An Improved Immunoaffinity Reagent for Quantitative Profiling of Lysine Acetylation

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Introduction

Acetylated Lysine (Ac-K) was originally identified in histone proteins and was found to have a functional role in gene regulation. Since then many other proteins have been shown to be Ac-K modified, and this reversible post-translational modification (PTM) has been shown to play a regulatory role in cancer, muscle contraction, cell cycle machinery and enzymatic metabolism. Immunoaffinity purification (IAP) of peptides containing Ac-K followed by LC-MS/MS analysis provides a specific and sensitive way to identify and quantify these modification sites across samples. The results of such experiments largely depend on the specificity, affinity, and sequence bias of the Ac-K antibody (Ab) used.

Here we introduce a new Ac-K reagent from Cell Signaling Technology (CST), which consists of seven monoclonal Abs. Each of these Abs covers a unique sub-population of the acetylome, and by combining the seven monoclonal Abs at an optimized ratio, the new Ac-K reagent is capable of covering a broad range of Ac-K sites with over 50% enrichment specificity. We performed a comprehensive analysis of the performance between CST's new Ac-K reagent with a reagent from ImmuneChem Pharmaceuticals Inc (ICP) by comparing the number of MS2 identifications, enrichment specificity, and relative intensity of enriched peptides at the MS1 level. We found that CST's new Ac-K reagent generated twice as many unique Ac-K sites as the ICP reagent. In addition, we observed that over 65% of the Ac-K sites identified by the ICP reagent were covered by the Ac-K reagent from CST. Based on the comparison of the relative intensity of common peptides in the MS1 channel, we found that the CST Ac-K reagent exhibited better affinity for the majority of the enriched Ac-K peptides.

Protein function analysis was conducted on the proteins identified from mouse liver: the top five protein function groups for Ac-K were mitochondrial proteins, enzymes, cell surface proteins, matrix proteins, and proteins responsible for translation. Pathway analysis of identified proteins carrying Ac-K showed possible involvement of acetylation in various diseases, including Alzheimer's, Huntington's, and Parkinson's as well as a role in common metabolic pathways such as fatty acid degradation, glycolysis/glyconeogenesis, and the PPAR signaling pathway.

Methods

Antibody Development and ELISA Analysis

Two degenerate peptide libraries were used to immunize New Zealand White rabbits. Test bleeds were screened by ELISA for Ac-K specificity using an Ac-K peptide library and a non-Ac-K peptide library as a control. Rabbits that showed good reactivity and unique Ac-K motif specificity were identified and used to generate monoclonal antibodies using CST's XMT® protocol. ELISA analysis of each clone in the mixture was performed according to a previously described procedure (1).

Cell Culture, Mouse Tissue and Western Blots

HCT 116 cells were cultured in DMEM supplemented with 10% FBS. Cells were treated with 1 μM trichostatin A (TSA) for 6 hours at 37°C. Mouse tissues including brain, liver, testis, spleen, lung, heart, brown adipose tissue (BAT), and gastrocnemius muscle were obtained from mature BALB/c mice. Mouse embryos were harvested at embryonic day 16 from BALB/c mice. Equal amounts of total protein (20 μg for cell lysate; 30 μg for tissue lysate) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with Ac-K antibody overnight at 4°C.

Immunoaffinity Purification of Lysine Acetylated Peptides

IAPs of Ac-K peptides from mouse liver tryptic peptides were performed using the PTMScan® protocol as described previously (2). Briefly, 80 μg of each clone in Ac-K Ab (CST #13416) was conjugated to Protein A beads (Roche) overnight at 4°C and then the beads were washed extensively with PBS. A total of 10 mg of mouse liver tryptic peptides were dissolved in 1.4 ml of IAP buffer, mixed with Ac-K Ab beads and incubated for 2 hours at 4°C. The beads were washed twice with 1 ml of IAP buffer and three times with 1 ml of HPLC grade water. Peptides were eluted from beads with 0.15% TFA. Eluted peptides were desalted over tops packed with Empore™ C18 and eluted with 40% acetonitrile in 0.1% TFA. Eluted peptides were dried under vacuum. For Ab comparison, beads conjugated with the same amount of Ac-K antibody (200 μg) from CST and ICP (ICP0388) were mixed with tryptic peptides from 10 mg mouse liver followed by similar steps to those mentioned above.

