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## A monoclonal antibody cocktail as an enrichment tool for acetylome analysis

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### Abstract

The availability and robustness of methods to analyze phosphorylated proteins has greatly expanded our knowledge of phosphorylation-based cell signaling. A key ingredient to the success of these studies is the ability to enrich phosphopeptides using antibodies or other chemical approaches. Most other post-translational modifications, such as lysine acetylation, are still poorly characterized because of the lack of availability of such enrichment methods. Recently, some groups have reported identification of acetylation sites in a global fashion by enriching acetylated peptides with a polyclonal antibody from a single source that was raised against pan-acetylated lysine. Instead of using this polyclonal antibody, we used a cocktail of monoclonal antibodies where each was directed against acetylated lysine in different contexts. Using high resolution Fourier transform mass spectrometry, we observed that the majority of acetylated lysine residues identified using the monoclonal antibody cocktail were distinct from those enriched by the polyclonal antibody used by the other groups. Our study demonstrates that immunoaffinity enrichment of acetylated peptides is somewhat limited by substrate specificity and that an optimal yield of enrichment can be achieved by employing a broader array of affinity reagents.

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Post-translational modifications (PTMs) of proteins are integral to the regulation of cellular processes but have been tedious to characterize and quantify in a global fashion until recently. The greatest advances have been made in the field of phosphorylation for which tens of thousands of unique sites have now been described by enriching phosphopeptides based on their chemical characteristics and through immunoaffinity approaches for the low abundance phosphotyrosine peptides<sup>(1)</sup>. Enrichment techniques for other PTMs, such as acetylation, have also been pursued with some success. These approaches have used isoelectric focusing<sup>(2)</sup> or immunoaffinity<sup>(3, 4, 5, 6, 7, 8)</sup> based methods to identify acetylated peptides or proteins, and one study identified 3,600 acetylation sites in human cancers using both methods in tandem<sup>(9)</sup>. The analysis described here examines the potential to further

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broaden the number of acetylation sites identified in an experiment by using a diverse set of antibodies for enrichment of acetylated peptides.

The enzymatic regulation of lysine acetylation of histones has been known for some time<sup>(10)</sup>. Mounting evidence suggests that lysine acetylation of non-histone proteins plays a major role in protein stability, localization and cellular signaling<sup>(11, 12, 13)</sup>. Acetylation results in dynamic changes in protein function by neutralizing the positive charge of lysine residues and through the interplay of competing lysine modifications such as methylation, sumoylation and ubiquitination<sup>(13)</sup>. Early studies suggesting that non-histone proteins are regulated by lysine acetylation examined the regulation of p53 and other proteins of critical importance to diseases such as cancer<sup>(11, 11, 14, 15)</sup>. Similar to the regulation of histone lysine acetylation, non-histone proteins are acetylated by histone acetyltransferases (HAT) and deacetylated by histone deacetylase (HDAC) enzymes, the latter of which are now promising therapeutic targets in the treatment of cancer. The histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) or vorinostat is an FDA-approved drug for the treatment of cutaneous T-cell lymphoma<sup>(16, 17)</sup> and is currently under investigation for use in breast cancer. Although the rationale for inhibition of HDACs as a cancer therapy was aimed at the targeted re-expression of tumor suppressor genes commonly silenced in cancer progression, the diverse array of non-histone proteins regulated by lysine acetylation underpins the potential pleiotropic effects of HDAC inhibitors as cancer therapies. Identification of these molecules can lead to the development of more targeted therapies and elucidate mechanisms of drug efficacy and resistance.

Recent studies using mass spectrometry to analyze acetylated proteins and peptides enriched using an anti-acetyl lysine polyclonal antibody from a single commercial source have demonstrated the high prevalence of non-histone protein acetylation in human cancers, including breast cancer<sup>(4, 6, 9)</sup>. It should be noted that the immunoprecipitation step in these studies was performed using the rabbit polyclonal pan-acetylated lysine antibody from a single source, which is potentially limited by selectively enriching peptides with a narrow sequence diversity because they bear similarity to the antigen used to generate the antibody. In addition, the supply of the antibody is not unlimited and more difficult to standardize in terms of specificity. To circumvent these limitations, we used a monoclonal antibody cocktail in which the monoclonal antibodies were raised against a variety of antigens containing acetylated lysine (Table 1). Previous studies have demonstrated that pan-acetylated lysine antibodies derived from multiple sources have different affinities for given acetylated peptide sequences<sup>(18)</sup>. Therefore, a cocktail of monoclonal antibodies has the potential to enrich a significant subset of acetylated peptides which might otherwise go unidentified using only the conventional polyclonal antibody.

