Sequential Immunoaffinity Purification of Post-Translationally Modified Peptides for Improved Enrichment Specificity

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INTRODUCTION

Post-translational modification (PTM) of proteins, including phosphorylation, acetylation, methylation, and ubiquitination, is critical to all aspects of cellular signaling. Antibody-based and enrichments of post-translationally modified peptides combined with LCMS have proven to be powerful methods for the study of PTMs in a wide variety of cells and tissues, and in profiling various disease states. Here, PTM peptides were sequentially enriched from samples twice with the same antibody. This sequential enrichment both qualitatively and quantitatively reduced the number of non-specific, unmodified peptides identified in the samples with minimal loss of PTM peptides of interest. This increase in enrichment specificity (% modified peptides) allows more instrument time for identification of peptides of interest as well as improving quantitative accuracy of the method.

METHODS

Mouse liver (phosphotyrosine, acetyl-lysine) or human HCT-116 cells (ubiquitin K-GG) were, lysed, digested with trypsin, and desalted over C18 columns. Post-translationally modified peptides were enriched using Phosphotyrosine pY-1000 (Cell Signaling Technology #8803), Acetyl Lysine (Cell Signaling Technology #13416), or Ubiquitin Remnant K-GG (Cell Signaling Technology #5562) antibodies. Peptide eluates were purified using STAGE tips. Half of each eluate was re-immunoprecipitated with the same antibody and re-purified on STAGE tips. Immunoprecipitated peptides were run in LC-MS/MS on an LTQ Orbitrap Velos or Elite mass spectrometer using a top 20 data-dependent analysis method. MS/MS spectra were assigned to peptide sequences using SEQUEST. Data was filtered using the Linear Discriminant module of Core (Harvard University). Label-Free quantification was performed using Progenesis (Nonlinear Dynamics) and manual review of ion chromatogram files.

RESULTS

A single enrichment of post-translationally modified peptides using Cell Signaling Technology Motif Antibodies and the PTMScan method was compared to two sequential enrichments with the same antibody. Sequential enrichment using all antibodies tested, including phosphotyrosine, acetylated lysine, and ubiquitin remnant K-GG resulted in equivalent or higher numbers of modified peptides identified from samples. The number of unmodified, non-specific peptides was significantly lower using the sequential enrichment than the single enrichment. Relative quantification of both modified and unmodified peptide levels was performed using Progenesis and manual review of ion chromatogram files. The quantitative data showed dramatic reduction of non-specific, unmodified peptide abundance relative to a single enrichment, while the level of the target, PTM peptides was largely unchanged.

This increase in enrichment specificity has multiple advantages including more instrument time devoted to identification of peptides of interest (those that are post-translationally modified), and a simplified mixture of peptides to be identified and quantified. A more highly enriched, less complex sample should result not only in better identification rates of peptides of interest, but also more accurate quantification of those peptides. Isobaric tagging methods such as iTRAQ or TMT in particular will benefit from this reduced sample complexity as a way to limit ratio compression.

