Proteomics

International Edition: DOI: 10.1002/anie.201810569
German Edition: DOI: 10.1002/ange.201810569

A Quantitative Chemical Proteomics Approach for Site-specific Stoichiometry Analysis of Ubiquitination

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Abstract: *Stoichiometric analysis of post-translational modifications is an emerging strategy for absolute quantification of the fractional abundance of the modification. Herein, a quantitative chemical proteomic workflow for stoichiometric analysis of ubiquitination is reported, named isotopically balanced quantification of ubiquitination (IBAQ-Ub). The strategy utilizes a new amine-reactive chemical tag (AcGG-NHS) that is structurally homologous to the GG remnant of ubiquitin on modified lysine after trypsin cleavage and therefore enables the generation of structurally identical peptides from ubiquitinated and unmodified lysine residues following trypsin digestion and secondary stable isotopic labeling. The strategy is highly robust, sensitive, and accurate with a wide dynamic range using either protein standards or complex cell lysates. Thus, this work provides an efficient chemical proteomics tool for quantitative stoichiometric analysis of ubiquitination signaling pathways.*

Ubiquitination (Ub) is an essential pathway in eukaryotic cells that controls the signaling flux in diverse biological processes.[1] The countering activities of the elegant E1-E2-E3 enzymatic cascade and the deubiquitinases (DUBs) determine the physiological stoichiometry of ubiquitination at the site-specific level.[1a,b] Advances in the quantitative proteomics, such as stable isotope labeling by/with amino acids in cell culture (SILAC) and isobaric tagging,[2] have enabled system-wide discoveries of Ub dynamics during signaling processes.[3] More recently, absolute quantification of ubiquitination has been achieved using spike-in stable-isotope labeled synthetic peptide standards (UB-AQUA)[4] or recombinant proteins (PSAQ),[5] which allowed targeted analysis of polyubiquitin linkages in ER stress, DNA damage response, mitophagy, and *in vitro* enzyme activities.[4,6]

Stoichiometry analysis is an emerging approach to measure the fractional abundance of post-translational modifications (PTMs).[6b,7] It allows the quantitative comparison of PTM abundance between different sites on the same, or different, target proteins. Systematic analysis of PTM stoichiometries does not have to rely on the synthesis of *in vitro* isotopically labeled standards and therefore, potentially enables global untargeted discoveries of modification abundance at the physiological levels. Recent advances in quantitative proteomics have enabled stoichiometric analysis of phosphorylation, lysine acetylation, and succinylation on a global scale.[7a–c,f–h,j–n] Despite these advances, accurate and site-specific stoichiometric analysis of ubiquitination has been challenging. In this study, the development of an efficient chemical-based quantitative proteomic approach (termed IBAQ-Ub) is reported, which enables the determination of the absolute site-specific stoichiometry of ubiquitination.

Following our previously successful experience in stoichiometry analysis of lysine acetylation,[7k] we designed an isotopically balanced quantification strategy for stoichiometry analysis of ubiquitination, based on the chemical signature of the glycylglycine (GG) remnant on Ub sites after trypsin digestion (Figure 1A). The major challenge of this workflow was to identify an effective strategy that enables the derivatization of lysine with an isotopically labeled GG remnant. To overcome this challenge, we developed an acetyl glycylglycine (AcGG) tag that is structurally similar to the GG remnant (Figure 1B). The N-terminal acetylation of the AcGG tag allowed the tag to be activated through *N*-hydroxysuccinamide(NHS) esterification, thereby protecting the tag from self-conjugation. Therefore, all unmodified lysines can be labeled with an AcGG tag (Figure 1C). In the next step, trypsin cleaves the ubiquitin and generates a GG remnant on the deubiquitinated lysine with a fresh N-terminus. The second labeling with heavy acetyl-NHS ($^{13}CD_3$-^{13}CO-NHS) can then be applied, which serves two purposes. First, it balances the chemical structure of deubiquitinated

