amount. This IMAC dataset, though massive, does not comprehensively profile the phosphoproteome in MKN-45 cells. In order to capture other key changes in protein phosphorylation in response to drug treatment, antibody-based enrichments were needed. Phosphotyrosine enrichment identified thousands of sites regulated by SU11274 treatment. Ser/Thr antibody enrichment identified certain key sites on c-Met signaling proteins such as Raf1. The Multi-Pathway enrichment, though giving the lowest overall number of identifications, provided many of the most critical observations for the dataset, such as both key regulatory sites on Akt1, T308, and S473. The Multi-Pathway enrichment also measures protein-level changes for proteins in the HGF signaling pathway, such as c-Met, Akt1, and Erk1/2. Taken together, these data demonstrate the importance of complementary enrichment strategies in order to build a complete picture of cellular signaling. These metal affinity and antibody-based enrichment reagents will all continue to be critical for the deconvolution of cellular signaling in normal cell growth and development, as well as in the study of disease biology.

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