## EXPERIMENTAL SECTION

### Materials

Acetonitrile was obtained from Fisher Scientific. All other chemicals were obtained from Sigma unless stated otherwise. Antibodies were obtained from the suppliers listed in Table 1.

### Cell Culture and Sample Preparation

MDA-MB-231 cells were obtained from the American Tissue Culture Collection (ATCC) and grown in Dulbecco's Modified Eagle Medium with 5% FBS at 37°C and 5% CO<sub>2</sub>. Cells were plated in 15cm culture dishes, grown to 80% confluency and treated with 10 μM suberoylanilide hydroxamic acid (SAHA, Cayman Chemical) for 24 hours prior to harvesting by scraping in lysis buffer (9.0 M Urea, 20 mM Hepes pH 8.0, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate and 0.5 μM

trichostatin A), yielding roughly 3 to 5 mg of protein lysate per culture dish. Cells were lysed by sonication and centrifuged at  $20,000 \times g$  for 15 minutes at  $15^{\circ}\text{C}$ , followed by reduction in 5 mM DTT and alkylation of cysteines by incubation in 10 mM iodoacetamide in the dark. The protein lysate was then digested using 1mg of trypsin (Worthington) per 50mg of protein after diluting 5-fold with 20 mM Hepes pH 8.0. The peptide mixture was acidified using trifluoroacetic acid (TFA) at final concentration of 1% and purified by  $\text{C}_{18}$  Sep-Pak filtration (Waters) followed by elution with 40% acetonitrile, 0.1% TFA and lyophilization at  $-60^{\circ}\text{C}$ .

### Immunoprecipitation

Dried peptides were resuspended in immunoprecipitation buffer (50 mM MOPS pH 7.2, 10 mM sodium phosphate and 50 mM NaCl) and incubated with either polyclonal antibody (ImmuneChem) or a monoclonal cocktail (four monoclonal antibodies combined in equal ratio) coupled to protein G agarose beads according to the manufacturer's instructions (Roche). Immunoaffinity enrichment was carried out by incubating 100  $\mu\text{g}$  of total antibody with 10 mg of peptide with constant mixing at  $4^{\circ}\text{C}$  overnight. Peptides were washed 3 times with immunoprecipitation buffer and then eluted with 0.1% TFA and purified using the Stage-tip method as previously described<sup>(19)</sup>.

### Mass Spectrometry

Peptides were analyzed by direct online injection into an LTQ-Orbitrap XL ETD mass spectrometer (Thermo) from an Agilent 1100 autosampler coupled to an Eksigent nanopump, or an LTQ-Orbitrap Velos mass spectrometer (Thermo) with a NanoAcquity UPLC (Waters). Chromatographic separation was performed over a gradient from 9–55% acetonitrile containing 0.1% formic acid over 120 minutes on a  $50 \mu\text{m} \times 15 \text{cm}$  analytical column packed in-house (Magic 5.0  $\mu\text{m}$ , 300 $\text{\AA}$  pore size, Michrom). Using optimized resolution parameters<sup>(20)</sup>, survey scans were acquired in the Orbitrap from a range of  $m/z$  350–1800 at 60,000 resolution with polydimethylcyclsiloxane from ambient air as a lock mass ( $m/z$  445.120025)<sup>(21)</sup>. In each cycle, the 8 most intense ions were fragmented by collisionally induced dissociation (CID) prior to ion trap detection using the LTQ-Orbitrap XL and the 10 most intense ions were fragmented by high energy collision dissociation (HCD) prior to Orbitrap detection at 7500 resolution using the LTQ-Orbitrap Velos. Ions were isolated at a target of 10,000 ions or 150 ms maximum injection time before being excluded for the following 30 seconds.

### Data Processing

Spectra were processed using Proteome Discoverer Version 1.2 (Thermo) to merge search results from Mascot (v.2.2, Matrix Science), with and without deconvolution, and Sequest (Thermo), and subjected to a strict cutoff of 1% false discovery rate (FDR). Spectra were searched against the RefSeq45 human protein database using a 20 ppm mass cutoff for MS and either a 0.5 Da (CID) or 0.05 Da cutoff for MS/MS spectra. Carbamidomethyl cysteine was searched as a fixed modification while methionine oxidation, pyroglutamine, protein N-terminal acetylation and lysine acetylation were used as variable modifications. Full details of search results and analysis are available as part of the Supporting Information (Table S1–S4). Sequence logos for acetylation sites were generated using enoLOGOS<sup>(22)</sup>. The parameters were used such that weight type was 'alignment counts' and LOGO plot method was based on frequency.

