# Comprehensive Quantitative Profiling of the Mouse Kinome using Data-independent LC-MS-MS/MS$^{ALL}$ with SWATH™ Acquisition

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## Introduction

Proteomic analysis of post-translational modifications (PTMs) can often be facilitated by the use of affinity techniques to enrich for peptides having the appropriate PTM. Cell Signaling Technology (CST) has developed an immunoaffinity-based LC-MS/MS method, called **PTMScan® Direct**, for multiplexed analysis of 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. Coupling PTMScan Direct and a data-independent LC-MS acquisition methodology (MS/MS$^{ALL}$ with SWATH™ Acquisition), we were able to profile levels of serine, threonine, and tyrosine kinase substrate phosphopeptide abundances in mouse liver, brain, and embryo. An MS/MS spectral library was generated from a ProteinPilot™ software search of data-dependent acquisitions on each sample type. The ion library was then used to produce targeted extracted ion chromatograms (XICs) from the high resolution MS/MS data collected during SWATH™ Acquisition.

## PTMScan® Direct Method

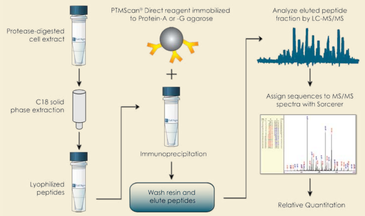



**Figure 1:** ***Flow Diagram for the PTMScan® Direct Method.*** The PTMScan Direct method is adapted from the original PhosphoScan® method developed at Cell Signaling Technology (Rush et al, 2005, Patent Allowed, U.S. Patent Publication 20030044848).

## Methods: Immunoaffinity Enrichment & LC-MS

Tissue samples are lysed under denaturing conditions (9 M Urea, 20 mM HEPES pH 8.0). The resulting soluble protein is digested with trypsin, and the resulting peptides are desalted over a C18 column and dried under vacuum. Peptides are resuspended in CST Immunoaffinity Purification (IAP) buffer (50 mM MOPS pH 7.2, 10 mM $KH_2PO_4$, 50 mM NaCl) and target peptides are immunoprecipitated using both PTMScan Direct Tyrosine and Serine/Threonine Kinases Reagents. Immunoprecipitated peptides were analyzed using the Eksigent nanoLC-Ultra™ 2D System with the cHiPLC®-nanoflex system using trap and column chips packed with ChromXP C18-CL, 3 µm, 300 Å reverse phase media. Eluant was analyzed using the TripleTOF® 5600 system (AB SCIEX) using MS/MS$^{ALL}$ with SWATH™ Acquisition mode. Q1 was scanned from 400-1000 m/z using 25 Da steps and full scan MS/MS was acquired for 100 msec accumulation time (total cycle time of 2.5 sec). All data were processed using the SWATH Acquisition MicroApp PeakView® Software and MarkerView™ Software.

## PTMScan® Direct: Serine/Threonine Kinase Targets

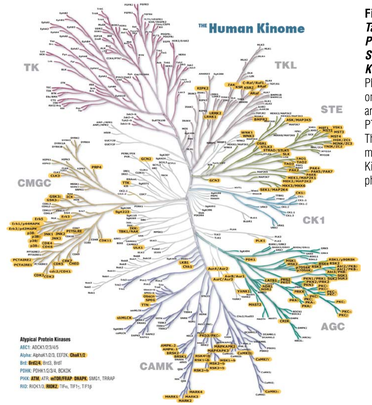



**Figure 2:** ***Proteins Targeted by the PTMScan Direct Serine/Threonine Kinases Reagent.*** Phosphorylation sites on highlighted proteins are detected by the PTMScan Direct Serine/Threonine Kinase Reagent, monitoring 130 Ser/Thr Kinases and 385 unique phosphorylation sites.

