# Revival of deuterium-labeled reagents for protein quantitation†‡

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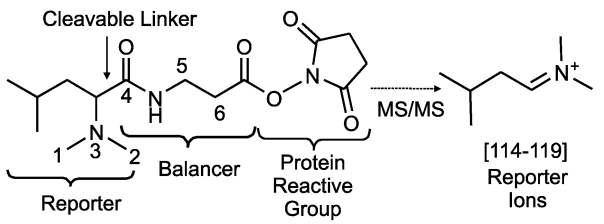
**A group of deuterium-labeled molecules that can be used for MS-based protein quantitation are synthesized, providing a cost-effective replacement of more expensive $^{13}C$- and $^{15}N$-labeled reagents.**

Stable isotope labeling methods, such as ICAT, iTRAQ, and SILAC, are widely used for the quantitative comparison of proteins, providing versatile tools for proteomics research and biomarker discovery.[1] All of these approaches use reagents that are coded with common isotope pairs, including $^{2}H/^{1}H$, $^{13}C/^{12}C$, and $^{15}N/^{14}N$, to label identical peptides or proteins to make them distinguishable by MS.[2] However, $^{2}H$-labeled molecules have recently been phased out and replaced by $^{13}C$- or $^{15}N$-coded tags because they can cause chromatographic shift in reverse phase HPLC and compromise the accuracy of quantitation by LC-MS/MS.[3] Nevertheless, as $^{2}H$-labeled compounds are usually easier and less expensive to synthesize than their $^{13}C$- or $^{15}N$-coded counterparts, it is still of great interest to develop $^{2}H$-based technologies for protein quantitation, provided $^{2}H$-related chromatographic shift could be eliminated. For instance, a study to identify structural features of $^{2}H$-containing molecules that are responsible for their isotope effects indicates that placing $^{2}H$ atoms next to hydrophilic groups and minimizing the number of $^{2}H$ atoms in a molecule can reduce their contribution to isotope effects, providing useful insight into the design of $^{2}H$-based tags that are irresolvable by HPLC.[4]

Here we report the development of a new type of $^{2}H$-labeled reagent, named DiART, which can be used to quantitate up to six protein samples concurrently (Fig. 1). Similar to commercially available iTRAQ[5] and TMT[6] isobaric tags, DiART reagents are a set of six structurally identical molecules consisting of a reporter, a balancer, and a protein reactive group. The mass of the reporter in a set is in the range of 114–119, while the balancer in each molecule is changed accordingly to offset the mass difference in the reporter to keep the total mass of the reporter and the balancer in all reagents the same. These reagents can covalently attach to the free amine group of tryptic peptides and label them for MS/MS analysis. During MS analysis, identical peptides differentially labeled with DiART reagents are indistinguishable from each other, thereby exhibiting a single peak. Once these precursor ions are fragmented in tandem MS, the cleavable linker in the tags is easily broken apart to produce a series of strong reporter ions ranging from 114 to 119, allowing protein quantitation by comparing the intensities of the six reporter ions in the MS/MS spectra. Each DiART reagent also contains the same number of $^{2}H$ atoms (four per molecule) that are placed next to hydrophilic groups, in order to eliminate $^{2}H$-related chromatographic isotope effects. By using $^{2}H$ as a coding isotope, the synthesis of DiART is greatly simplified compared to other $^{13}C$- and $^{15}N$-labeled tags.

The synthesis of DiART reagents began with the preparation of β-alanine benzyl ester **1** (Scheme 1), which was then coupled with Boc–leucine–NHS to yield compound **2**. After the Boc group was removed from compound **2**, the primary amine was treated under standard reductive methylation conditions with formaldehyde and sodium cyanoborohydride, followed by the removal of the benzyl protecting group by hydrogenation to produce compound **3**. The newly exposed carboxylate group in compound **3** was activated by reacting with NHS and DCC to yield the final product, compound **4**. Because all of these reactions have good yields, we were able to synthesize DiART reagents within six steps in an overall yield of 30%–40%, in contrast to 14-step and less than 1% yield of TMT reagents.[7] Furthermore, all of the isotope-labeled starting materials can be purchased at low cost, making this synthetic route a very cost-effective approach to prepare DiART reagents.