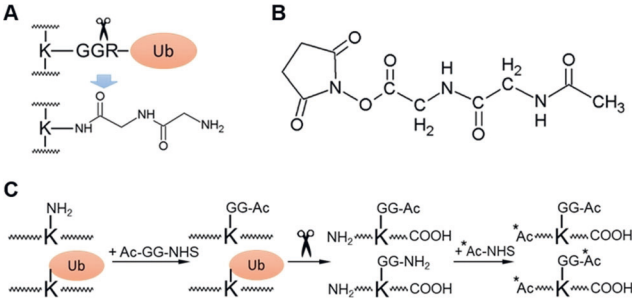

Figure 1. A chemical proteomics workflow for quantitative analysis of Ub stoichiometry. A) A glycylglycine remnant after trypsin digestion of a Ub site. B) The molecular structure of acetyl glycylglycine *N*-hydroxysuccinamide (AcGG-NHS) for Ub stoichiometry analysis. C) The IBAQ-Ub workflow that involves the derivatization of unmodified lysines with AcGG-NHS, trypsin digestion, and stable isotopic labeling with heavy acetyl-NHS.

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Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201810569.

lysine (with GG remnant) and previously unmodified lysine (with AcGG tag). Second, it incorporates stable isotopes only at the lysines with a GG remnant, thereby differentiating deubiquitinated lysines from unmodified lysines. Following these steps, originally ubiquitinated peptides and their unmodified counterparts will have exactly the same chemical structure, but differ in stable isotope labeling (Figure 1C). Therefore, these peptides can be analyzed by LC-MS for direct stoichiometry quantification based on the MS intensities.

To validate this workflow, we first synthesized the AcGG-NHS and heavy acetyl-NHS (Supporting Information, Figure S1). The ability of these compounds to label lysine residues has been previously tested[7k] or demonstrated in this study using the Ninhydrin test (Supporting Information, Figure S2).

Next, we determined if the IBAQ-Ub strategy allows the accurate determination of Ub stoichiometry. To this end, we applied this strategy to analyze the recombinant di-Ub standard with K48 linkage (Figure 2). To generate ubiquitin peptides with suitable lengths for LC-MS analysis after AcGG labeling and trypsin digestion, a second digestion with a different protease, such as Asp-N, was included based on the analysis of the ubiquitin primary sequence (Figure 2A). Our results showed that IBAQ-Ub analysis precisely determined the absolute stoichiometry of K48-linked di-Ub standard with a measurement of $50.14 \pm 0.24\,\%$ (Figure 2B–D).

To determine the dynamic range and sensitivity of the IBAQ-Ub workflow, we performed serial dilution experiment by mixing recombinant K48-linked di-Ub with recombinant-free ubiquitin at various ratios. These mixtures gave a wide range of theoretical Ub stoichiometries from 0.5% to 50% (Figure 3A). Each mixture was analyzed using IBAQ-Ub