## PTMScan® Direct: Tyrosine Kinase Targets

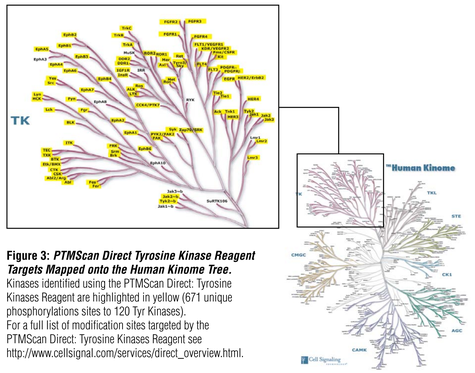



**Figure 3:** ***PTMScan Direct Tyrosine Kinase Reagent Targets Mapped onto the Human Kinome Tree.*** Kinases identified using the PTMScan Direct: Tyrosine Kinases Reagent are highlighted in yellow (671 unique phosphorylations sites to 120 Tyr Kinases).
For a full list of modification sites targeted by the PTMScan Direct: Tyrosine Kinases Reagent see http://www.cellsignal.com/services/direct_overview.html.

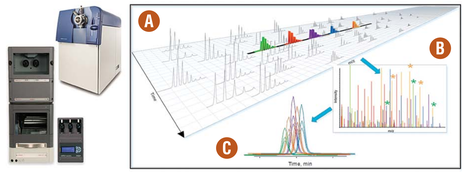



**Figure 4:** ***Targeted Quantification using MS/MS$^{ALL}$ with SWATH™ Acquisition.*** In the MS/MS$^{ALL}$ with SWATH Acquisition, instead of the Q1 quadrupole transmitting a narrow mass range through to the collision cell, a wider window containing more analytes is passed (**A**). This produces a more complex MS/MS spectrum which is a composite of all the analytes within that Q1 m/z window (**B**). Because the fragment ions are high resolution, high quality XICs can be generated post-acquisition to produce the MRM-like data (**C**). This Q1 window can be stepped across the mass range, collecting full scan composite MS/MS spectra at each step, with an LC compatible cycle time, enabling a ***data-independent LC-MS workflow.***

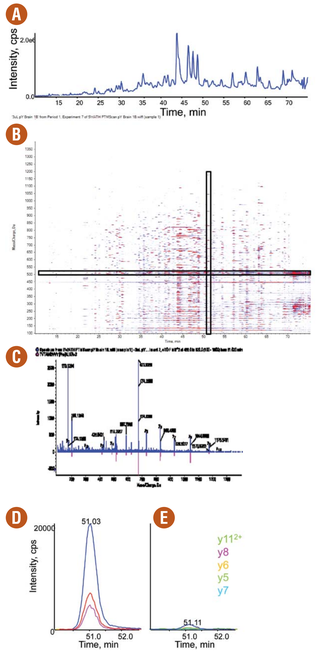
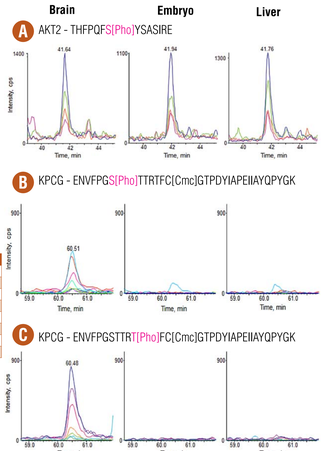



**Figure 5A & 5B:** ***Single SWATH™ Acquisition Window.*** (**A**) The TIC of a single SWATH acquisition window illustrates the number of peptide precursors that are transmitted through the Q1 window, which is set to transmit 500 to 525 m/z in this case. (**B**) This same window is shown as a LCMS contour plot and all of the MS/MS fragments across the LC run can now be visualized. The horizontal orange box highlights all the unfragmented precursors that have transferred through Q1. The vertical strips of data are the MS/MS fragment ions of the peptides with precursor masses between 500 to 525 m/z that have eluted at that point in the LC run. A peptide is highlighted at 51 mins and the spectrum shown in Figure 5C.

**Figure 5C, 5D, & 5E:** ***Quantitative Fragment Ion XICs from the Full Scan MS/MS.*** (**C**) The full scan MS/MS of Histone H4 peptide TVTAMDVVY[Pho]ALKR 3+ from 51 mins is shown in blue. The corresponding MS/MS fragment pattern as seen in a parallel IDA experiment is shown in pink. (**D** & **E**) The most intense fragment ions based on the pink spectrum are extracted at a width of 0.05 Da to generate MRM-like data for qualitative detection and quantification. The same fragments for this peptide from brain and embryo are compared, this peptide is more abundant in brain.