REFERENCES

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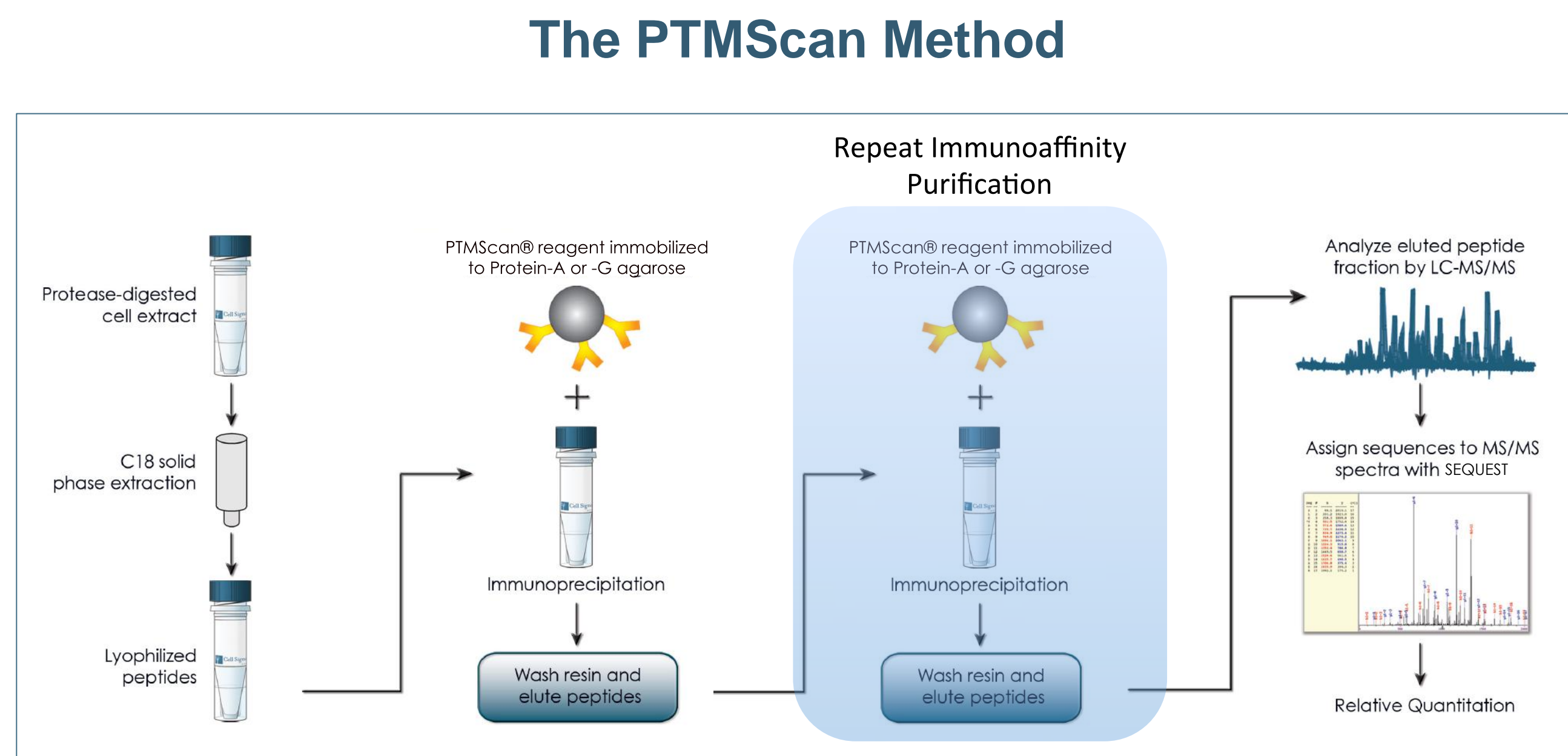
The PTMScan Method

