PTMScan® Direct: Quantitative Profiling of Critical Nodes From Cellular Signaling Pathways

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Introduction

Proteomic analysis of post-translational modifications (PTMs) often employs data-dependent analysis, providing rich datasets that consist of randomly sampled identified peptides due to the dynamic response of the mass spectrometer. This can complicate the primary goal of programs for drug development, mutational analysis, and kinase profiling studies, which is to monitor how multiple nodes of known, critical signaling pathways are affected by a variety of treatment conditions. Cell Signaling Technology (CST) has developed an immunaffinity-based LC-MS/MS method, called PTMScan® Direct, for multiplexed analysis of these important signaling proteins. PTMScan Direct enables the identification and quantification of hundreds of peptides from a particular signaling pathway, across multiple pathways, or from a particular protein type (such as serine/threonine kinases). Cell lines, tissues, or xenografts can be used as starting material. PTMScan Direct is amenable to quantification using labeling methods such as SILAC or label-free quantification studies.

PTMScan® Direct Reagents

Multi-Pathway: detects key nodes of multiple signaling pathways

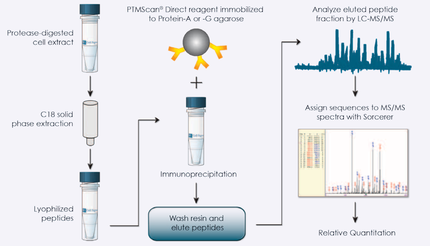
Ser/Thr Kinases: detects critical modification sites on 130 serine/threonine protein kinases

Under Development: Akt/PI3K Signaling, Tyrosine Kinases, Cell Cycle/DNA Damage, and Apoptosis/Autophagy.

Methods

PTMScan® Direct Method Flow Diagram

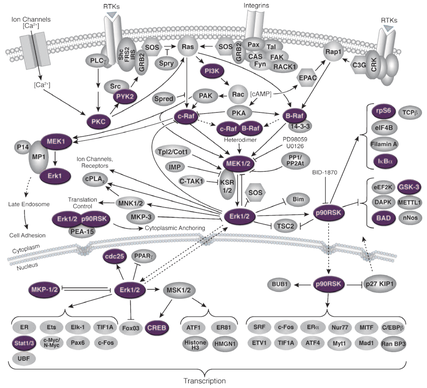
Adapted from the original PhosphoScan® method developed at CST (Rush, J. et al, 2005, U.S. Patent No.s 7,198,896 and 7,300,753).



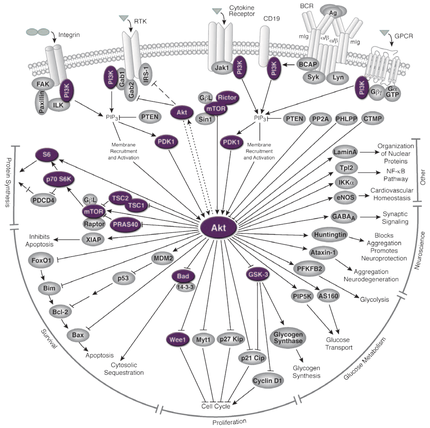
Immunoaffinity Enrichment: Cell lines, tissues, xenografts, or other biological starting materials are lysed under denaturing conditions, digested with trypsin, and the resulting peptides are desalted over a C18 column and dried under vacuum. Peptides are resuspended in Immunoaffinity Purification (IAP) buffer and target peptides are immunoprecipitated using one PTMScan Direct reagent or sequentially with multiple PTMScan Direct reagents. Immunoprecipitated peptides are separated on a reversed-phase Magic C18 AQ column and data-dependent MS methods are performed with an LTQ-Orbitrap Velos mass spectrometer. MS/MS spectra are evaluated using SEQUEST 3G and the SORCERER 2 v4.0 platform from Sage-N Research (Lundgren et al., 2009). Quantification is performed using chromatographic peak apex intensities.

PTMScan® Direct: Multi-Pathway

HIGHLIGHTED PURPLE NODES designate targeted protein modification sites within each sample signaling pathway that can be monitored with PTMScan® Direct: Multi-Pathway Reagent.