LC-MS/MS Analysis and Database Searching

Enriched Ac-K peptides were separated on a 100 μm X 15 cm reversed-phase column and eluted using a 72-min linear gradient of 5%–30% acetonitrile in 0.125% formic acid delivered at 300 nl/min using an Easy nLC™. Tandem mass spectra were collected in a data-dependent manner with either an Orbitrap Elite™ or a Q Exactive™ mass spectrometer. All MS2 spectra were searched using SEQUEST® (v. 28 (rev. 12), 1998–2007) against the NCBI mouse database and their reversed complements. Static carbamidomethylation of cysteine (+57.0215) was required, and appropriate Ac-K (+42.0106) and methionine oxidation (+15.9949) were dynamically allowed with a maximum of four modifications of one type per peptide. Peptide spectral matches were filtered to a 1% false discovery rate using linear discriminant analysis in combination with the target-decoy strategy. The 15-mer sequences of all identified Ac-K peptides were generated for motif analysis of each clone by Motif-X (3).

Peptide Quantification and KEGG Pathway Analysis

Peptides enriched by Ac-K beads from CST and ICP were subject to LC-MS2 analysis by Orbitrap Elite™ over a gradient of 90 min in analytical duplicates. Filtered pepXML files containing Ac-K peptides only and raw data were processed by Skyline v2.1 according to ref. 4 and online tutorial. Quantitative data were evaluated and clustered in TIBCO™ Spotfire® DecisionSite v 9.1.2. KEGG pathway analysis of identified proteins with Ac-K was done through DAVID Bioinformatics Resources 6.7 (5).

References

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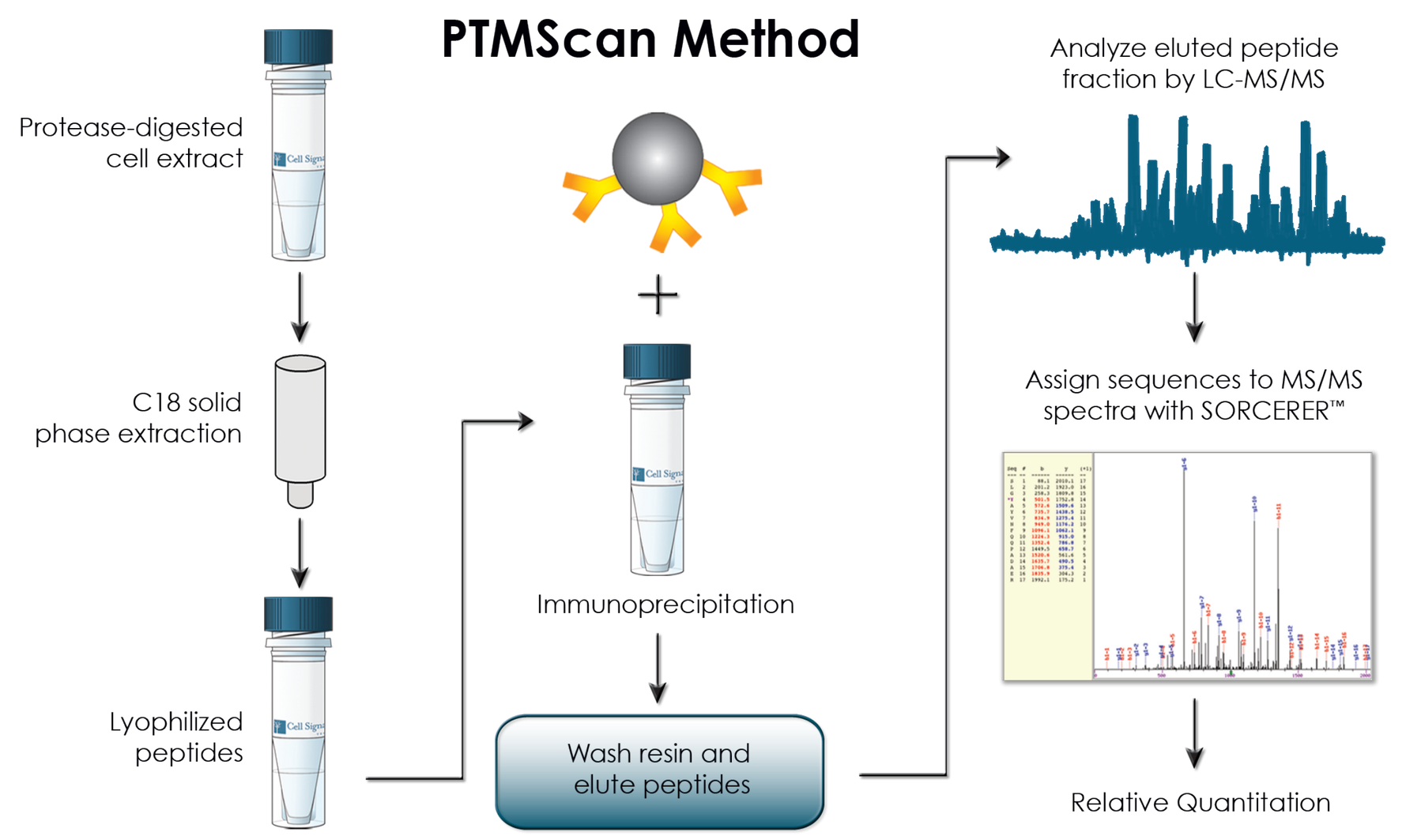
2. Guo, A., and Comb, M.J. (2014) *Mol. Cell. Proteomics* 13, 372–387.
3. Schwartz, D., and Gygi, S.P. (2005) *Nat. Biotechnol.* 23, 1391–1398.
4. Schilling, B., and Gibson, B.W. (2012) *Mol. Cell. Proteomics* 11, 202–214.
5. Huang, D.W., and Lempicki, R.A. (2009) *Nat. Protoc.* 4, 44–57.