Figure 1: The PTMScan Method with sequential immunoaffinity purification. Proteins from cells, tissues, or biofluids are digested to peptides and post-translationally modified peptides are enriched using motif/PTM antibodies. The sequential method incorporates an additional enrichment of peptides prior to LC-MS/MS analysis (blue shading).
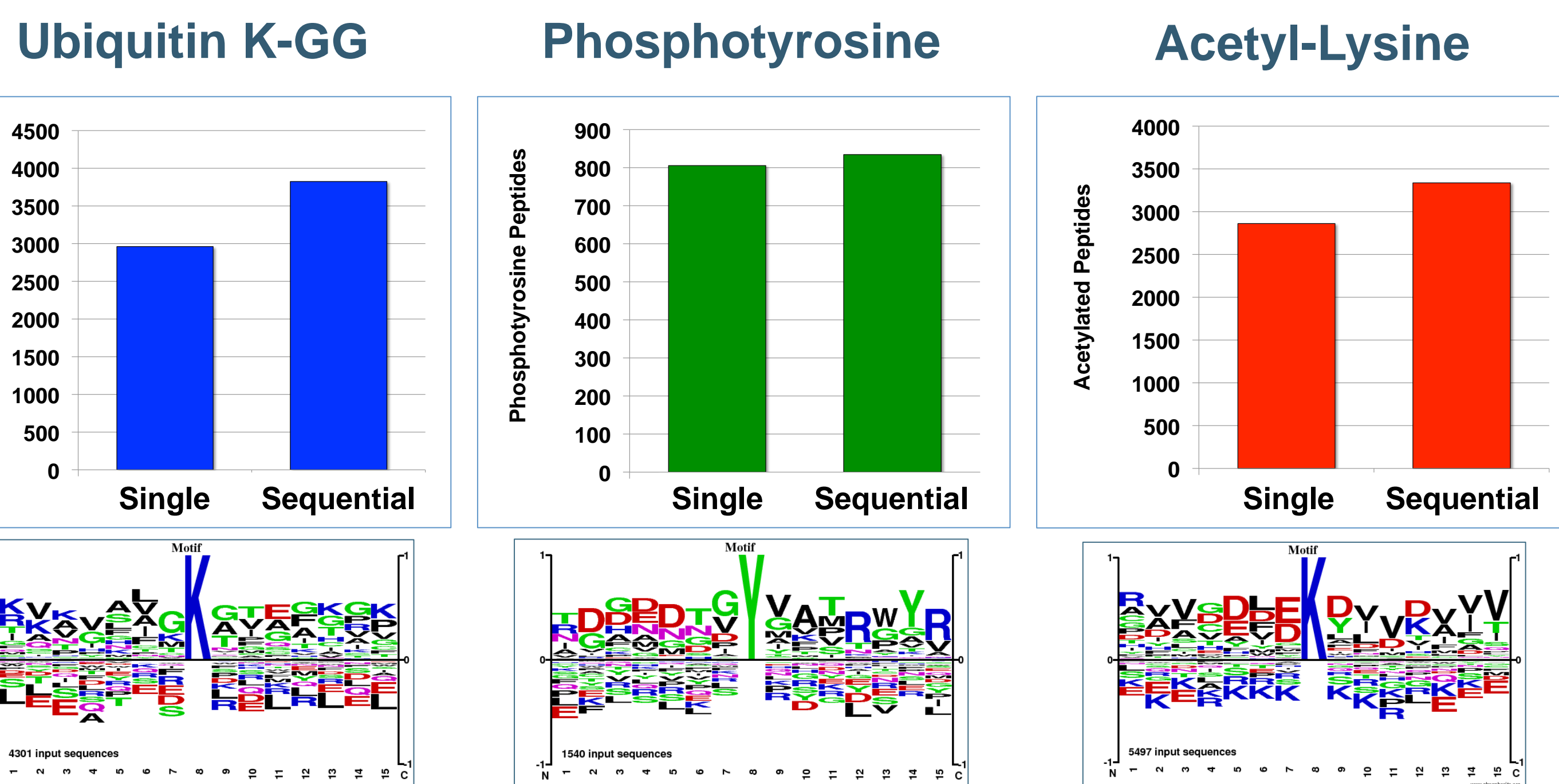

Figure 2: Single versus sequential enrichment. Number of peptide identifications from a single immunoaffinity purification versus two sequential enrichments using the same antibody for the Ubiquitin Remnant K-GG Motif Antibody (**blue**), the Phosphotyrosine pY-1000 Antibody (**green**), and the Acetyl-Lysine Antibody (**red**). Sequence logos generated using PhosphoSitePlus (www.phosphosite.org) for each enrichment are shown