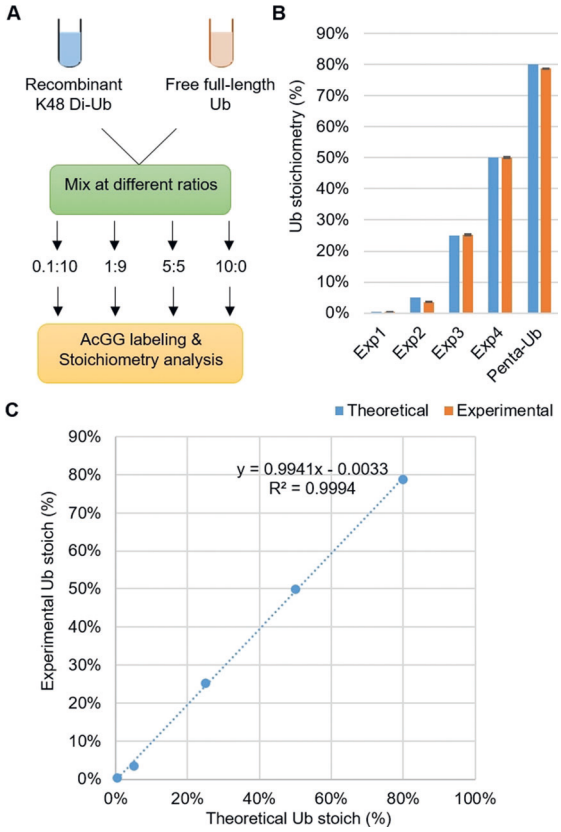

Figure 3. Dynamic range of detection for the IBAQ-Ub workflow. A) An experimental strategy for the measurement of the dynamic range of quantification for the IBAQ-Ub workflow. B) A bar graph comparing the theoretical stoichiometry values in each sample with experimentally measured values. C) A linear correlation line graph showing the correlation between theoretical stoichiometry values and the experimental measurements.

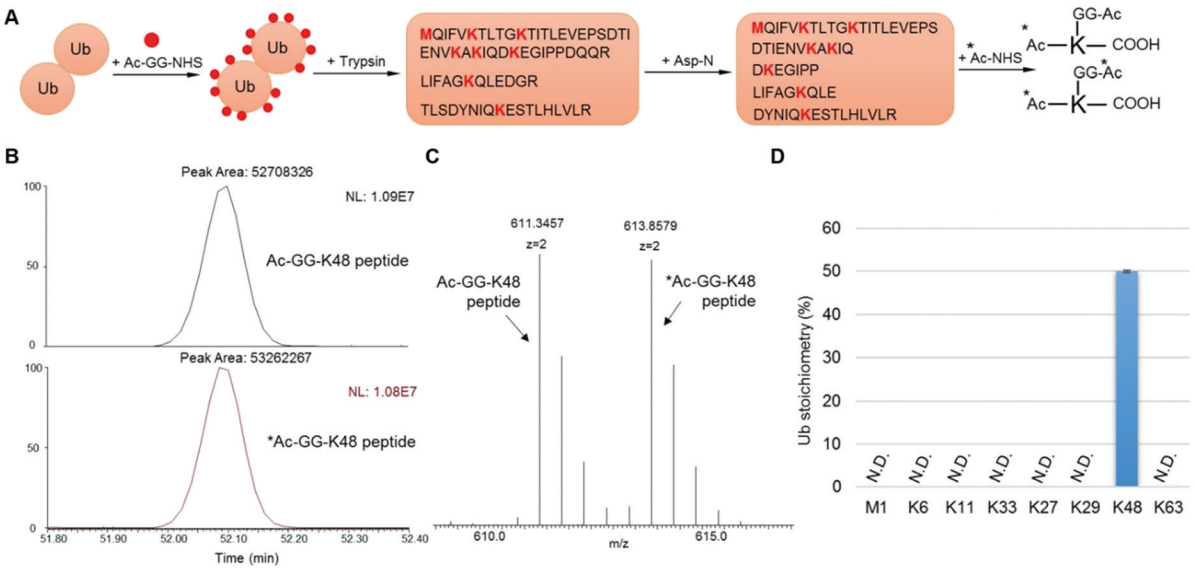

Figure 2. Stoichiometric analysis of the K48-linked di-Ub standard. A) A diagram for quantitative analysis of polyubiquitin linkage stoichiometry using IBAQ-Ub strategy. B) Extracted ion chromatograms of K48 peptides with AcGG, and heavy AcGG labeling. C) A representative mass spectrum to show the precursor ions of K48 peptides with AcGG, and heavy AcGG labeling. D) Stoichiometry quantification of ubiquitination linkages in K48-linked di-Ub standards. *Ac indicates heavy Ac.

workflow independently with three replicates. To determine if our strategy can measure longer polyubiquitin chains, we obtained the commercially available penta-Ub protein with K48 linkage. Analysis of the di-Ub mixtures and the purified penta-Ub standard showed that the experimental measurements of Ub stoichiometry were highly accurate and reproducible when comparing to the expected values with a linear correlation coefficient R^2 of 0.9994 (Figure 3B,C). Our data suggest that IBAQ-Ub workflow is highly sensitive and capable of measuring a wide range of Ub stoichiometries with high accuracy.

