Targeting critical signaling nodes using multiplexed antibody based phosphopeptide enrichment with iMRM validation

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INTRODUCTION

A challenge for biomedical researchers is to develop assays to analyze complex systems that interrogate whole cellular signaling networks. Here we employ antibody based enrichment combined with liquid chromatography tandem mass spectrometry to identify and quantify post-translationally modified peptides (PTMScan® Discovery, 1). Site specific antibodies can be multiplexed on beads to profile critical signaling nodes across multiple cellular pathways (PTMScan® Direct, 2). These same reagents, or custom mixtures of a subset of the antibodies used, can be combined with synthetic heavy isotope labeled peptides to perform targeted analysis of proteins/sites of interest (iMRM, 3). This targeted method allows rapid, quantitative profiling of the proteins/sites across hundreds or thousands of samples with lower input protein amounts such as from primary cell populations, sorted cells, or biopsies.

METHODS

Human cells or mouse tissues were lysed, digested with trypsin, and desalted over C18 columns. Post-translationally modified peptides were enriched using the indicated antibodies. Peptide eluates were purified using STAGE tips (4). Immunoprecipitated peptides were run in LC-MS/MS on a QExactive mass spectrometer using a top 20 data-dependent analysis method or PRM targeted method. MS/MS spectra were assigned to peptide sequences using SEQUEST (5). Data was filtered using the Linear Discriminant module of Core (Harvard University). Label-Free quantification for discovery experiments was performed using Progenesis (Nonlinear Dynamics) and manual review of ion chromatogram files. Quantification for targeted analyses was performed using Skyline (6).

CONCLUSIONS

Multiplexed pathway enrichment is a powerful tool for the study of hundreds to thousands of endpoints in a single assay covering critical cellular signaling nodes. These multiplexed enrichment tools can be used in discovery mode assays to broadly profile signaling. Subsets of the proteins/sites covered by the reagent can be analyzed using targeted assays with immuno-multiple reaction monitoring (iMRM) methods. The assays can be configured using the multiplexed reagent itself, or with custom panels of site specific/protein specific antibodies. These targeted assays can be run reproducibly, rapidly, and on biopsy level sample amounts, allowing screening of thousands of samples for the proteins/sites included.

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PTMScan®: Target Discovery by PTM

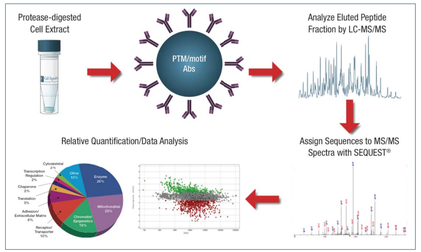


Figure 1. PTMScan® Discovery allows identification and quantification of hundreds to thousands of novel sites of post-translational modification (PTM) in a single LC-MS/MS analysis. This method can be used to profile protein phosphorylation, ubiquitination, acetylation, methylation, and many other critical PTMs.

PTMScan® Application: Tyrosine Kinase Profiling in Lung Cancer Patient Samples

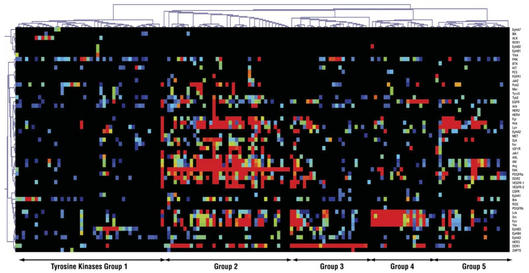
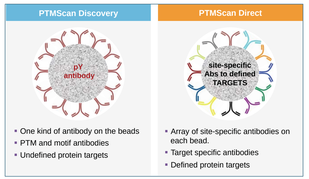


Figure 2. Application of PTMScan® to identify phosphorylated tyrosine kinases in non-small cell lung cancer patients. Tumors are clustered by tyrosine kinase phosphorylation, with color representing increased signal intensity. Clustering produced five groups of tumors with different sets of tyrosine kinases predominating (Rikova et al., Cell 2007).

PTMScan® Direct: Target Discovery by Pathway



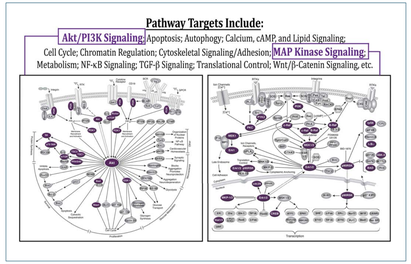


Figure 3. PTMScan® Direct reagents combine antibodies to target multiple signaling pathways in a single experiment. These same antibodies were used to immunoprecipitate the targeted phosphopeptides for the iMRM assays.