Ubiquitin K-GG

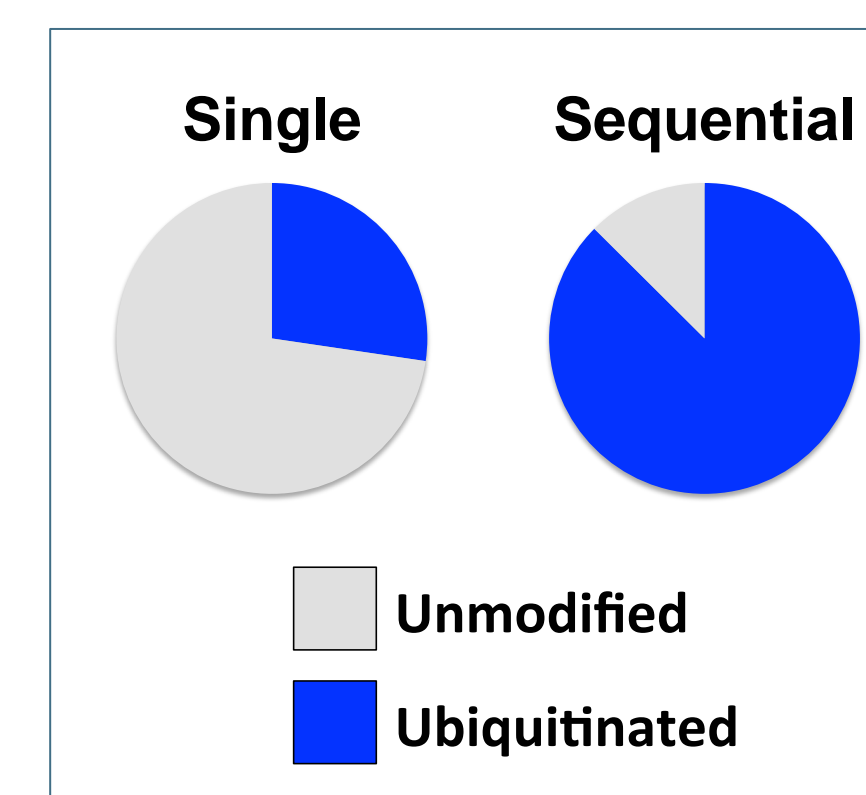| Method	Antibody	Ub K-GG	Unmodified
1 IAP	Ubiquitin	2979	7921
1 IAP	Ubiquitin	2944	7958
2 IAPs	Ubiquitin	3731	535
2 IAPs	Ubiquitin	3918	623

Figure 3: Increased Ubiquitin K-GG enrichment specificity from sequential IAP. Number of unmodified (grey) and ubiquitinated (**blue**) peptide identifications from single versus sequential enrichment for the Ubiquitin Remnant K-GG Motif Antibody. The pie chart reflects the decrease in unmodified peptide identifications with sequential enrichment.
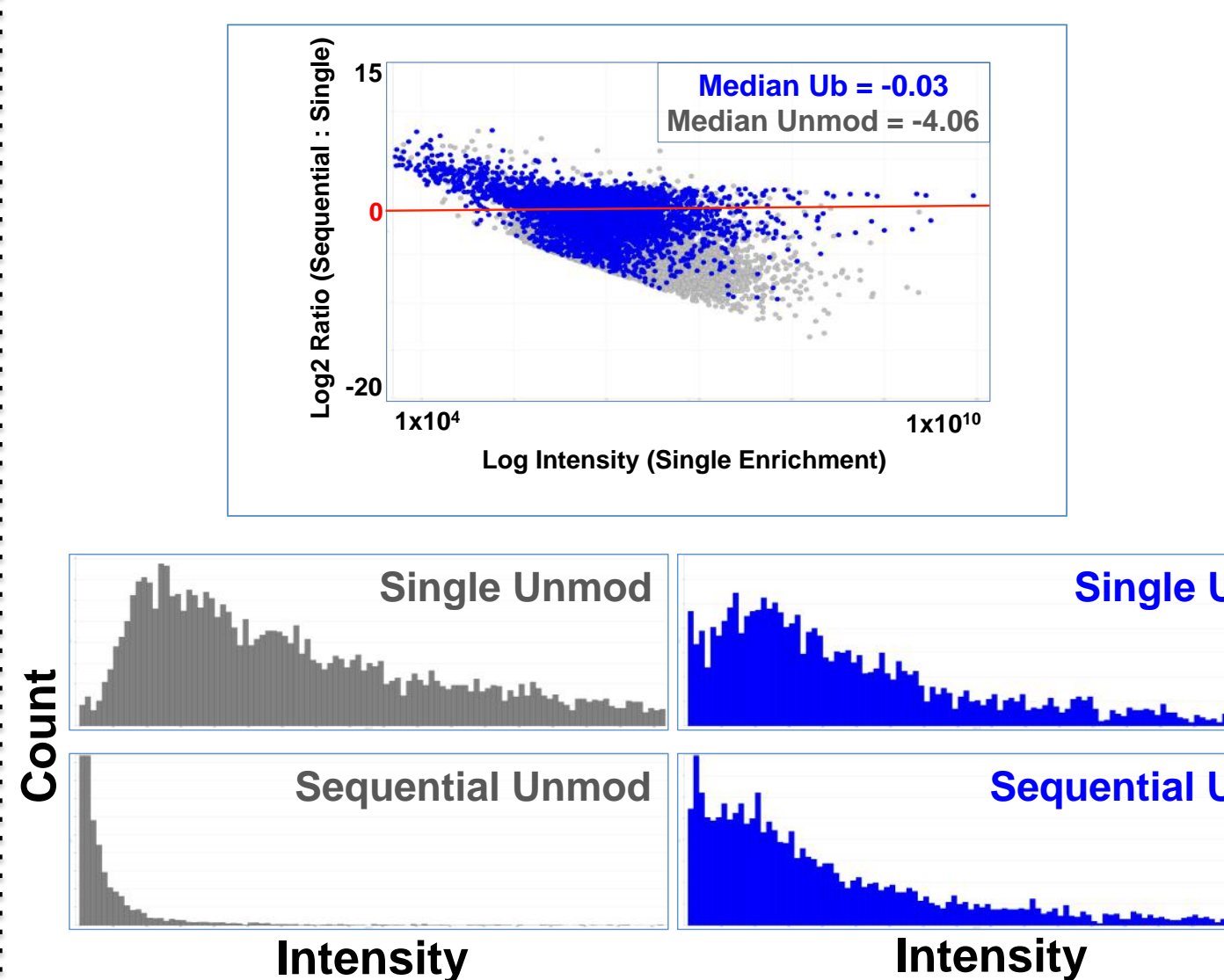

