

# Immunoaffinity Enrichment and Quantitative Profiling of Protein Phosphorylation, Methylation, and Acetylation in Receptor Tyrosine Kinase Pathway-addicted Cancer Cell Lines

## Abstract

Somatic mutation and altered protein expression patterns cause inappropriate activation and deactivation of key players in cellular signaling, resulting in the oncogenic phenotype. Phosphorylation patterns of oncogenes and other signaling proteins have been studied extensively in the last several years, resulting in significant advances in our understanding of cancer and our ability to identify and therapeutically target tumor drivers.

Here we take the next step in this process, expanding the enriched, identified, and quantified post-translational modification (PTM) space to include not only phosphopeptides but also acetylated, methylated, and ubiquitinated peptides, resulting in a multifaceted analysis of cellular protein modification to better understand the complex signaling events of cancer biology.

Variation in cellular signaling measured by ten different candidate motif antibodies was evaluated by performing western blot analysis of cell lysates from 22 different lung cancer cell lines. This screening tool allowed us to select those antibodies with the most diverse staining patterns across the cell lines for global proteomic PTM profiling. Phosphotyrosine, acetyl-lysine, methyl-arginine, ATM/ATR substrate (s/tQ), and AGC/CAMK/STE family kinase motif, which specifically enriches close to 100 active kinase phosphopeptides, were all selected for use in peptide immunoprecipitation, allowing us to characterize and quantify a broad PTM space.

Initial method development and PTM profiling were performed using a set of six lung cancer cell lines (A549, H1650, H1703, H3122, H3255, and MKN45) that have different signaling pathways driving their growth and transformation. Phosphotyrosine profiling of these cell lines with tandem mass tag (TMT) quantification revealed clear quantitative differences in phosphorylation, successfully identifying their activated signaling pathways.

The established method of TMT labeling coupled with serial peptide immunoprecipitation was applied to profile drug-treated and untreated RTK pathway-addicted cancer cell lines, with quantitiative analysis of the effects of SU11274 on the Met-addicted MKN45 cell line, Crizotinib on the Alk-addicted H3122 cell line, Gleevec on the PDGFR $\alpha$ -addicted H1703 cell line, and Iressa on the EGFR-addicted H3255 cell line. These samples were combined in 6-plex groups for TMT analysis with the A549 cell line serving as a non-responsive control. Serial fractionation and LCMS/MS analysis resulted in identification and quantification of hundreds of sites of phosphorylation, arginine methylation, and lysine acetylation. Those sites found to be drug-sensitive, including all PTM types, were used for pathway analysis of signaling downstream of each receptor tyrosine kinase (RTK) disease driver.

#### Objectives

:: Establish method for quantitative analysis of multiple classes of post-translational modifications from a single sample

:: Apply method to investigate signaling downstream of several different oncogenic receptor tyrosine kinases



#### Figure 1. Western blots used to select motif antibodies for phosphopeptide immunoprecipitation.

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### Evaluating LCMS methods

METHOD OF QUANTIFICATION



**Figure 2. Qualitative comparison of TMT labeling quantified by MS2 or MS3 with label-free quantification**. Six cell lines (A549, H1650, H1703, H3122, H3255, and MKN45) either were processed individually and analyzed separately using 45 minute LCMS gradients and CID fragmentation (label-free), or were TMT-labeled and combined for immunoprecipitation. The TMT-labeled samples were analyzed using 150 minute LCMS gradients, either using HCD MS2 for both peptide identification and quantification in a single step (TMT-HCD only), or using CID MS2 for peptide identification with HCD MS3 for TMT quantification (TMT-MS3). The number of modification sites, unique peptides, and total redundant peptides identified using each method is displayed for acetyl-lysine and methyl-arginine immunoprecipitation samples. In all cases, the TMT method with HCD peptide identification and quantification resulted in discovery of the highest number of PTM sites, and this method was selected for further studies.