PTMScan Method
Protease-digested cell extract
C18 solid phase extraction
Lyophilized peptides
Immunoprecipitation
Wash resin and elute peptides
Analyze eluted peptide fraction by LC-MS/MS
Assign sequences to MS/MS spectra with SORCERER™
Relative Quantitation

Results

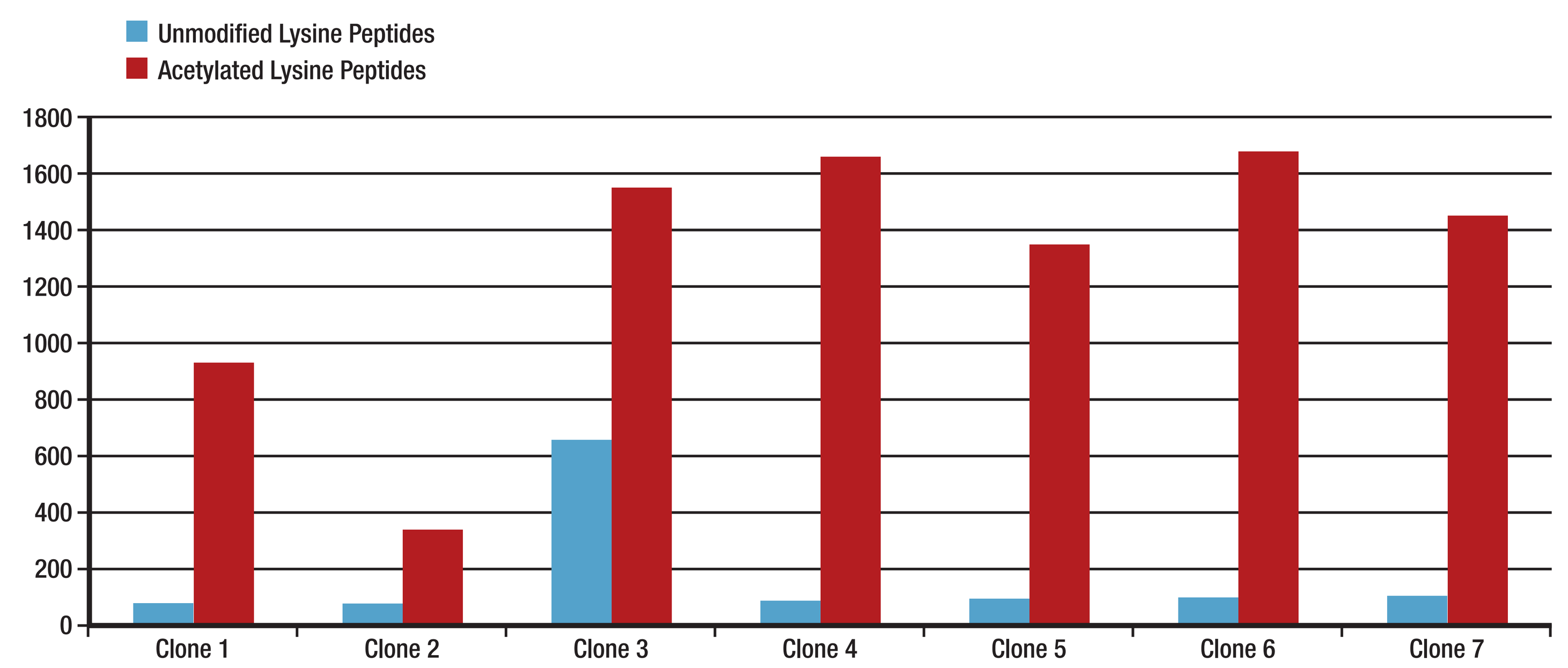


Figure 1: ELISA analysis of seven monoclonal Abs in the acetyl-lysine reagent (CST #13416). Same peptide library without acetylation on lysine was used as control.