Figure 4: Quantitative analysis of single versus sequential Ubiquitin K-GG enrichment. Log2 ratio plot (top panel) and intensity histograms (bottom panels) for single versus sequential enrichment. Unmodified (grey) and ubiquitinated (**blue**) peptides are shown separately. Median Log2 ratios for unmodified and ubiquitinated peptdes are indicated. Quantification was performed using Progenesis (Nonlinear Dynamics).

Phosphotyrosine

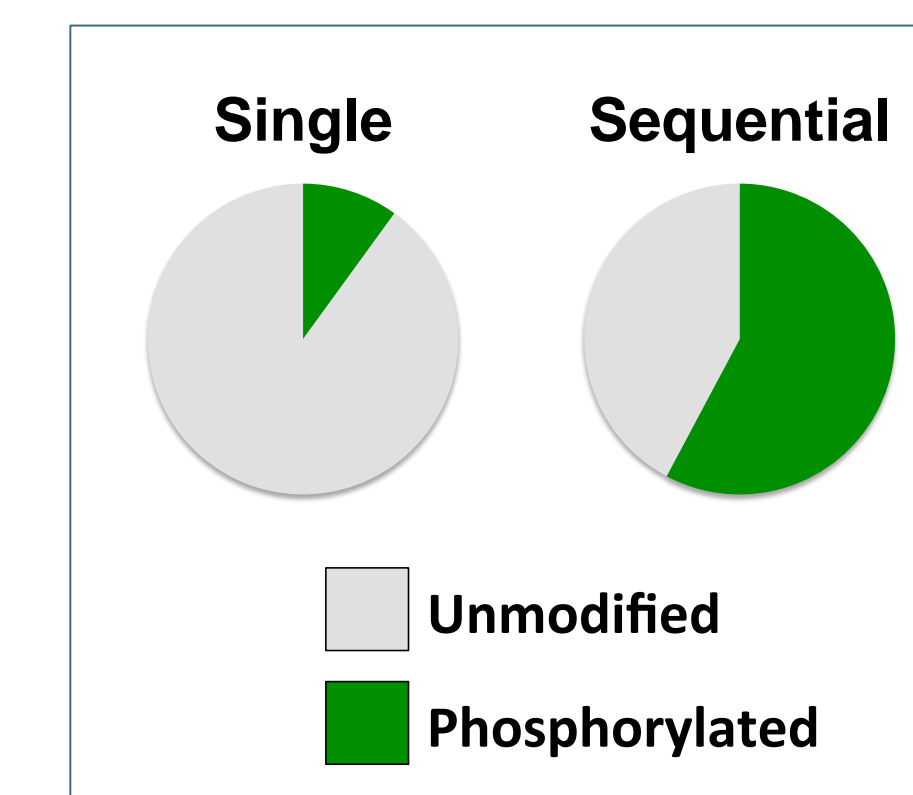| Method	Antibody	Phospho	Unmodified
Single	pY-1000	915	8230
Single	pY1000	820	8098
Sequential	pY1000	824	1267
Sequential	pY1000	976	713