To determine if the IBAQ-Ub workflow is suitable for the analysis of a complex protein mixture, we estimated the site-specific labeling efficiency of the strategy using a SILAC-based approach (Figure 4A), where proteins from heavy

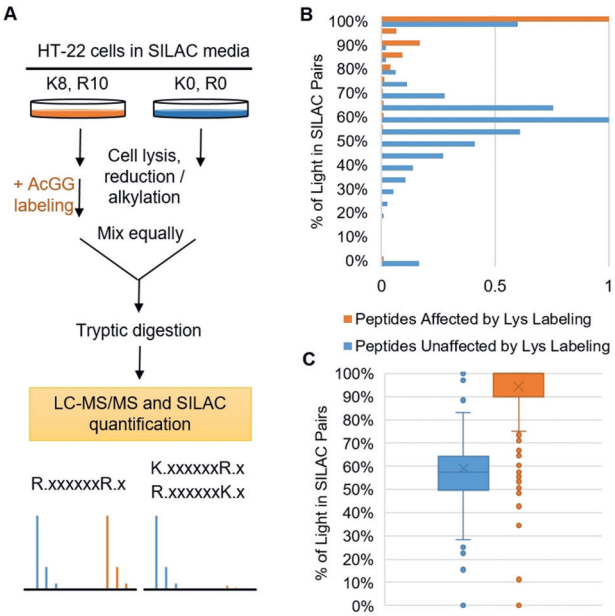

Figure 4. Analysis of the AcGG labeling efficiency in the complex cell lysate using a SILAC-based approach. A) A SILAC-based strategy to measure the global site-specific labeling efficiency of AcGG-NHS in HT22 whole cell lysate. B) Bar graphs and C) Box-Whisker plots comparing the distributions of SILAC ratios between peptides that are theoretically affected by AcGG-NHS labeling on lysines (orange) and peptides that are not affected by AcGG-NHS labeling on lysines (blue).

SILAC-labeled HT22 cells were subjected to AcGG-NHS labeling before mixing with light-labeled proteins for quantitative analysis (Figure 4A). By comparing the SILAC ratios of peptides affected and not affected by AcGG labeling, we found that derivatization with AcGG-NHS achieved more than 90% site-specific labeling efficiency in a complex cell lysate (Figure 4B,C).

We applied the IBAQ-Ub workflow to the site-specific stoichiometric analysis of known Ub sites on acid-extracted histones from 293T cells treated with DMSO (as control) or MG132 (a proteasome inhibitor) (Figure 5A). To ensure the reliability of our analysis, we checked the labeling efficiencies using LC-MS. Our data show that the labeling efficiencies of

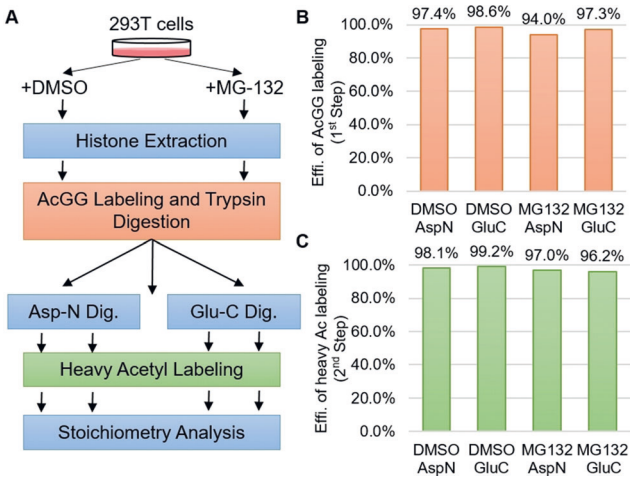

Figure 5. Comparative analysis of ubiquitination stoichiometries in the histone enriched fractions of 293T cells treated with and without the proteasome inhibitor MG132. A) A diagram of the experimental workflow. B) Bar graphs showing the labeling efficiencies of AcGG-NHS at the protein level across different whole cell lysate samples. C) Bar graphs showing the labeling efficiencies of heavy acetyl-NHS at the peptide level across different samples.

both AcGG-NHS and heavy Ac-NHS were very high and consistent across different samples (Figure 5B,C), suggesting that IBAQ-Ub workflow is highly robust for complex protein mixtures, including proteins with a large number of lysines, such as histones.