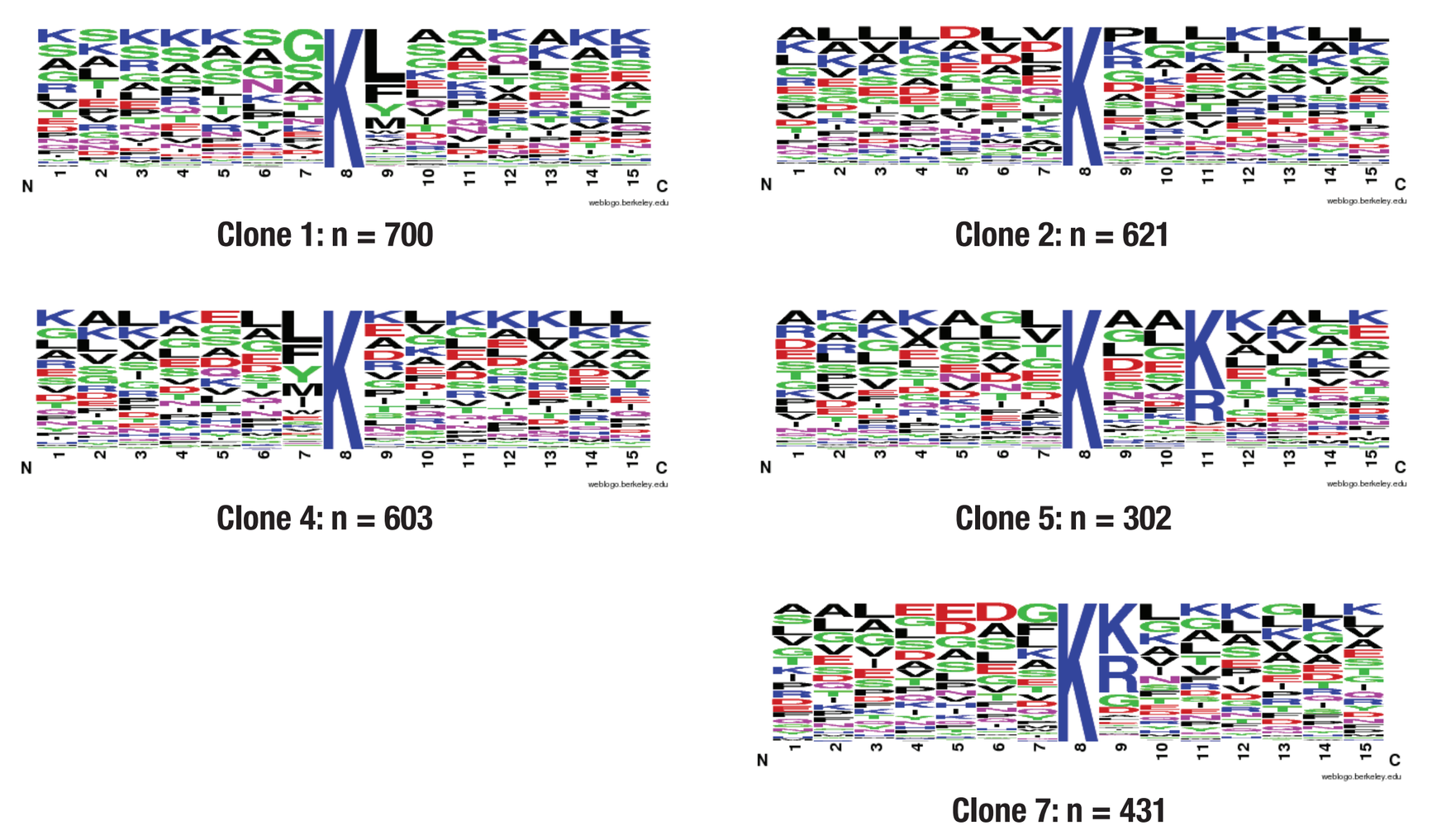


Figure 2: Motif logo analysis of the seven monoclonal Abs in the acetyl-lysine reagent (CST #13416). Acetyl-peptides were enriched from peptides of mouse liver, and identified by LC-MS/MS analysis using a Q-Exactive™ mass spectrometer over a 72 min gradient.