Figure 5: Increased phosphotyrosine enrichment specificity from sequential IAP. Number of unmodified (grey) and phosphorylated (**green**) peptide identifications from single versus sequential enrichment for the Phosphotyrosine Motif Antibody. The pie chart reflects the decrease in unmodified peptide identifications with sequential enrichment.
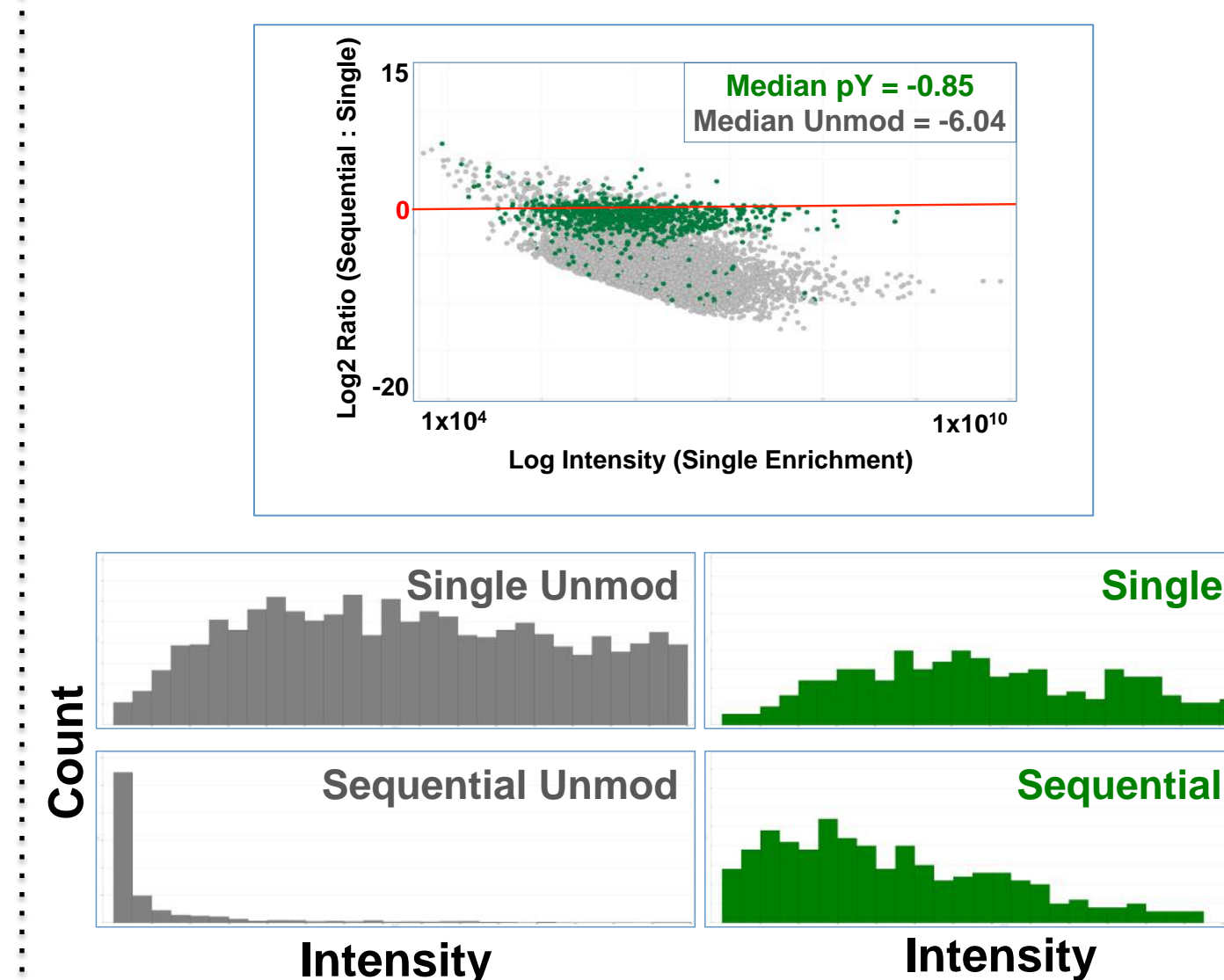