## PTMScan® Direct: STY Kinases & SWATH™ Acquisition

| Ion | m/z |
|---|---|
| b3 | 386.18228 |
| b5 | 611.29362 |
| y6 | 662.34678 |
| y4 | 504.27764 |




PTM Specific Fragment Ions

| pThr | m/z | pSer | m/z2 |
|---|---|---|---|
| b7 | 731.3359 | b8 | 912.3499 |
| b8 | 832.3836 | b10 | 1169.499 |
| b9 | 933.4312 | b9 -98 | 915.4207 |
| $y22^{2+}$ | 1321.106 | b10 -98 | 1071.522 |
| $y21^{2+}$ | 1243.055 | $y23^{3+}$ | 1331.647 |
| $y22^{2+}$-98 | 1272.117 | $y22^{2+}$ | 1281.123 |
| $y21^{2+}$-99 | 1194.067 | $y21^{2+}$ | 1203.072 |
| | | $y23^{3+}$ -98 | 1282.658 |

**Figure 6:** ***Single SWATH™ Acquisition Window.*** (**A**) Extracted fragment ion chromatograms (XICs) for a peptide from AKT2 that does not change significantly among all three tissues is shown. (**B** & **C**). Extracted fragment ion XICs for the unique fragment ions that distinguish the two different peptides from protein kinase C gamma with different localized phosphorylation sites. The peptides have the same precursor mass and therefore XICs are generated from the same SWATH acquisition window. There is approximately 2x more of the pThr form and both pThr and pSer are observed in brain but not detected in embryo and liver

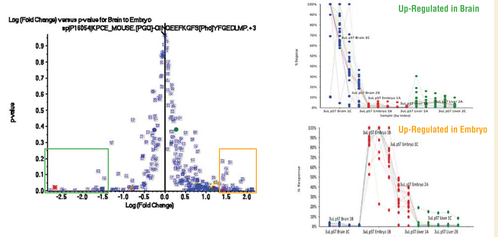






**Figure 7:** ***T-test Comparison of Mouse Tissues.*** A T-test was performed between the different sample group pairs. Shown here is the volcano plot from the comparison between brian vs embryo. A number of phosphopeptides are observed that show statistically significant change in occupancy. The line plots show the relative % areas for each of these changing phosphopeptides across all the samples. Good analytical reproducibility is obtained on each sample and good agreement between the sample preparation replicates is observed.

## PTMScan® Direct: STY Kinases & SWATH™ Acquisition

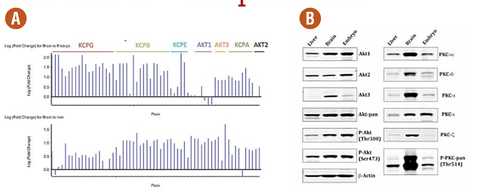



**Figure 8:** ***Single SWATH™ Acquisition Window.*** (**A**) The fold change for all the phosphopeptides analyzed for two protein families were plotted to illustrate the degree of change for each phosphorylation site. The sites are organized by protein and show that all KCP phosphorylation sites and all AKT proteins, except AKT1, are up-regulated in brain as compared to embryo (top). A similar plot was generated for brain vs liver (bottom), which again shows most phospho sites to be higher in brain. (**B**) Western blots for total and phosphorylated forms of AKT and PKC isoforms is consistent with the SWATH™ Acquisition data.

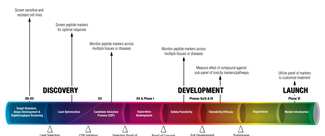



**Figure 9:** ***The Potential Use of PTMScan® Direct in Drug Discovery and Development.*** Stages in the drug discovery and development process at which PTMScan Direct/LCMS analysis can be used are indicated with arrows.

## Summary

- PTMScan Direct is a novel method that allows multiplexed monitoring of critical signaling nodes in the human kinome.
- PTMScan Direct is widely applicable in drug development and discovery, as well as in any application where monitoring of known signaling pathways is desired.
- MS/MS$^{ALL}$ with SWATH™ Acquisition provides a targeted quantitation strategy to survey biological samples for large numbers of specific peptides and proteins, with the specificity and accuracy approaching that of MRM analysis but with much higher multiplexing.

## References

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4. iIlet LC et al (2012) *Mol. Cell. Prot.* E-pub.

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