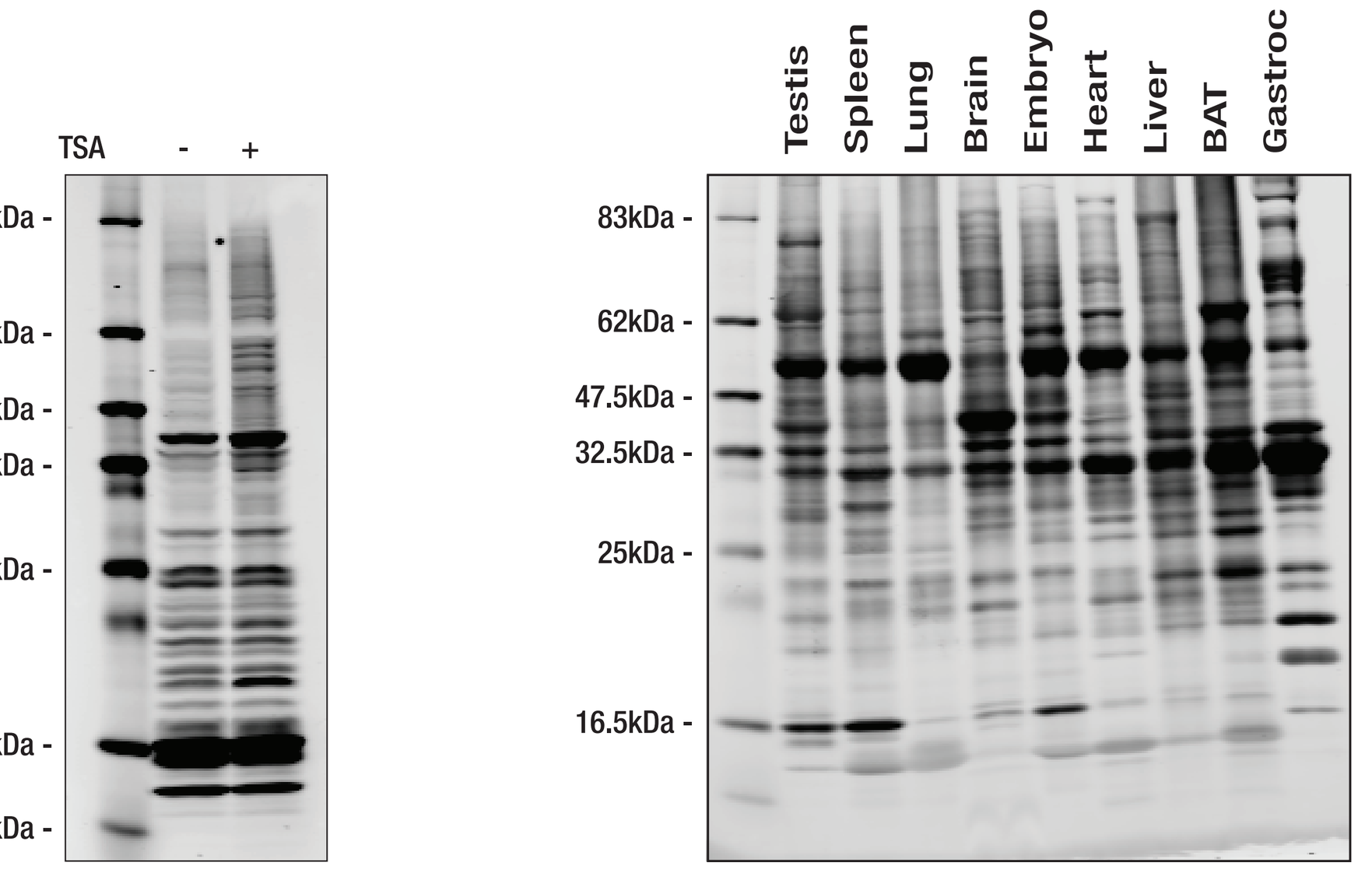


Figure 3: Western blot analysis of Ac-K in HCT 116 cell extracts (+/- Trichostatin A-TSA, left); and mouse tissue extracts (right) using acetyl-lysine reagent (CST #13416, 1:20,000).