Figure 6: Quantitative analysis of single versus sequential Phospho-tyrosine enrichment. Log2 ratio plot (top panel) and intensity histograms (bottom panels) for single versus sequential enrichment. Unmodified (grey) and phosphotyrosine (**green**) peptides are shown separately. Median Log2 ratios for unmodified and phosphotyrosine peptdes are indicated. Quantification was performed using Progenesis (Nonlinear Dynamics).

Acetyl-Lysine

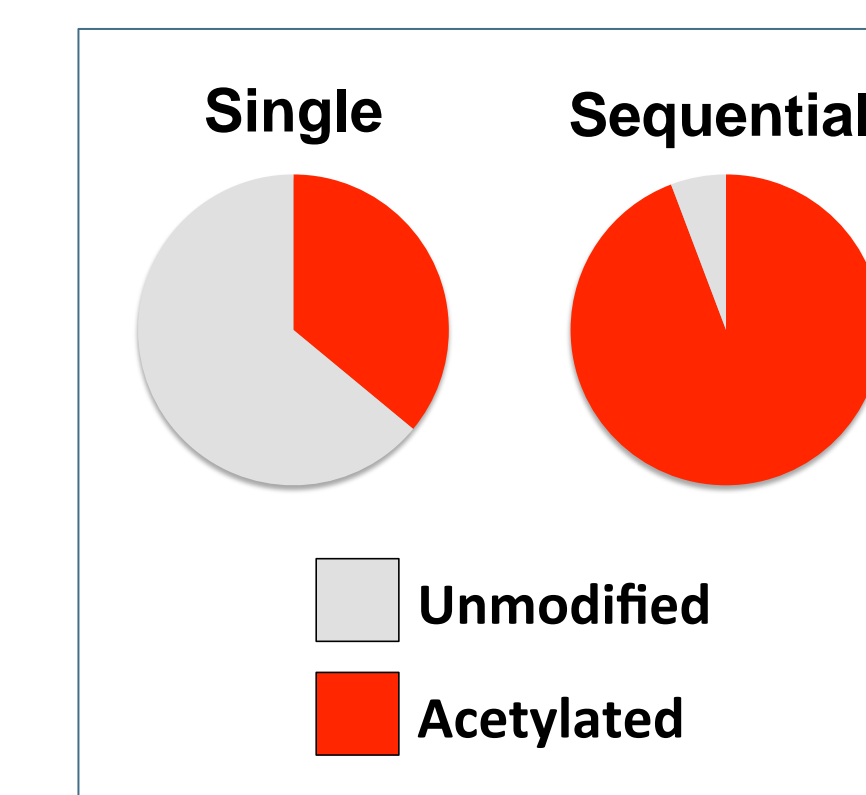| Method	Antibody	Acetylated	Unmodified
Single	AcK	3030	5387
Single	AcK	2696	4688
Sequential	AcK	3670	224
Sequential	AcK	3008	130

