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Improved CILAT reagents for quantitative proteomics

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ABSTRACT

Improved CILAT reagents have been developed, with which an unprecedented number of protein samples can be measured in high-throughput assays, providing a robust tool for MS-based quantitative proteomics.

MS-based quantitative proteomics is a robust tool for the systematic understanding of biological processes.[1] Many techniques, including both stable isotope-labeled and label-free methods, have been developed to improve the throughput and accuracy of protein quantitation. Stable-isotope labeled methods, such as ICAT[2] and iTRAQ[3], rely on the introduction of stable isotope tags that are chemically identical, but are distinguishable by MS, into different biological samples. On the other hand, label-free quantitation is achieved by spectral count and statistical analysis of unlabeled proteolytic peptides. Despite the availability of a wide choice of methods, there are no perfect ways for quantitative proteomics as each technique has its own benefits and drawbacks.[4] For instance, label-free methods are easy to implement, but suffer from interference from experimental variation and signal noise. Label-based strategies can provide more accurate quantitation, yet it is expensive and sometime impractical to label scarce biological samples. As a result, methods that can overcome limitations of present techniques have always been sought to improve the speed and reliability of quantitative analysis.

A major advantage of labeled over label-free approaches is the ability to process multiple specimens in parallel as demonstrated by commercially available 8-plex iTRAQ[5] and 6-plex TMT reagents.[6] However, as both iTRAQ and TMT reagents react with primary amine that is ubiquitously present in each proteolytic peptide, labeled peptides must be extensively fractionized to reduce their complexity to a level within the analytic capacity of MS. This requirement is usually alleviated when ICAT and other cysteine-targeting reagents are used because labeled cysteine-containing peptides, which represent about 10% of all proteolytic peptides from a proteome, can be enriched by affinity purification before MS analysis.[7] Nevertheless, a drawback of ICAT reagents is that they can only be used for pair-wise measurement.

Previously, we have developed another type of reagents, named CILAT[8], with attempts to combine benefits offered by both ICAT and iTRAQ together. However, our prototype reagents were limited to the comparison of only two samples as well. Here, we demonstrate the second generation CILAT reagents that allow the quantitation of up to 12 samples. These reagents are a set of 12 chemically identical compounds with each containing an isobaric tag comprised of a reporter and a balancer (compound **1**, Fig. 1a). They also share a common thiol-capture group to label cysteine-containing peptides and a cleavable alkyne tag that allows the solid-phase based enrichment of labeled peptides in a catch-and-release fashion. However, these reagents differ from one another by incorporating different isotope atoms such as ^{2}H, ^{13}C and ^{15}N into the different positions of isobaric tags. Consequently, the molecular weight of the reporters spans from 130 to 142 (except 136)[9] and that of the balancers changes concertedly from 167 to 155 to keep the molecular weight of all isobaric tags at constant 297. When they are applied for quantitative assay (Fig. 1b), these reagents can label up to 12 samples, respectively, which are then mixed together and digested with trypsin to generate a large population of peptides. Labeled peptides can then be covalently immobilized on beads coated with azide via a biologically compatible

Abbreviations: CILAT, cleavable isobaric labeled affinity tag; MS, mass spectrometry; ICAT, isotope coded affinity tags; iTRAQ, isobaric tags for relative and absolute quantitation; TMT, tandem mass tags.

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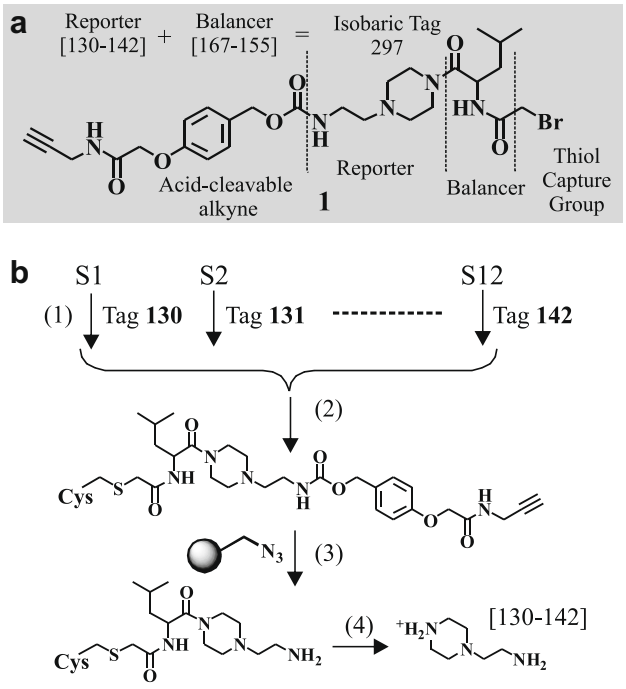
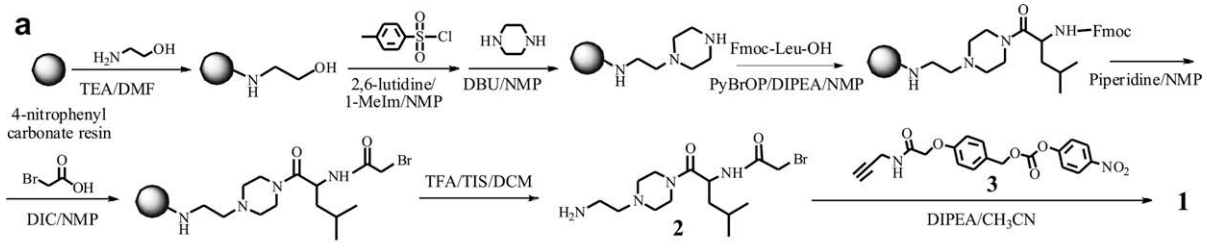