### Statistical Analysis

The proportion of acetylation sites common between two replicate runs using the same affinity reagent (either the monoclonal antibody cocktail or the polyclonal antibody) or

between runs using two different affinity reagents (i.e. monoclonal versus polyclonal) were calculated as the number of common sites divided by the average number of sites identified in the two runs. To assess whether the two different enrichment approaches contributed to a difference in the acetylation sites identified, a z-test for two independent proportions was performed. We tested the null hypothesis that the proportion of common sites identified between two runs of different enrichment approaches was no different than what would be observed between two replicate runs of the same enrichment approach. The results of analysis is summarized in Table S5.

## RESULTS AND DISCUSSION

Three independent peptide immunoprecipitations were performed using the monoclonal cocktail or the polyclonal antibody, for which one sample was analyzed by ion trap detection of CID fragments and the other two were analyzed by Orbitrap detection of MS/MS ions from HCD fragmentation. In total, the three biological replicates led to identification of 181 and 244 acetylation sites from enrichment with the monoclonal antibody cocktail and the polyclonal antibody, respectively (Figure 1). Both immunoprecipitation procedures yielded acetylated lysines on histones and a variety of non-histone proteins, suggesting that both reagents possess binding affinity for a broad spectrum of acetylated peptide sequences. Sample analysis with the high resolution MS/MS detection of HCD fragments dramatically outperformed the ion trap CID method, yielding considerably more acetylation sites identified in each HCD experiment compared to the corresponding CID experiment (Figure 1A). The enhancement of acetylation sites identified in the HCD analysis relative to CID analysis is due to multiple factors, including differences in ion accumulation and scan speed between the two instruments used. In particular, high resolution detection of HCD fragments is critical to the identification of large, highly charged acetylated peptides as seen in Figure 2. In addition to accurate mass measurement, large highly charged peptides can be confidently identified by several doubly charged fragments when using high resolution detection of MS/MS fragments, that would not be identified by ion trap detection.

Interestingly, the overlap of identified acetylated residues was limited to 65 lysines belonging to only 37 different proteins, indicating distinctive antigen specificities between the different reagents. Statistical analysis of the replicate HCD datasets shows that the proportion of common acetylation sites identified between the monoclonal antibody cocktail and polyclonal antibody ranged from 28.8% to 37.6%, while the proportions between replicate runs of the same enrichment approach were 52.5% for monoclonal antibody cocktail and 58.9% for the polyclonal antibody. The difference between the proportion of common sites from runs of the same approach is significantly higher than that from runs of different approaches, with p-values ranging between 0.038 to <0.001. This indicates that the monoclonal antibody cocktail enrichment identified a unique subset of acetylation sites compared to the sites identified using the polyclonal antibody with statistical significance, even when adjusting for the run-to-run variation. (Table S5).

One of the reasons why the monoclonal cocktail led to identification of acetylated lysines not identified by the polyclonal antibody is likely due to the specificity of the monoclonal antibodies for a distinct subset of peptides. To assess this possibility, we compared the peptide sequences surrounding the acetylation sites identified by each method (Figure 3). The only major sequence motif which stands out in this data is the occurrence of histidine (H) at the +1 position at a 35% frequency in the monoclonal dataset but with only a 6% frequency in the polyclonal dataset. This motif difference has not been described by other dataset analyses searching for acetylation motifs<sup>(23, 24)</sup>, suggesting that it is specific to the peptide population enriched by the monoclonal cocktail. Additionally, it is possible that subsets of more complex motifs are hidden in these datasets. The significant number of

acetylation sites unique to the monoclonal-derived dataset, together with the identification of a new acetylation motif that is exclusive to the monoclonal cocktail enrichment, is consistent with the possibility that a unique subset of acetylated peptides was enriched using the monoclonal antibody cocktail.

## CONCLUSIONS

Although current methods using a polyclonal antibody from a single supplier have been used to identify a considerable number of acetylation sites in the human proteome, this work demonstrates that a cocktail of multiple monoclonal antibodies has the potential to complement the breadth of acetylated peptides enriched for this type of analysis. Clearly, further biological insights will be necessary to determine the full benefit of additional acetylation sites identified using the monoclonal cocktail.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

