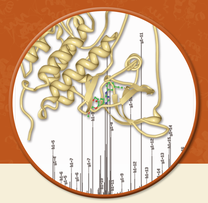

Motif Antibody Enrichment Enables the Identification of a Large Discrete and Complementary Set of Phosphorylation Sites

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Abstract

In this report we describe how commonly used phosphopeptide enrichment strategies like metal affinity chromatography followed by fractionation and phosphospecific motif antibodies are surprisingly complementary in the phosphorylation sites they identify. Using equal amounts of HCT116 cell lysate, we were able to identify over 9,500 unique phosphorylation sites using TiO_2 followed by basic reverse-phase fractionation. Separately, using four technical replicates and three different phosphospecific motif antibodies in series, we identifed over 1,900 unique phosphorylation sites. However, despite identifying over 10,000 sites with the two methods, the total overlap in unique phosphorylation sites was less than 4%. Surprisingly, only 267 of phopho-Ser/Thr containing sites overlapped between these approaches, suggesting combining these two strategies might be necessary for maximum coverage, an observation not previously described.

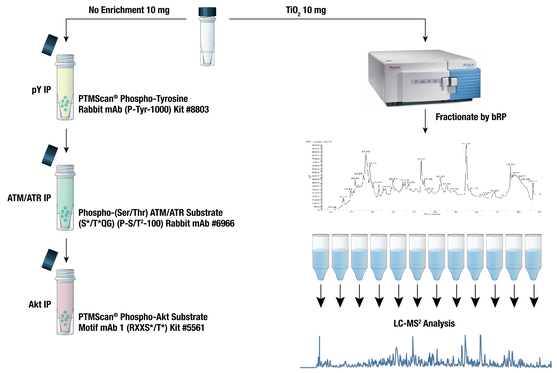
Methods

HCT116 cells were harvested in a 9M urea lysis buffer. The lysate was reduced alkylated, and digested with trypsin. After digestion, samples were purified over SepPak® C18 columns and divided into 10 mg aliquots. Using the same amount of starting material (10 mg) samples were processed using two distinct phosphopeptide enrichment strategies in parallel: bulk enrichment by TiO_2 or phosphospecific motif antibody immunoprecipitation (IP) using antibodies against phosphotyrosine, AKT, and ATM/ATR substrate motifs. Sample runs were searched using Sequest® and filtered to a 1% FDR at the protein level. The number of unique identified sites from each method was evaluated using AScore. Overlap between the two datasets was determined only for confidently localized sites (Ascore ≥ 13).

Preliminary Data

Following the bulk enrichment method described by Gerber et al (2011), HCT116 peptides were subjected to bulk TiO_2 enrichment before being fractionated using a Zorbax C18 column. A total of 24 fractions analyzed by LC-MS/MS using a 90 minute gradient resulted in the identification of 52,664 total phosphopeptides, 20,360 of which were unique, mapping to a total of 9,597 unique sites with an Ascore ≥ 13. Sequential IPs on four technical replicates were performed using three different phosphopeptide motif antibodies (Rush et al. 2005) resulting in the identification of 11,790 total phosphopeptides, 4,453 of which were unique, mapping to 1,975 unique sites with an Ascore ≥ 13. Both of these approaches performed well at protein level, identifying ~1000 protiens by IP and over 3400 proteins using the bulk enrichment method. The overlaps at the protein level were quite high, having an overlap of nearly 70% when comparing IP identified proteins to those identified by the TiO_2 method. However, when comparing the over 10,000 combined unique phosphorylation sites seen using both approaches, the overlap was less than 4%. This surprising finding showed the necessity in using a combination of these strategies when trying to obtain a comprehensive phosphorylation dataset.

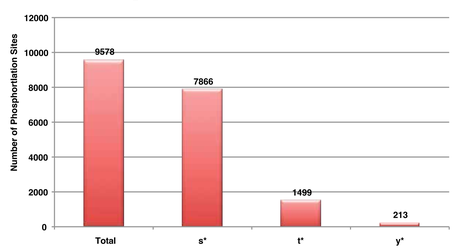
Experimental Workflow



Methods	Total Peptides	Total Phospho-peptides	Unique Phospho-peptides	AScore Passing Sites (>13)
Bulk TiO_2	117362	52664	20360	9578
pY IP	22455	5366	2226	939
Akt IP	16728	2552	1120	398
ATM/ATR IP	16040	3872	1286	502