We then sought to determine the stoichiometries of known histone Ub sites. Histone H2B monoubiquitination has been well studied for its roles in gene expression and is required for subsequent H3K4 and H3K79 trimethylation in transcriptional activation.[1c] It was previously estimated that roughly 1% of histone H2B in eukaryotic cells carries this modification.[8] Using IBAQ-Ub strategy, we were able to measure the absolute stoichiometry of C-terminal histone H2B ubiquitination by measuring the precursor ion intensities of the peptide containing K117/K120 (E.GTKAVTKYTSSK.-). Our analysis showed that histone H2B K117/K120 Ub stoichiometry was 0.91% in 293T cells (Figure 6A), which is in close agreement with previous estimates, and also suggests that C-terminal K117/K120 H2B ubiquitination are the major Ub events on histone H2B. MG132 treatment significantly decreased the abundance of H2B K117/K120 ubiquitination to a level that was below our detection limit (Figure 6B). Such a finding agrees well with previous results obtained using orthogonal approaches such as Western blotting or SILAC-based quantitative proteomics.[3b,c,f,9]

We further measured the K48 polyubiquitin linkage stoichiometry on extracted histones. Our results showed that K48 linkage stoichiometry was only 0.85% in DMSO treated control samples and increased significantly to 2.93% upon proteasome inhibition (Figure 6C,D). This observation agrees well with the knowledge that K48-linked polyubiquitination leads to proteasome-mediated substrate degradation.[1a,6a] Despite this large increase, the absolute stoichiometry change of K48 ubiquitination on extracted histone proteins was very small, suggesting that the major functional role of

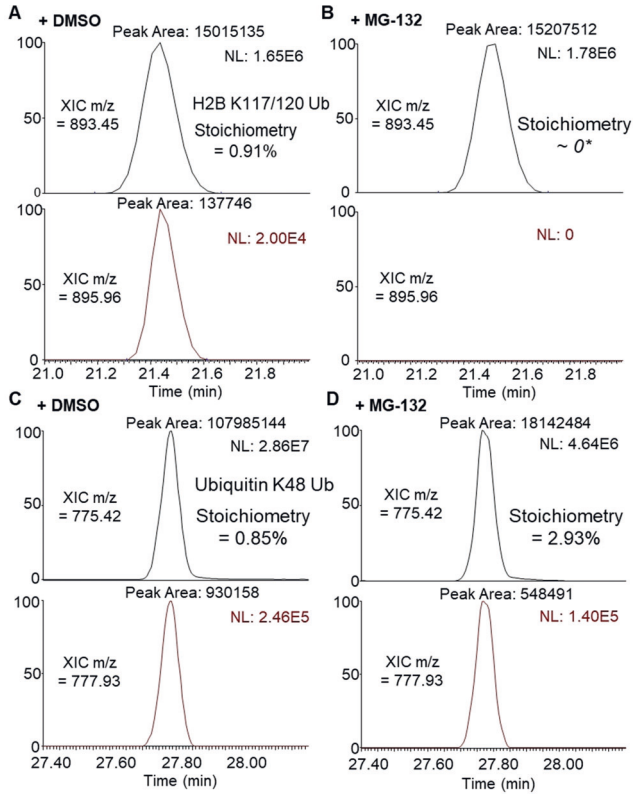

Figure 6. Representative analysis of endogenous ubiquitination stoichiometries. A,B) Stoichiometry analysis of the histone H2B peptide E.GTKAVTKYTSSK.-(containing the known K117 and K120 ubiquitination sites; protease cleavage sites are indicated with a "." and the "-" represents the C-terminus) based on the extracted ion chromatograms of AcGG (upper panels), and heavy AcGG (lower panels) labeled peptides under (A) DMSO-treated and (B) MG132-treated samples. C,D) Stoichiometry analysis of the ubiquitin peptide R.LIFAGKQLEDGR.T (containing the known K48 ubiquitination site; protease cleavage sites are indicated with a ".") based on the extracted ion chromatograms of AcGG (upper panels), and heavy AcGG (lower panels) labeled peptides under (C) DMSO-treated and (D) MG132-treated samples.

histone ubiquitination is unlikely to be the targeting of histones for proteasome degradation.