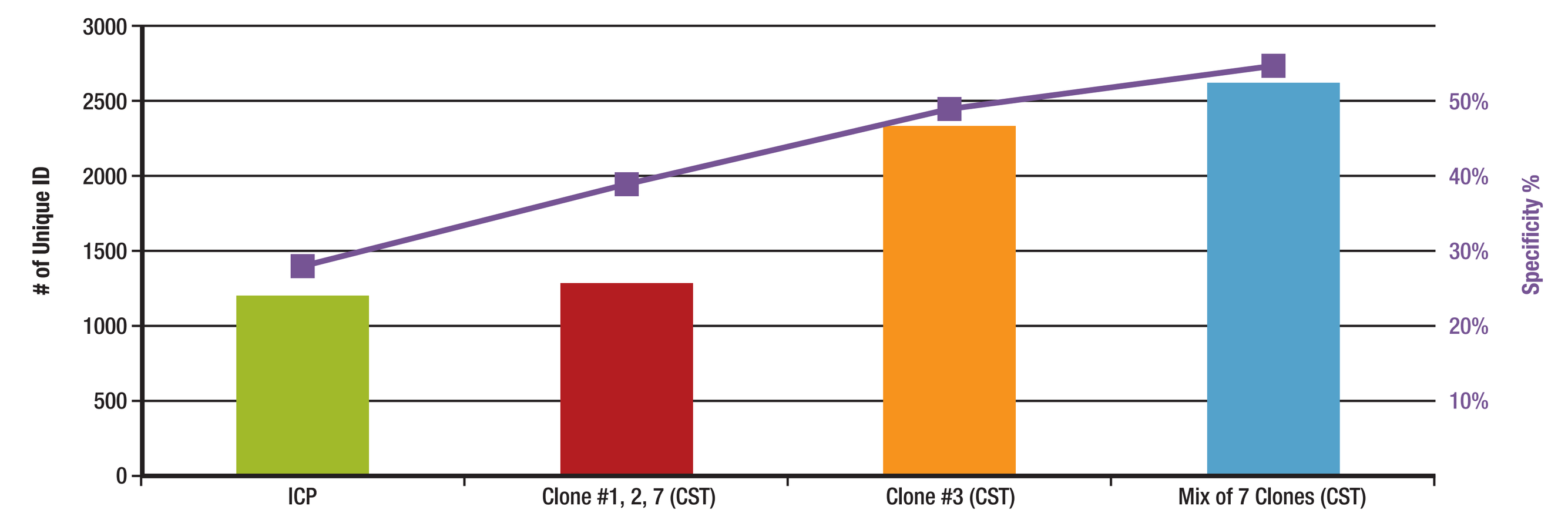


Figure 4: Comparison of acetyl-lysine reagent from ICP (ICP0388) and CST (#13416) by LC-MS/MS analysis. Parallel enrichments were performed from mouse liver tryptic peptides (10 mg). These were analyzed by Orbitrap-Velos™ over a 120 min gradient. Specificity was defined as number of unique acetylated peptides over the total number of identified peptides.