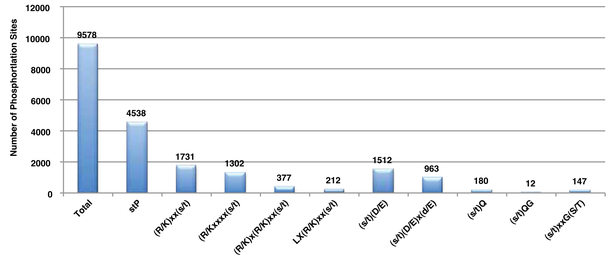
The 24 TiO_2 enriched fractions were run using a 90 minute gradient. Each of the immunoprecipitate samples were run using a 90 minute gradient. All peptides used for this analysis had an FDR of less then 2.0%, and an Ascore value of no less then 13.

TiO_2 Phosphorylation Breakdown



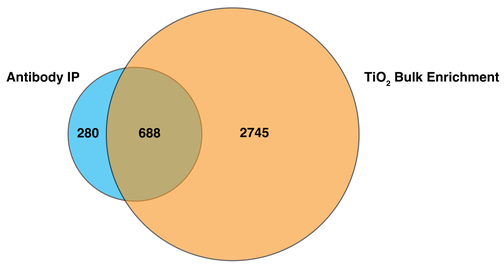
A breakdown of the passing phosphorylation sites seen in the TiO_2 sample set showed a dominance in the serine space followed by threonine and a small population of phospho-tyrosine. This was expected based on the normal population of these sites within the cell.

TiO_2 S/T Motif Breakdown



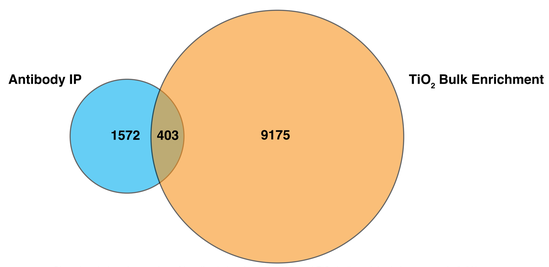
Common S/T motifs observed by TiO_2 enrichment showed the dominance in the proline directed peptides. Followed by an almost equal abundance of basophilic and acidic type peptides.

Protein Level Overlap



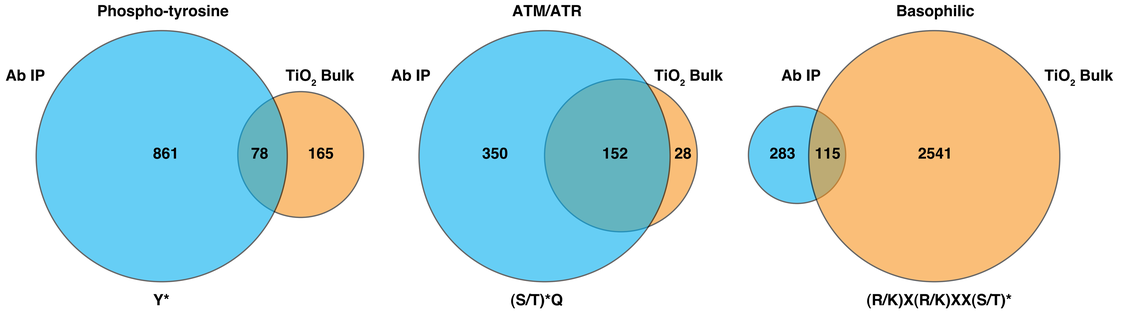
Overlap at the protein level was ~70% when looking at proteins identified with the TiO_2 bulk enrichment method vs antibody IP.

All Sites Level Overlap



Phosphorylation site overlap of localized sites between IAP and TiO_2 bulk enriched sites was only 3.6%.

All Sites Level Overlap



Phosphorylation-specific analysis of identified sites reveal discrete and complementary identification by IP vs bulk enrichment. The majority of pY sites were identified by IP and were >90% discrete, ATM/ATR sites identified by IP were complementary, and the relatively small number of basophilic sites identified by IP were >70% discrete.

Conclusion

Standardized methods of phospho-peptide enrichment such as TiO_2 have been thought to be relatively comprehensive. In this study we show that in comparison with an approach which identified ~10,000 unique phosphorylation sites, phospho-specific motif antibody immunoprecipitation allows for the identification of a complementary set of phosphorylation sites.

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