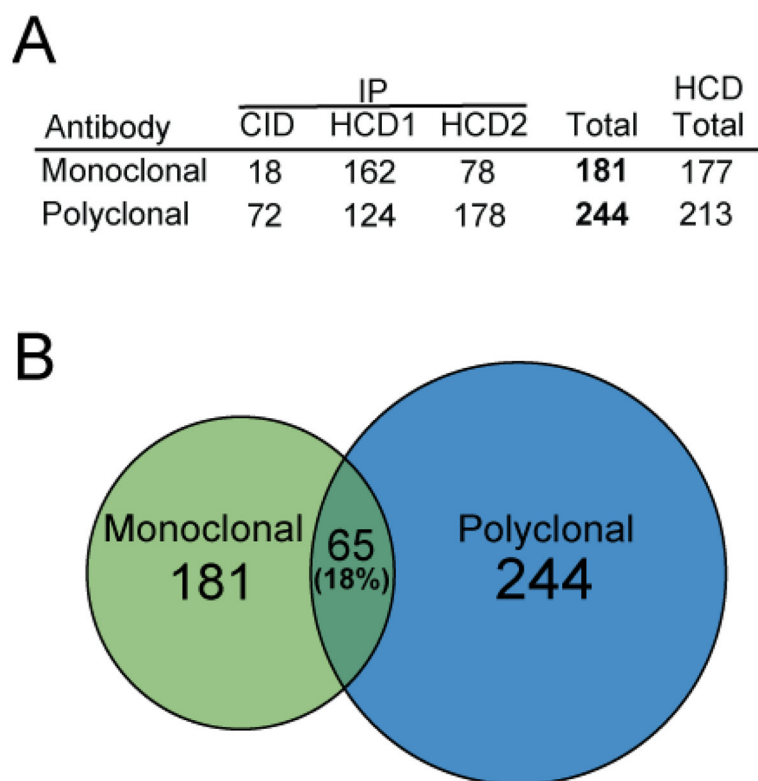
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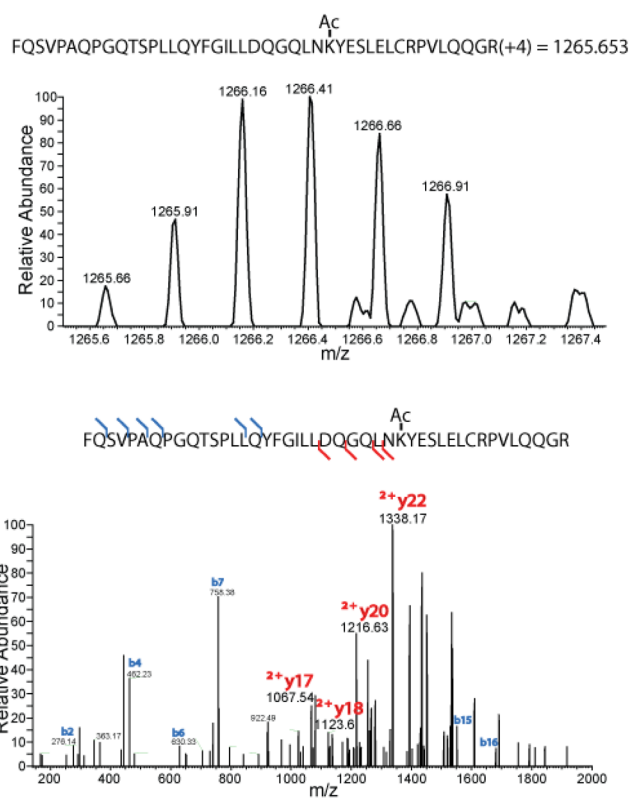
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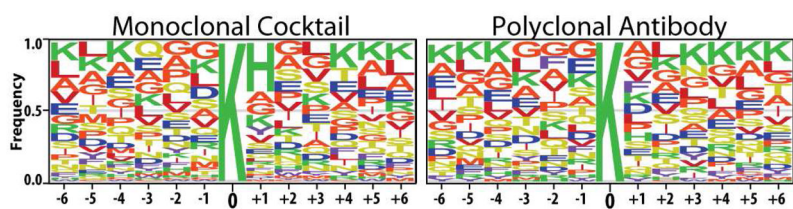


**Figure 1.** The number of sites identified from each immunoprecipitation experiment using various fragmentation schemes as indicated is listed in **(A)**. The relative overlap of the datasets is displayed as a Venn diagram with the circles drawn to scale in **(B)**.



**Figure 2.** The high resolution MS spectrum of a large peptide with a +4 charge (**top**) is unambiguously identified by high resolution detection of both singly and doubly charged MS/MS fragments (**bottom**).





**Figure 3.** Sequence alignment of the regions six amino acids from either side of the acetylated lysine residues identified using the monoclonal cocktail (**left**) and the polyclonal antibody (**right**).

**Table 1**

Comparison of pan-acetyl lysine antibodies

<b>Immunogen</b>	<b>Clone</b>	<b>Antibody Class</b>	<b>Supplier</b>
Acetylated peptide coupled KLH	Not applicable	Rabbit Polyclonal	ImmuneChem
Acetylated KLH	AKL5C1	Mouse Mono IgG <sub>1</sub>	Santa Cruz
KLH coupled XXX(Kac)XXX	Ac-K-103	Mouse Mono IgG <sub>2a</sub>	Cell Signaling
Acetylated protein mixture	15G10	Mouse Mono IgG <sub>2b</sub>	Novus
Synthetic Ac K9 of Histone H3	1C6	Mouse Mono IgG	Pierce