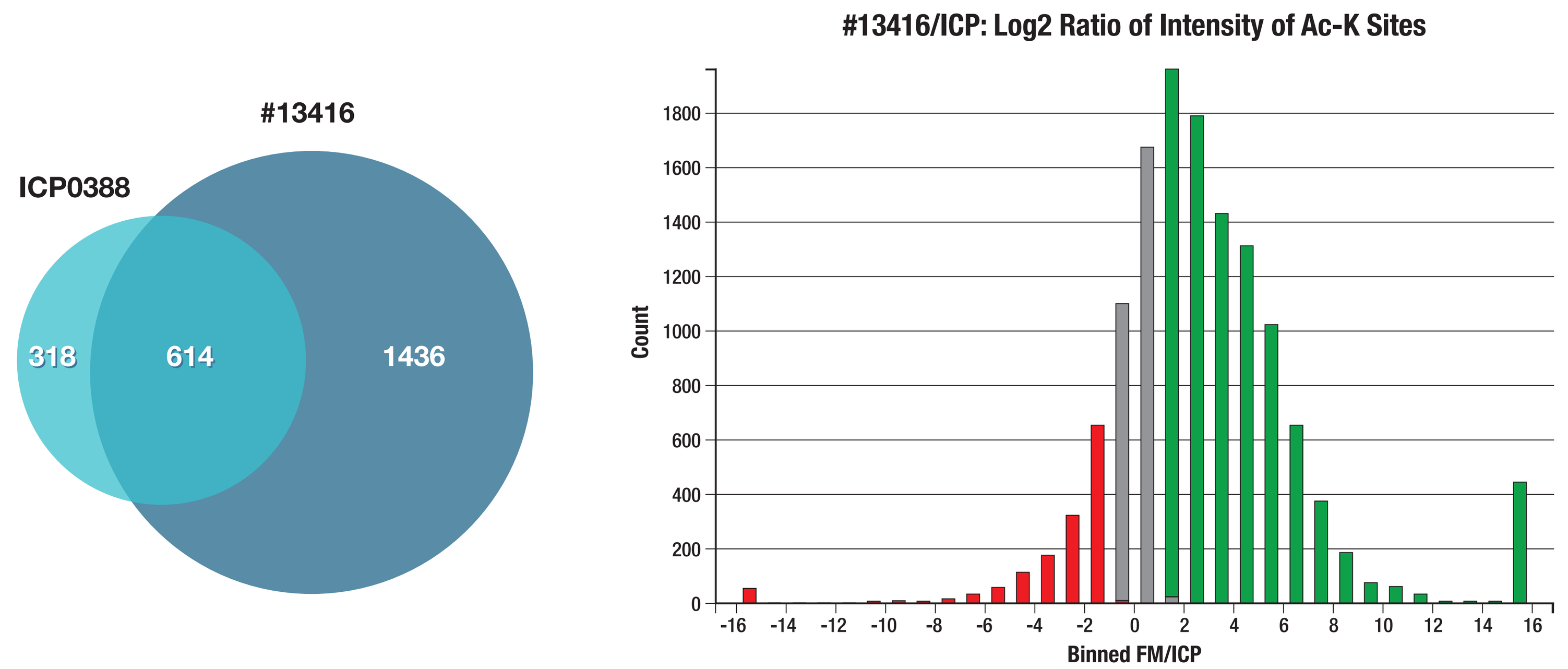


Figure 5: Comparison of MS2 identification and MS1 intensity of Ac-K peptides enriched by CST and ICP antibodies. A total number of 2,368 unique Ac-K sites were identified by combining the identifications of CST and ICP antibody IP results. (left) About two-thirds of sites identified by the ICP reagent were also covered by the CST reagent. There were 1,436 and 318 peptides uniquely enriched by the CST and ICP antibody, respectively. (right) Distribution of Log2 ratio of MS1intensity of Ac-K peptides identified by CST and ICP antibodies. (right) CST Ac-K antibody has affinity for a broad range of Ac-K peptides with higher enrichment efficiency.

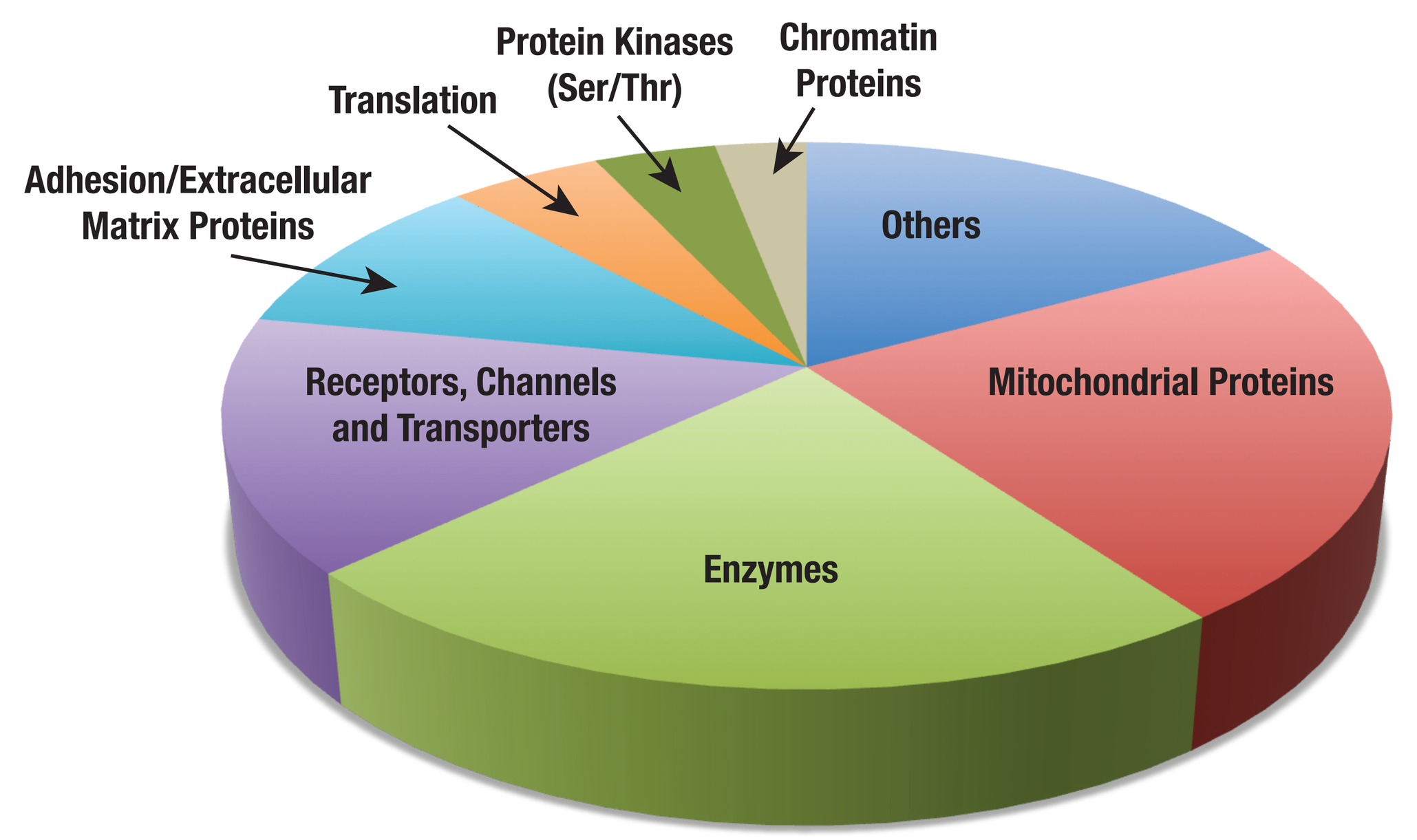


Figure 6: Protein function distribution of identified proteins with Ac-K from mouse liver using the CST acetyl-lysine reagent (#13416).