Figure 1. (a) Structure of CILAT reagents (Compound **1**). Each CILAT reagent contains a thiol capture group, an isobaric tag composed of a reporter and a balancer, and an acid-cleavable alkyne group as an enrichment tag. The molecule weight of the reporters in all 12 reagents ranges from 130 to 142, except 136, and the balancer in each reagent changes correspondingly to keep the molecular weight of the isobaric tag at 297. (b) Procedure to use CILAT reagents. (1) Up to 12 protein samples (S1 to S12) can be labeled on cysteine residues with Tag **130** to Tag **142**, respectively. (2) All samples are mixed together and digested with trypsin. (3) The resulting peptide mixtures are treated with solid support beads coated with azide. Only cysteine-containing peptides that are labeled with CILAT reagents can couple to the beads while other unlabeled peptides are washed off. Then, these immobilized peptides are cleaved from beads with acid treatment via the acid-cleavable linker in CILAT reagents. (4) The labeled peptides are analyzed by MS/MS to generate signature ions for quantitation.

[3+2] cycloaddition (click chemistry) between azide and alkyne.[10] After extensive washing to remove any unlabeled peptides, tagged peptides are released from beads by acid treatment and directly delivered for MS/MS analysis. As the name of isobaric tag suggests, the same peptides labeled with different CILAT reagents have the same molecule weight, thus exhibiting a single peak in MS. When this peak is fragmented in MS/MS, the amide bond between the reporters and the balancers breaks apart to yield a series of strong signature ions with *m/z* ranging from 130 to 142 for quantitation. Other larger fragments can be used for *de novo* sequencing of this peptide.

These CILAT reagents were prepared by a simple semi-solid-phase synthesis (Fig. 2). First, ethanolamine was loaded onto 4-nitrophenyl carbonate resin via its primary amine group, and its hydroxyl group was activated by tosylation, which was displaced with piperazine and coupled with Fmoc-leucine subsequently. Next, after Fmoc was removed to generate an amine group to couple with a bromoacetic acid, an intermediate product (compound **2**) was cleaved from beads by acid treatment and was used to react with another intermediate (compound **3**) to obtain compound **1**. By using various isotope labeled building block molecules including ethanolamine, piperazine, Fmoc-leucine, and bromoacetic acid, all 12 CILAT reagents were synthesized similarly.

To examine if the CILAT reagents could indeed be used for 12-plex measurement, bovine catalase, a 60 kD protein containing four cysteines, was tested as an example. 12 samples of this protein with predetermined ratios (2:2:3:3:5:6:7:6:3:3:4:4) were labeled with individual CILAT reagents, respectively, mixed together, precipitated to remove excess labeling reagents, and then digested with trypsin. After the catch-and-release enrichment of labeled peptides, three out of four cysteine-containing peptides (*m/z* = 1392.0, 1575.1 and 2041.4) exhibited strong signals in MS and the last one (calculated *m/z* = 4043.0) was not observed due to its poor ionization efficiency (Fig. 3a). When one of peptides (1392.0) was selected to fragment at low collision energy condition, the sequence of this peptide (H-LC*ENIAGHLK-OH)[11] was easily identified (Fig. 3b). At high collision energy condition, a series of signature ions from 130 to 142 appeared as designed (Fig. 3c). The other two peptides (1575.1 and 2041.4) also performed similarly when they were fragmented. Before these peaks were used for quantitation, however, two issues that might potentially lead to miscalculation of peptide ratios should be addressed: 1) are these signature peaks exclusively generated from the isobaric tags? and 2) how can we calibrate quantitation if CILAT reagents are not 100% isotopically pure? To help answer the first question, the same peptide (1392.0) from bovine catalase solely labeled with Tag **130** was fragmented. A single peak at 130 within the range of 130–142 (Fig. 3d) was observed, implying the signature ions were indeed produced from the reporters, not from the peptide itself.