RTK Inhibitor Study in Human Cancer Cell Lines

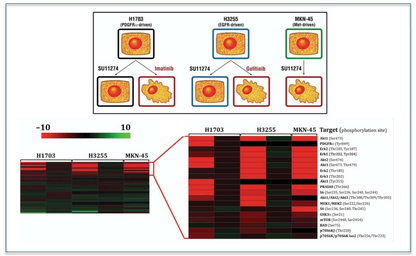


Figure 4. PTMScan® Direct analysis of human cancer cell lines treated with RTK inhibitors. Top panel: experimental design. Bottom panels: heat map of fold-change values derived from immunoprecipitated phosphopeptides using the PTMScan® Direct Multipathway reagent. Green = increase relative to DMSO control, red = decrease relative to DMSO control.

iMRM: Targeted Quantitative Analysis

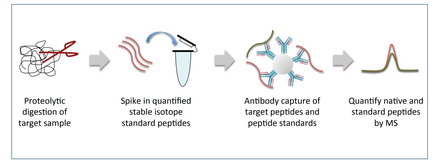
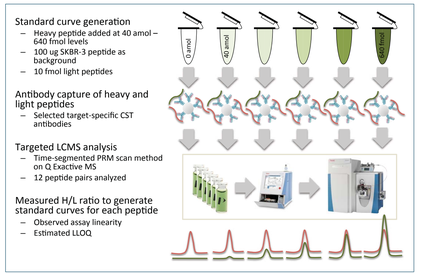


Figure 5. iMRM workflow. Site specific antibodies are used to immunoprecipitate multiple phosphopeptides simultaneously. Heavy versions of the endogenous tryptic phosphopeptides are synthesized and spiked into peptides from cells, tissues, serum, or other biological materials. Endogenous and synthetic peptides are enriched with antibodies, analyzed by LCMS, and quantified across samples.

iMRM Assay Linearity and Sensitivity



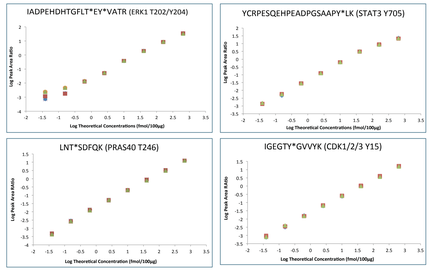


Figure 6. iMRM results for representative phosphopeptides. The heavy/light peptide signal ratio is plotted for triplicate peptide enrichments. Many peptides have assay linearity across three orders of magnitude with LLOQ less than 1 fmol/100 ug protein. Assay CVs are less than 20% at LLOQ and are typically 5% across the linear range.

iMRM Application to Cell Line Profiling

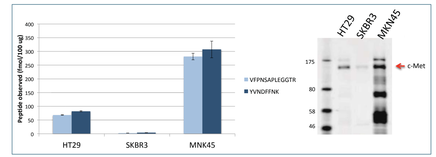
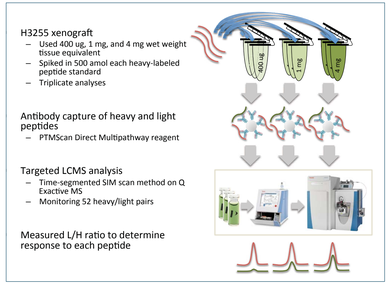


Figure 7, iMRM assay data (left panel) using antibodies raised to two different peptides in c-Met matches Western blot data (right panel). iMRM assays are advantageous as they can be highly multiplexed, provide quantitative data, and eliminate concerns relating to antibody specificity.

Application to Xenograft Tumor Profiling



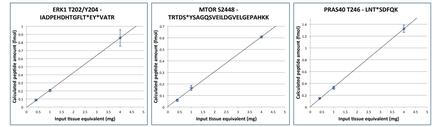


Figure 8. Application of immuno-MS to H3255 xenograft tumor tissue. Top panel: Experimental details and flowchart. Bottom panels: Representative data are shown, with observed target peptide amount plotted against input xenograft wet weight tissue amount. Linear curve fits for each peptide are presented. Error bars represent standard deviation of triplicate immunoassays. A value of 0.1 fmol observed target peptide at the 400 µg 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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