#### COMBINING IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC) WITH PHOSPHOPEPTIDE IMMUNOPRECIPITATION



## Figure 3. IMAC sample clean-up step reduces background of non-phosphorylated peptides and allows identification of more phosphopeptides. Identical tyrosine phosphopeptide immunoprecipitations were performed and analyzed with or without subsequent IMAC to further enrich phosphopeptides over background unphosphorylated peptides. IMAC was found to significantly reduce levels of contaminating unphosphorylated peptides and thus increase the number of phosphopeptides identified.

Validating methods



**Figure 4. Receptor tyrosine kinase disease drivers identified in appropriate cell lines using TMT HCD only method.** The expected patterns of expressed and phosphorylated RTKs is observed, with PDGFRα in H1703, ALK in H3122, EGFR overexpressed in H3255, and Met in MKN45. EGFR is expressed in all of these immortal cell lines at some level, and phosphorylation of some sites (Y1110 for example) is detected in all cases. GSK3B is also constitutively expressed and phosphorylated. MKN45 has an extremely high level of tyrosine phosphorylation, so some interference is detected in the TMT channel associated with that cell line.

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**Figure 5. Experimental flowchart.** Cell lysates were prepared from RTK inhibitor treated and untreated cell lines, proteins were digested with trypsin, peptides were TMT-labeled, and samples were mixed for 6-plex analysis. The peptide mixtures were serially fractionated through immunoprecipitation with antibodies specific for phosphotyrosine, ATM/ATR substrate motif, AGC/CAMK/STE family kinase motif, acetyl-lysine, and methyl-arginine, though any motif-recognizing antibody could be used with this protocol. The enriched peptides from each immunoprecipitation were analyzed by LCMS/MS using an LTQ<sup>®</sup> Orbitrap<sup>®</sup> Elite, with quantification of the TMT labels enabled by HCD fragmentation.

	Sample Mix 1	Sample Mix 2	Sample Mix 3	Total Sites (nonredundant)
phosphotyrosine	236	1111	572	1369
K-Ac	1928	1988	2492	3679
R-Me	757	986	900	1329
ATM/ATR substrate (pSQ)	264	384	135	574
ACG kinase motif	137	173	160	302

 Table 1. Number of modification sites identified and quantified for each PTM type analyzed.
 The 6-plex sample components are as follows:

 Sample Mix 1: A549 untreated, A549+Crizotinib, A549+Gleevec, A549+Iressa, A549+SU11274.

Sample Mix 2: A549 untreated, A549+Crizotinib, H3122 untreated, H3122+Crizotinib, MKN45 untreated, MKN45+SU11274.

Sample Mix 3: A549 untreated, A549+Iressa, H1703 untreated, H1703+Gleevec, H3255 untreated, H3255+Iressa.



**Figure 6. Percent of sites responsive for each PTM motif studied.** At least a two-fold change in reporter ion signal/noise was required to be classified as responding. As expected, phosphotyrosine sites are the most responsive to RTK inhibition, but many other phosphorylation, acetylation, and methylation responses were also observed.



**Figure 8. Pathway diagrams for each cell line were generated using Cytoscape**<sup>®</sup>. These diagrams include all proteins with modification sites increasing or decreasing at least 2x upon relevant drug treatment. Proteins with PTM site peptides decreasing upon treatment (dark green protein names) are distinguished from those with sites increasing upon drug treatment (red protein names). Proteins identified in different PTM classes (phosphotyrosine, ATM/ ATR substrate phosphorylation, AGC/CAMK/STE kinase family motif phosphorylation, lysine acetylation, and arginine methylation) are distinguished by different colored protein symbols. Shapes of protein symbols signify protein functionality (cytoskeletal, adaptor, kinase, adhesion protein, etc.). First order interacting partners with the driving RTK are presented in bright yellow.

#### Summary

- : Intelligent fractionation through sequential immunoprecipitation can be used to probe numerous PTM spaces, allowing in-depth pathway mapping of valuable samples
- : Quantification and sample multiplexing with TMT labeling increases annotation rate over label-free quantification with lower sample analysis time
- Deep data sets generated through enrichment of different types of modified peptides allow elucidation of novel biological relationships

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