Multiplexed cellular signaling pathway profiling through targeted peptide immunoenrichment and quantitative analysis by LCMS

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INTRODUCTION

It is clear that robust methods are needed to monitor the activity of cellular signaling pathways in all diseases and in all steps of drug discovery and development. Current methods such as IMAC are often utilized for broad phosphorylation enrichment, but sample complexity results in stochastic peptide identification. Bead-based arrays and ELISA assays are available only to a select few commonly studied targets, and with limited multiplexing.

METHODS

We have developed the PTMScan® Multi-Pathway Enrichment Kit to bridge this gap between highly targeted assays and broad, complex samples, providing pathway-based peptide enrichment upstream of LCMS analysis. This reagent contains an array of site-specific antibodies representing key signaling nodes from multiple pathways, including cell cycle and checkpoint control, AKT/PI3K signaling, T-cell and B-cell receptor signaling, MAPK and JNK cascades, and many others.
To profile pathway activity, proteins are proteolytically digested, and enrichment is performed at the peptide level. Enriched peptides and phosphopeptides are analyzed through data-dependent LC-MS/MS for total pathway profiling or through targeted MS analysis in multiplexed assay format.

RESULTS

We have used the PTMScan® Multi-Pathway Enrichment Kit to quantify peptides from mouse tissues and multiple human cell lines. Using data-dependent LC-MS/MS, thousands of peptides are identified representing hundreds of sites of phosphorylation, enabling measurement of activity of dozens of signaling pathways. We compare use of this reagent with IMAC phosphopeptide enrichment to assess breadth and depth of coverage of key signaling nodes.
This enrichment reagent has also been utilized for peptide purification upstream of targeted LCMS analysis, quantifying dozens of peptides with attomole sensitivity.

CONCLUSIONS

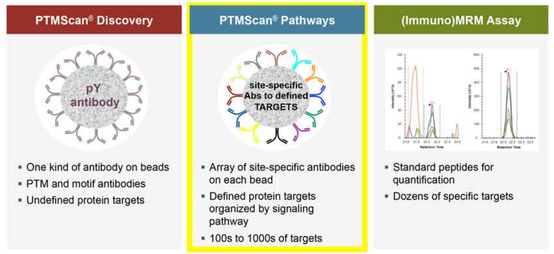
- PTMScan® Multi-Pathway Enrichment Kit enables identification of thousands of target peptides in defined signaling pathways
- Target-specific enrichment significantly reduces required instrument time
- Pathway-based enrichment enables hypothesis-driven, targeted studies with data acquisition and quantitative flexibility

REFERENCES

- Stokes MP, Farnsworth CL, Moritz A, Silva JC, et al. PTMScan direct: identification and quantification of peptides from critical signaling proteins by immunoaffinity enrichment coupled with LC-MS/MS. *Mol Cell Proteomics*. 2012;11(5):187-201.

PTMScan® Multi-Pathway Enrichment Kit (#75676)

This reagent provides a bridge between broad discovery studies and highly targeted assays.



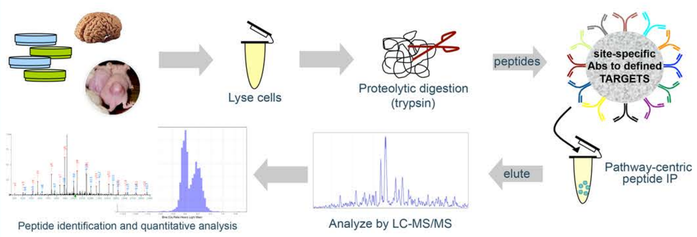
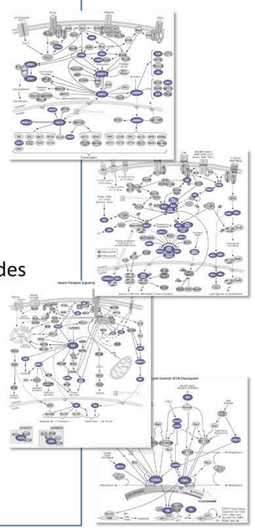
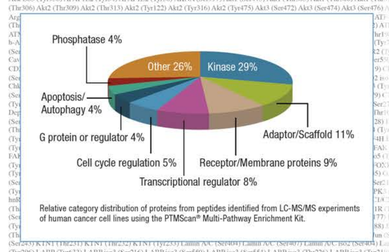