b

Monomer \ Tag No.	130	131	132	133	134	135	137	138	139	140	141	142
H_2N~OH	0	1	2	3	4	1	3	0	1	2	3	4
HN NH	0	0	0	0	0	4	4	8	8	8	8	8
Fmoc-Leu-OH	10	10	10	7	7	7	3	3	3	0	0	0
Br~O OH	2	1	0	2	1	0	2	1	0	2	1	0

Figure 2. (a) Semi-solid-phase synthesis of CILAT reagents. (b) Isotope-labeled building block monomers used for the synthesis of Tag **130** to Tag **142**. A number (2nd–5th rows) indicates the molecular weight difference (Daltons) between this isotope-labeled monomer and its unlabeled form. The structures of these starting materials are illustrated in Supplementary data.

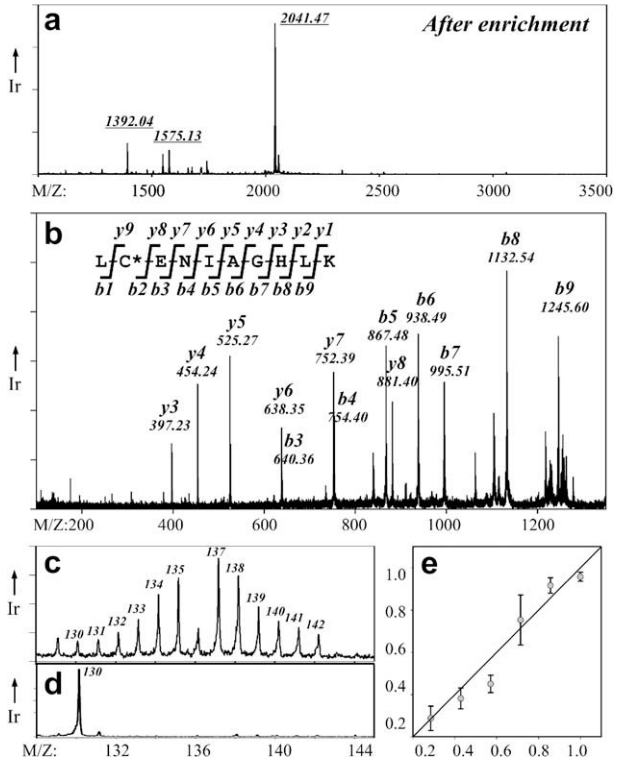


Figure 3. (a) MS spectrum of labeled peptides after enrichment. Cysteine-containing peptides are underlined. Ir = % relative intensity (b) MS/MS spectrum of the precursor ion (1392.04) labeled with all 12 tags at low collision energy condition. The sequence of this peptide and its y/b-series fragments are shown. An asterisk on cystein indicates it is labeled with tags. (c) MS/MS spectrum of the precursor ion (1392.0) labeled with all 12 tags at high collision energy condition. (d) MS/MS spectrum of the precursor ion (1392.0) solely labeled with Tag **130** at high collision energy condition. (e) Linear regression relationship between the measured ratios (*y*-axis) of labeled peptides and their normalized predetermined ratios (*x*-axis). Standard error bars are shown.

Nevertheless, it is noteworthy that the *b1* ion of a peptide containing an N-terminus Glu/Met/His residue is in this range and may introduce errors during quantitation. This problem can be solved by identifying these peptides and removing interfering peaks from quantitation. For example, the m/z of the *b1* ion of a peptide containing an N-terminus Glu residue is 130, so the peak at 130, together with the peak at 131 and 132 for the sake of isotope effect, will be excluded during quantitation and the remaining peaks within the range will be used for a 9-plex measurement, rather than a 12-plex quantitation. The second concern can be addressed by slightly modifying an algorithm already developed for iTRAQ reagents, whose isotope purity also affects the quantitation of peptides in a similar way.[12] Thus, a computer program was developed to calibrate quantitation (see Supplementary data). Finally, when all 36 adjusted ratios of these three cysteine-containing peptides in the 12-plex measurement were plotted versus their normalized predetermined ratios (2/7:3/7:4/7:5/7:6/7:7/7), the linear regression relationship between them indicated the CILAT reagents were indeed able to determine protein ratios in this high-throughput assay (Fig. 3e).

The second generation CILAT reagents would enable us to process an unprecedented number of samples with an additional benefit to reduce biological complexity. In addition, solid-phase enrichment could potentially eliminate problems associated with affinity purification based on conventional biotin–avidin system, such as high background. Although these reagents are designed to complement, rather than replace existing techniques such as ICAT and iTRAQ, the ease and robustness of this technique would make it a preferred choice in many applications.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.022.

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