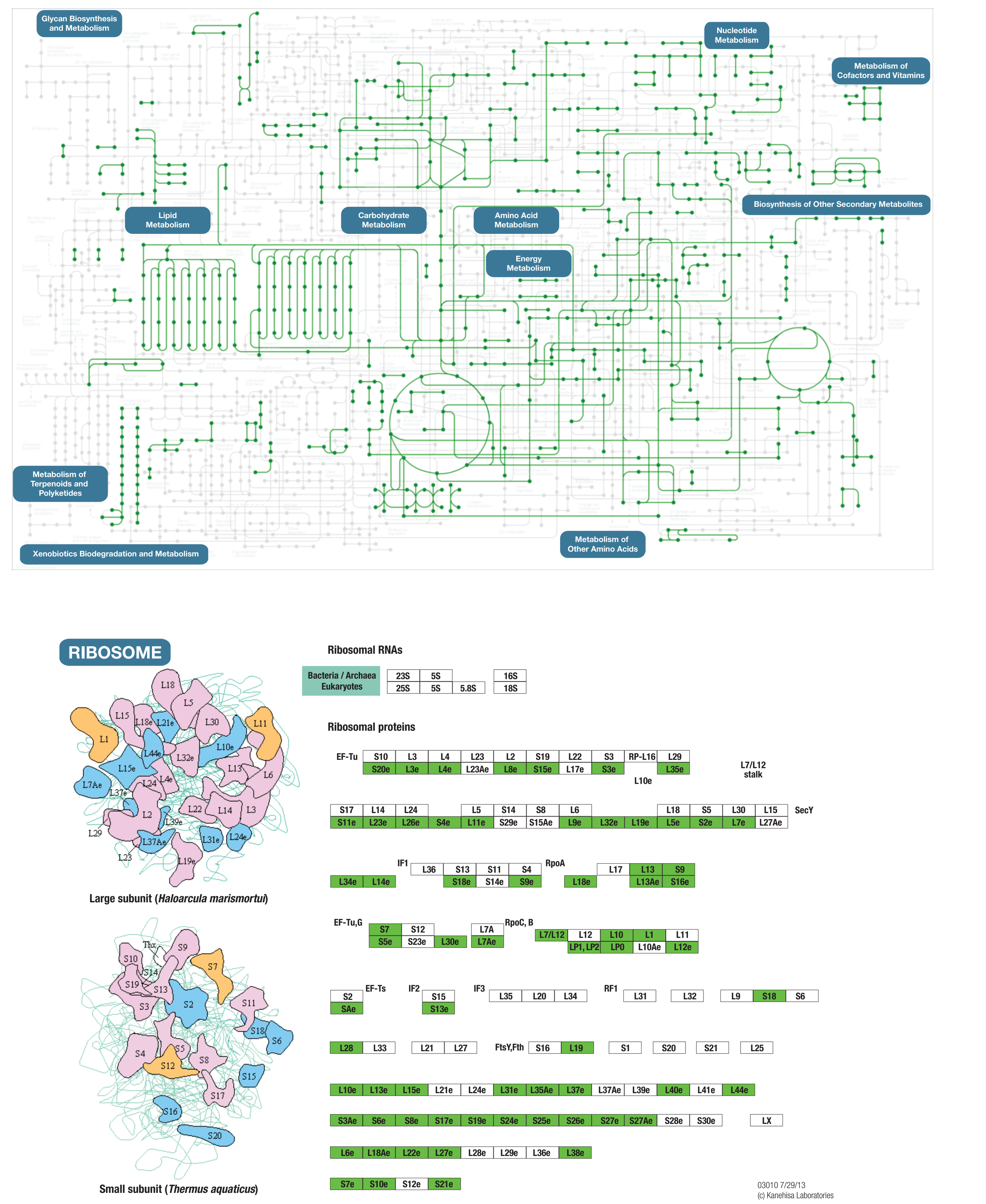


Figure 7: KEGG pathway analysis of identified proteins with Ac-K. Identified proteins covered the majority of enzymes in the metabolic pathway nodes (top) demonstrating the broad affinity of the reagent, and most ribosomal proteins (bottom) indicating involvement of acetylation in protein translation.

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