Figure 1 Pathway-based enrichment
Top panel: PTMScan® Pathways bridges discovery and targeted assays.
Bottom panel: Workflow diagram. Mouse or human tissue or cell line samples are lysed and digested with trypsin. Peptides are immunoenriched, eluted, and identified and quantified by LCMS/MS.

Pathway coverage in the Multi-Pathway Enrichment Kit:

- Adherens junction dynamics
- AMPK signaling
- B-Cell receptor signaling
- Cell cycle control: G1/S checkpoint
- Cell cycle control: G2/M DNA damage checkpoint
- ErbB/HER signaling
- G protein-coupled receptor signaling
- Insulin receptor signaling
- Jak/Stat signaling: IL-6 receptor family
- Mitogen Activated Protein Kinase signaling cascades
- MAPK/Erk in growth and differentiation
- NF-κB signaling
- PI3 Kinase/Akt signaling
- Regulation of actin dynamics
- Regulation of microtubule dynamics
- SAPK/JNK signaling cascades
- Signaling pathways activating p38 MAPK
- T-cell receptor signaling
- Toll-like receptor signaling





- Validated for >4000 proteoforms of >800 proteins
- Targeted phosphorylation sites are key regulatory nodes for more than 20 signaling pathways
- Designed for study of human and mouse cells and tissues
- Full list of validated sites and peptides available online

Figure 2 Multi-Pathway Enrichment Kit details
Top panel: Key nodes of more than 20 signaling pathways are enriched with this kit.
Bottom panel: The 800+ proteins profiled represent diverse protein classes relevant to cell signaling.

Application to T-cell signaling

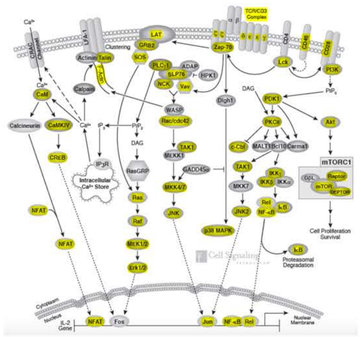


Figure 3 T-cell signaling pathway
Proteins represented in Multi-Pathway Enrichment Kit are highlighted in yellow.

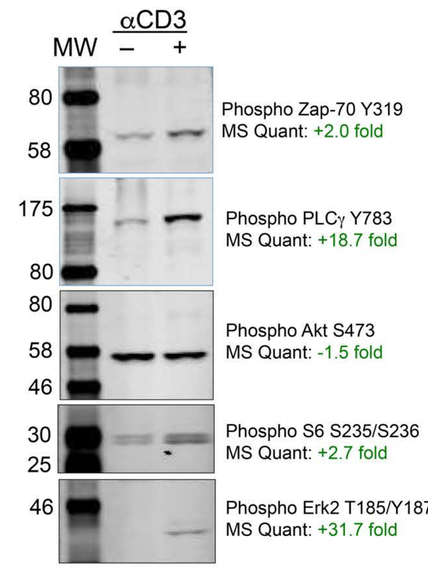
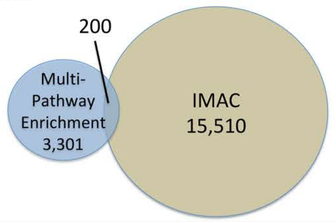


Figure 4 T-cell signaling quantitation
Jurkat T-cells were treated with and without anti-CD3 to stimulate the T-cell signaling pathway. Signaling changes were measured either using the Multi-Pathway Enrichment Kit (green numbers on right) or by Western blot. Kit provides data similar to thousands of Western blots in a single experiment.