Monitor phosphorylation in MAP Kinase Signaling: ATF-2 (Thr52, Thr69, Thr71) • Bad (Ser75) • B-Raf (Ser446) • c-Raf (Ser338) • cdc25C (Ser168, Ser216) • c-Jun (Ser73) • GSK-3α/β (Ser21) • GSK-3β (S9) • HeBir (Ser32, Ser36, Tyr42) • JNK3 (Thr221, Thr223) • JunD (Ser90, Ser100) • MEK1 (Ser217, Ser221) • MEK2 (Ser222, Ser226) • MKP-2 (Ser386) • p42 MAPK (Erk2) (Thr184, Tyr186) • p44 MAPK (Erk1) (Thr202, Tyr204) • p90RSK (Ser363, Thr359) • PI3K p85α (Tyr467) • PI3K p85β (Tyr464) • PKCA (Thr496) • Pyk2 (Tyr579, Tyr580) • RSK2 (Ser227) • RSK4 (Ser389) • S6 (Ser235, Ser236, Ser240, Ser244) • SAPK/JNK1 (Thr183, Tyr185) • Stat3 (Ser705, Tyr705, Ser727)

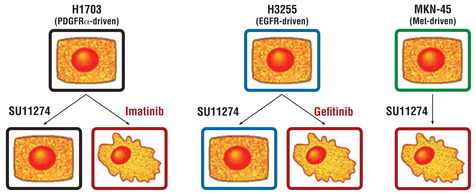


Monitor phosphorylation in PI3 Kinase/Akt Signaling: 4EBP-2 (Thr37, Thr46) • Akt1 (Thr308, Thr312, Ser473, Thr479) • Akt2 (Thr309, Ser474, Ser478) • Bad (Ser75) • GSK-3αβ (Ser21) • GSK-3β (Ser9) • MDM2 (Ser166) • mTOR (Ser2448) • p70 S6 Kinase (Thr252, Thr412) • p70 S6 Kinase β (Thr229) • PDK1 (Ser241) • PI3K p55 (Tyr199) • PI3K p85α (Tyr467) • PI3K p85β (Tyr464) • PRAS40 (Thr246) • Rictor (Ser1370, Ser1373, Thr1376) • S6 (Ser235, Ser236, Ser240, Ser244) • Wee1 (Ser642)

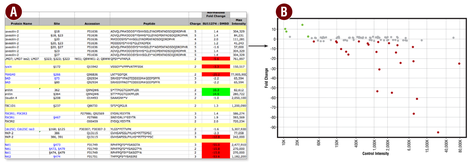
Additional Pathway Targets Include: Apoptosis; Autophagy; Calcium, cAMP, and Lipid Signaling; Cell Cycle; Chromatin Regulation; Cytoskeletal Signaling/Adhesion; Metabolism; NF-κB Signaling; TGF-β Signaling; Translational Control; Wnt/β-Catenin Signaling.

PTMScan® Direct: Multi-Pathway RTK Inhibitor Study

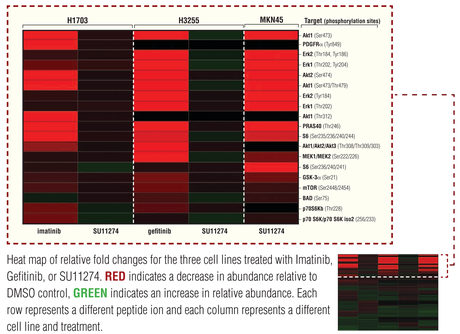
The PTMScan Direct: Multi-Pathway Reagent was used to assess receptor tyrosine kinase (RTK) inhibitor sensitivity in 3 cell lines whose growth is dependent on RTK activity: H1703 cells (PDGFRα$_{cc}$-dependent), H3255 cells (EGFR-dependent), and MKN-45 cells (Met-dependent). The effects of PDGFR inhibitor (Imatinib), EGFR inhibitor (Gefitinib) and Met inhibitor (SU11274) treatment were assessed using label-free quantification.



Experimental design for RTK inhibitor sensitivity study using PTMScan Direct. All treatments were 3 hrs with 1 μM of indicated compound.



PTMScan Direct results for MKN-45 cells treated with DMSO or SU11274 **(A)**. Tables were generated containing qualitative and quantitative information. Peptide ions that showed a fold-change >2.5 in response to treatment are colored in **RED** (decrease) and **GREEN** (increase) **(B)**. Scatter plot of fold change versus DMSO sample intensity for MKN-45 cells –/+ SU11274. Each point represents a unique peptide ion. Peptide ions that changed in abundance with SU11274 treatment are highlighted in **RED** and **GREEN**.

