

Phosphopeptide enrichment methods for mass spectrometry analysis

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	RXXRX(s/t)
#5564	Phospho-AMPK Substrate Motif Kit	LXXRX(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	PXiP
#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	SiP
#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 1 μ M 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, 500 μ g 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.

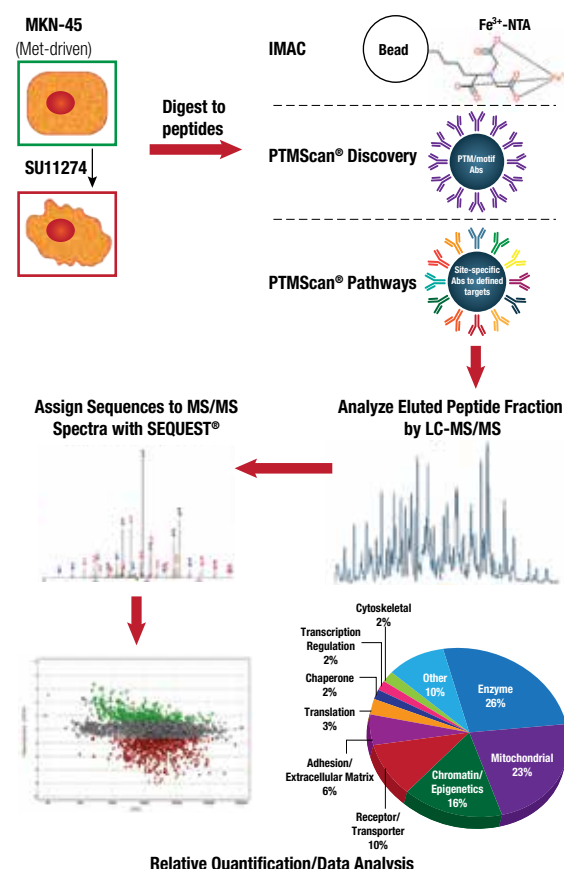


Figure 2: The PTMScan Method and experimental design.

Learn more at:

cst-science.com/enrichment-app-note



Cell Signaling
TECHNOLOGY®

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.

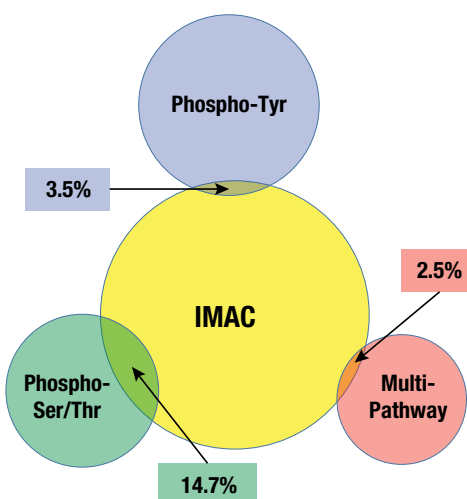


Figure 3: Venn diagram overlap of enriched peptides identified by each method.

Figure 4.

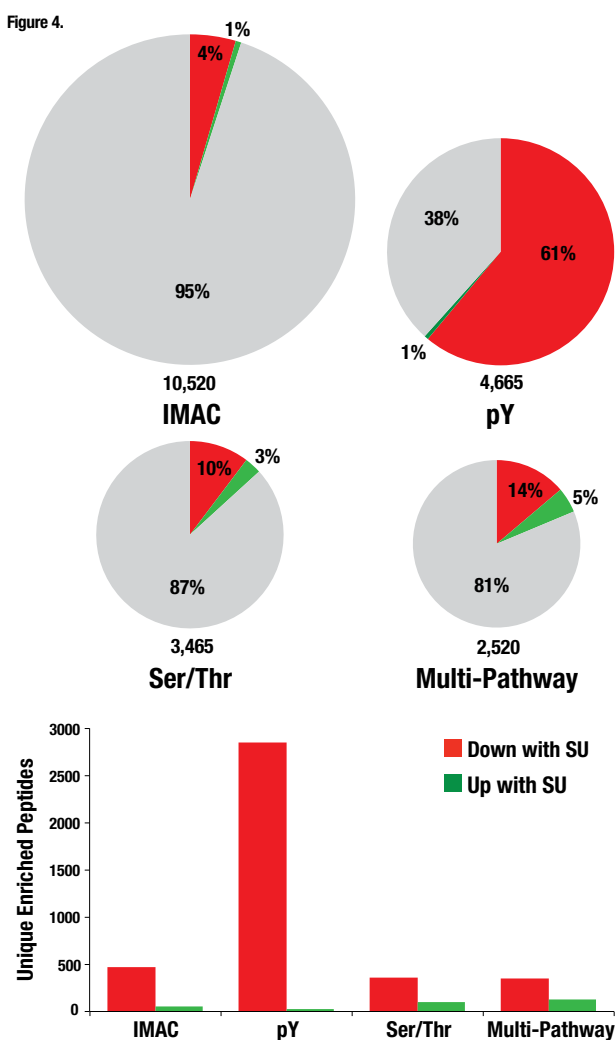


Figure 4: Quantitative overview. Number of unique enriched peptides, percentage of those peptides changing with treatment, and number of changes up/down with treatment for each method.

Phosphopeptide enrichment methods for mass spectrometry analysis

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
Akt1 T308				•	-10.4
Akt1 S473				•	-2.0
Total Erk1				•	-1.2
Gab1 Y373				•	-32.5
Gab1 Y472				•	-8.3
Raf1 S339				•	1.7
Raf1 Y340				•	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.

References

1. Beltran L, Cutillas PR. (2012) *Amino Acids*. 43(3), 1009-24.
2. Zhang H, et al. (2002) *J Biol Chem*. 277(42), 39379-87.
3. Rush J, et al. (2005) *Nat Biotechnol*. 23, 94-101.
4. Stokes MP, et al. (2012) *Mol Cell Proteomics*. 11, 187-201.
5. Stokes MP, et al. (2015) *Proteomes*. 3(3), 160-83.
6. Rappsilber J, et al. (2003) *Anal Chem*. 75(3), 663-70.
7. Eng JK, et al. (1994) *J Am Soc Mass Spectrom*. 5, 976-89.
8. Villen J, et al. (2007) *Proc Natl Acad Sci*. 104(5), 1488-93.
9. MacLean B, et al. (2010) *Bioinformatics*. 26(7), 966-8.
10. Possemato AP, et al. (2017) *J Proteome Res*. 16(4), 1506-14.

Contributors:

Matthew P. Stokes, Charles L. Farnsworth, Jian Min Ren, Kimberly A. Lee

At CST, providing exceptional customer service and technical support are top priorities. Our scientists work at the bench daily to produce and validate our antibodies, so they have hands-on experience and in-depth knowledge of each antibody's performance. In the process, these same scientists generate valuable reference information that they use to answer your questions and help troubleshoot your experiment by phone or email.

For questions about how to customize your protocol using our full catalog of nearly 1000 antibodies approved for IHC, please contact technical support: Visit www.cellsignal.com/support or call 1-877-678-8324.

Ordering Information

www.cellsignal.com/orderinfo

For a complete list of CST offices and distributors, please visit www.cellsignal.com/contactus