Finally, peptides with known Ub sites were identified by analysis of histone-enriched chromatin fractions based on the Ub database.[10] Then the Ub stoichiometries of sites that were identified in both DMSO, and MG132 treated samples were compared (Supporting Information, Table S1). Overall, MG132 treatment increased the Ub abundance among 72% of the sites, while 27% of the sites showed decreased ubiquitination. Interestingly, despite the broad increase in the Ub level upon MG132 treatment, the absolute changes of Ub stoichiometries were very small, and the overall Ub fractional abundance remained very low after the proteasome inhibitor treatment. This suggests that the inhibition of proteasome activities may generally have limited impact on the absolute levels of site-specific ubiquitination, which may partially explain the lack of correlation between the changes in Ub levels and corresponding protein abundances upon proteasome inhibition, observed in a recent study.[3f]

To conclude, ubiquitination is a complex post-translational modification, which plays vital roles in cellular physiology and protein homeostasis.[1] Unlike small chemical modifications (e.g. acetylation and phosphorylation), it is very difficult to develop antibodies against specific Ub sites. Therefore, studying the dynamics and physiological significance of site-specific ubiquitination using traditional biochemical strategies is challenging. In this study, a chemical proteomics approach ("IBAQ-Ub") was developed for the stoichiometric quantification of site-specific Ub abundance. The high efficiency and quantification accuracy of the IBAQ-Ub workflow was demonstrated using protein standards and complex protein mixtures. Similar to other MS-based stoichiometry analysis strategies, IBAQ-Ub workflow may not be suitable for the analysis of peptides with multiple types of modifications in high abundance. Furthermore, the application of IBAQ-Ub strategy should be limited to known ubiquitination sites to avoid the interference of other ubiquitin-like proteins (e.g. Nedd8 and ISG15). Overall, the chemical-based IBAQ-Ub workflow proposed herein offers an efficient and generalizable approach to determine the stoichiometric dynamics of ubiquitination at the site-specific level, in either targeted or untargeted applications, and therefore, will be a valuable tool for the functional analysis of this important signaling pathway.

Acknowledgements

We greatly appreciate the Center for Mass Spectrometry and Proteomics and the Masonic Cancer Center at the University of Minnesota for LC-MS access and WuXi Apptec. Co. for the technical support on chemical synthesis. This work was supported by the research start-up fund to Y.C. from the University of Minnesota and National Science Foundation (CHE-1753154 to Y.C.).

Conflict of interest

The authors declare no conflict of interest.

Keywords: IBAQ-Ub · proteasome inhibition · quantitative proteomics · stoichiometry · ubiquitination

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Manuscript received: September 13, 2018
Revised manuscript received: November 7, 2018
Accepted manuscript online: November 15, 2018
Version of record online: ■■ ■■, ■■■■

Communications

Proteomics

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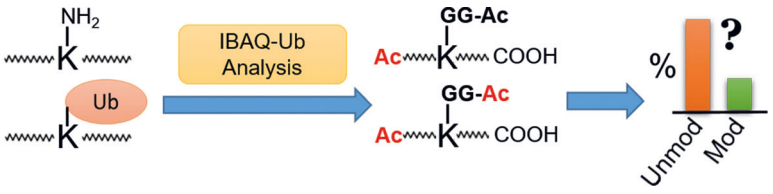
A Quantitative Chemical Proteomics Approach for Site-specific Stoichiometry Analysis of Ubiquitination



How much is ubiquitinated: A quantitative chemical proteomics strategy has been developed to determine the absolute stoichiometry of ubiquitination. The approach is accurate, sensitive, and has a wide dynamic range of quantification at the site-specific level. It can be applied to both targeted and untargeted analysis of complex protein mixture to quantify endogenous ubiquitination stoichiometries.