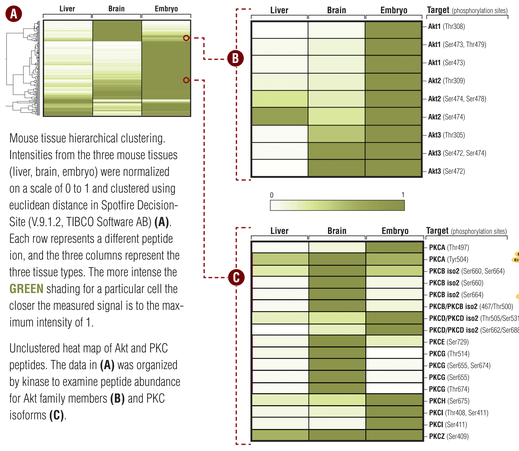


Heat map of relative fold changes for the three cell lines treated with Imatinib, Gefitinib, or SU11274. **RED** indicates a decrease in abundance relative to DMSO control, **GREEN** indicates an increase in relative abundance. Each row represents a different peptide ion and each column represents a different cell line and treatment.

PTMScan® Direct: Ser/Thr Kinases

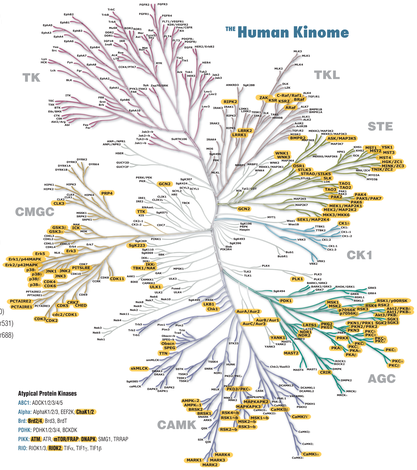
PTMScan® Direct: Ser/Thr Kinase Mouse Tissue Study

The PTMScan Direct: Ser/Thr Kinases Reagent was used to quantify differences in kinase activity between three mouse tissues: liver, brain, and embryo. Label-free quantification was performed and intensities were normalized for each peptide ion across the three samples based on a maximum intensity of 1. Normalized intensities were entered into Spotfire DecisionSite and displayed using hierarchical clustering.



Mouse tissue hierarchical clustering. Intensities from the three mouse tissues (liver, brain, embryo) were normalized on a scale of 0 to 1 and clustered using euclidean distance in Spotfire DecisionSite (V.9.1.2, TIBCO Software AB) **(A)**. Each row represents a different peptide ion, and the three columns represent the three tissue types. The more intense the **GREEN** shading for a particular cell the closer the measured signal is to the maximum intensity of 1.

Unclustered heat map of Akt and PKC peptides. The data in **(A)** was organized by kinase to examine peptide abundance for Akt family members **(B)** and PKC isoforms **(C)**.

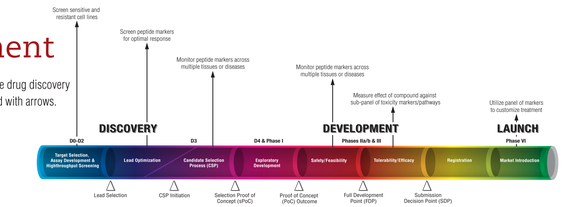


PTMScan® Direct in Drug Discovery and Development

The potential use of PTMScan Direct in drug discovery and development. Stages in the drug discovery and development process at which PTMScan Direct analysis can be used are indicated with arrows.

Applications Include:

- Identification of Biomarkers
- Mechanism of Action studies
- Validation of Targets
- Screening New Drugs
- Predict Therapeutic Outcomes
- Monitor Drug Therapy



Summary

PTMScan Direct is a novel method that allows multiplexed monitoring of critical signaling nodes from a single pathway or multiple pathways combined. PTMScan Direct is widely applicable in drug development and discovery, as well as in any application where monitoring of known signaling pathways is desired.

References

Rush, J. et. al. (2005) *Nat. Biotechnol.* 23, 94–101. • Lundgren, et. al. (2009) *Curr. Protoc. Bioinformatics.* 13: Unit 13-3. • Farnsworth, C.L. et. al. (2010) *Amer. Society for Mass Spect. Annual Confer.* Poster WP228. • Silva, J.C. et. al. (2010) *Amer. Society for Mass Spect. Annual Confer.* Poster TP244.

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