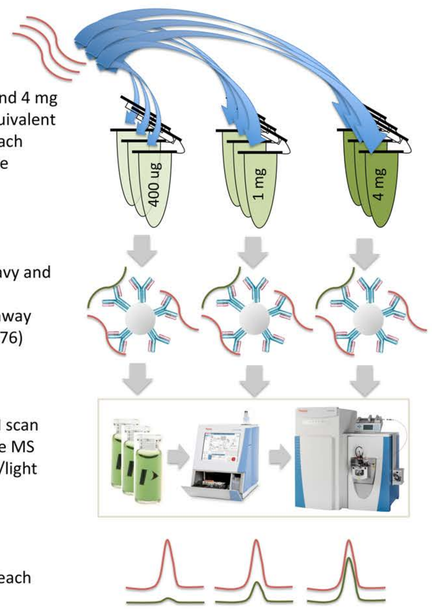
Protein-Site Enrichment Comparison

Protein Site	PTMScan® Multi-Pathway	vs	IMAC
Zap70 Total	✔		
pZap70 Y315	✔		
pZap70 Y292	✔		
pZap70 S301	✔		✔
pZAP70 Y315/Y319	✔		
pZAP70 Y319	✔		
pZAP70 Y492/Y493	✔		
pZap70 S493	✔		
ERK Total	✔		
pERK1 T202/Y204	✔		✔
pERK1 Y204	✔		✔
pERK2 T185/Y187	✔		✔
pERK2 Y187	✔		✔

Figure 5 Comparison with IMAC
Top panel: IMAC enrichment results in identification of more phosphorylation sites, but overlap is low.
Bottom panel: Relevant signaling proteins may not be sufficiently profiled without targeted enrichment.

Multiplexed LCMS immunoassay

The targeted enrichment of the multipathway reagent was utilized in tandem with a targeted LCMS assay to monitor specific phosphopeptides from low-level xenograft tumor samples.

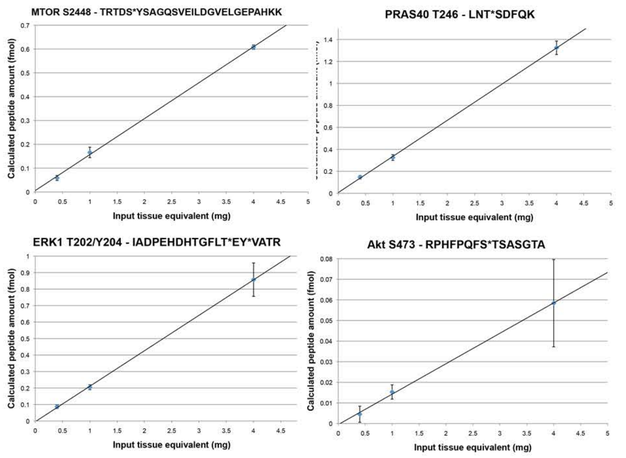


H3255 xenograft
- Used 400 ug, 1 mg, and 4 mg wet weight tissue equivalent
- Spiked in 500 amol each heavy-labeled peptide standard
- Triplicate analyses

Antibody capture of heavy and light peptides
- PTMScan® Multi-Pathway Enrichment Kit (#75676)

Targeted LCMS analysis
- Time-segmented SIM scan method on Q Exactive MS
- Monitoring 52 heavy/light pairs

Measured L/H ratio to determine response to each peptide



52-plex immunoassay provides successful quantification of key signaling protein phosphorylation from biopsy-level amounts of xenograft tumor tissue

Figure 6 Application of LCMS immunoassay to H3255 xenograft tumor tissue
Top panel: Experimental details and flowchart.
Bottom panel: Representative data are shown, with observed target peptide amount plotted against input xenograft wet weight tissue amount. Linear curve fits for each peptide are presented. Error bars represent standard deviation of triplicate immunoassays. A value of 0.1 fmol observed target peptide at the 400 ug input tissue level would be equivalent to 300 copies per cell if xenograft weight is assumed to consist entirely of H3255 cells.

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