Figure 7: Increased Acetyl-Lysine enrichment specificity from sequential IAP. Number of unmodified (grey) and acetylated (**red**) peptide identifications from single versus sequential enrichment for the Acetyl-Lysine Motif Antibody. The pie chart reflects the decrease in unmodified peptide identifications with sequential enrichment.
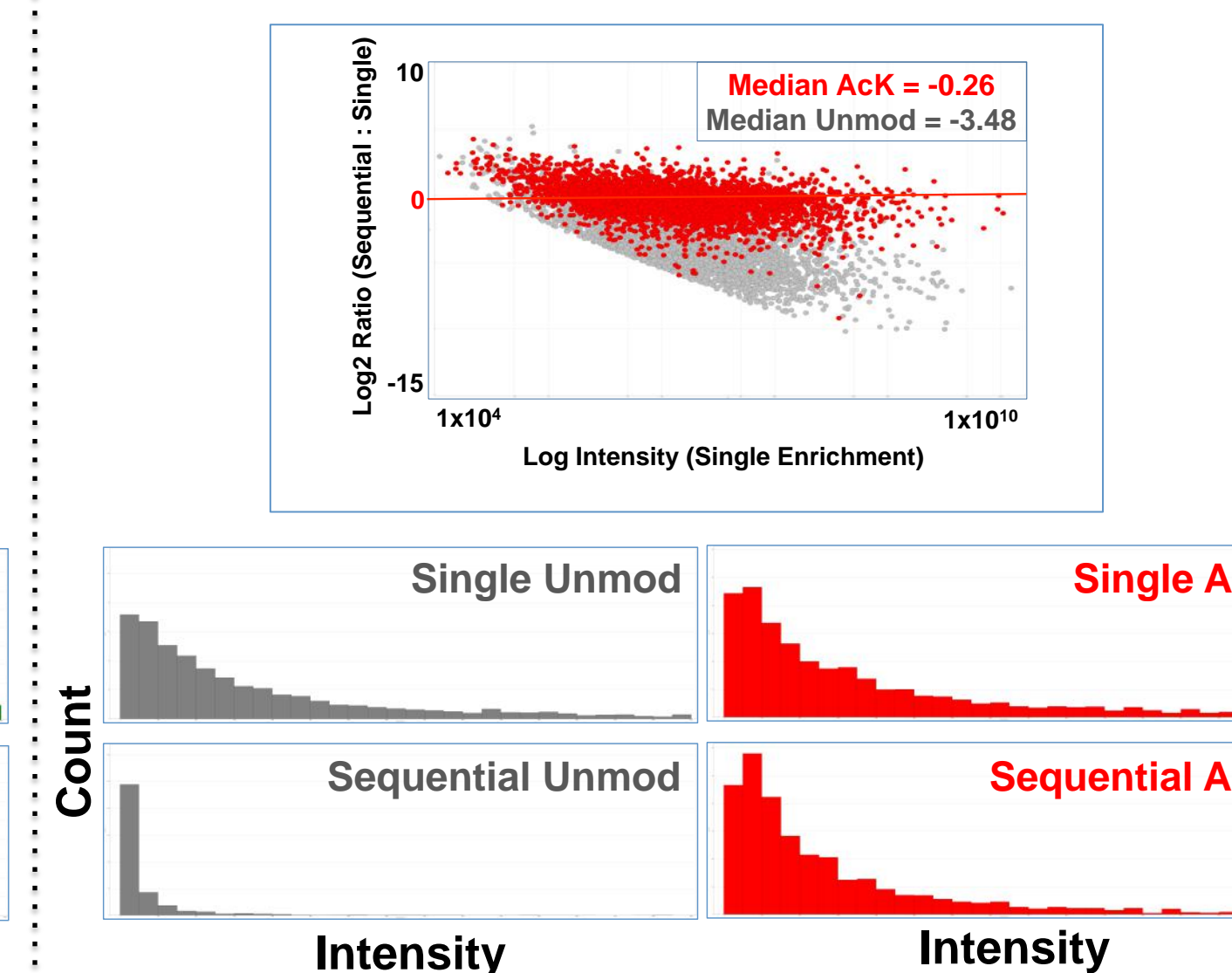

Figure 8: Quantitative analysis of single versus sequential Acetyl-Lysine enrichment. Log2 ratio plot (top panel) and intensity histograms (bottom panels) for single versus sequential enrichment. Unmodified (grey) and acetylated (**red**) peptides are shown separately. Median Log2 ratios for unmodified and phosphotyrosine peptdes are indicated. Quantification was performed using Progenesis (Nonlinear Dynamics).

Cell Signaling TECHNOLOGY

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