| Tag \ Position | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| DiART$^{6}$-114 | $CH_3$ | $CH_3$ | $^{14}N$ | $^{13}C$ | $CD_2$ | $CD_2$ |
| DiART$^{6}$-115 | $CH_3$ | $CH_3$ | $^{15}N$ | $^{12}C$ | $CD_2$ | $CD_2$ |
| DiART$^{6}$-116 | $CH_2D$ | $CH_2D$ | $^{14}N$ | $^{13}C$ | $CD_2$ | $CH_2$ |
| DiART$^{6}$-117 | $CH_2D$ | $CH_2D$ | $^{15}N$ | $^{12}C$ | $CD_2$ | $CH_2$ |
| DiART$^{6}$-118 | $CHD_2$ | $CHD_2$ | $^{14}N$ | $^{13}C$ | $CH_2$ | $CH_2$ |
| DiART$^{6}$-119 | $CHD_2$ | $CHD_2$ | $^{15}N$ | $^{12}C$ | $CH_2$ | $CH_2$ |

**Fig. 1** Structure of DiART tags consisting of a reporter, a balancer, and a protein reactive group. Those positions containing heavy isotope atoms ($^{15}N$, $^{13}C$, $^{2}H$ = D) in each reagent are underlined. When these reagents are fragmented in MS/MS, they generate strong reporter ions ranging from 114 to 119.

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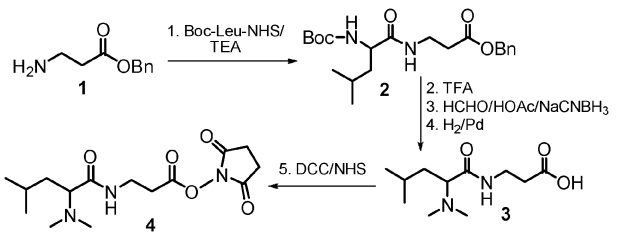
‡ Abbreviations: ICAT, isotope coded affinity tag; iTRAQ, isobaric tag for relative and absolute quantitation; SILAC, stable isotope labeling with amino acids in cell culture; DiART, deuterium isobaric amine reactive tag; TMT, tandem mass tag; MALDI, matrix-assisted laser desorption ionization; ESI, electrospray ionization; TFA, trifluoroacetic acid; NHS, *N*-hydroxysuccinimide; Boc, *tert*-butoxycarbonyl; TEA, triethylamine; DCC, *N*,*N*′-dicyclohexylcarbodiimide.




**Scheme 1** Synthesis of DiART reagents.

To demonstrate that the DiART reagents can eliminate $^{2}$H isotope effects in reverse phase HPLC, we labeled phenylalanine with each of the DiART reagents, mixed them together, and injected them into a HPLC (Fig. 2). We obtained a broad peak and collected three time fractions for MALDI-MS/MS analysis. All fractions generated the reporter ions with identical relative intensities, implying that DiART-labeled phenylalanines co-eluted and were irresolvable by HPLC. Because phenylalanine, as a small single-residue peptide, should be more prone to $^{2}$H-related isotope effects than larger peptides, this result confirmed that DiART-labeled tryptic peptides would not display any chromatographic shift.

To make DiART reagents useful for proteomics applications, they must be compatible with standard MS/MS data analysis programs used for protein identification, such as Mascot[8] and SEQUEST.[9] We decided to test DiART reagents with Mascot because this database search engine is widely used in the proteomics community and its latest release (version 2.2) includes new features for protein quantitation that can be easily modified to fit different needs. Therefore, we prepared a three-protein mixture, including bovine serum albumin, bovine catalase, and chicken ovalbumin. These proteins were digested with trypsin after their cysteine residues were reduced and alkylated under denaturing conditions. The resulting peptides were labeled with six DiART reagents, respectively, and then mixed at a 1 : 1 : 1 : 1 : 1 : 1 ratio. The labeled peptides were then fractionated with a capillary HPLC and analyzed with MALDI-MS/MS (Fig. 3a and b). The peak lists containing their $m/z$ values and intensities extracted from MS data were sent to a custom-configured Mascot server to obtain both the identity and the quantity of peptides simultaneously. For example, a peptide from chicken ovalbumin (GGLEPINFQTAADQAR) was identified with high confidence. At the same time, the relative abundance of this

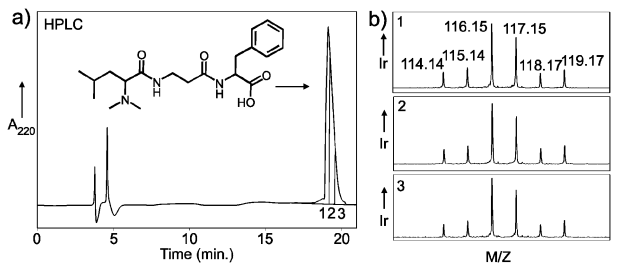



**Fig. 2** (a) HPLC chromatogram of a mixture of phenylalanines that are differentially labeled with six DiART tags. Three time fractions (1–3) were collected for MALDI-MS/MS analysis. $A_{220}$ = absorbance at 220 nm. (b) MS/MS spectra of three fractions. Each spectrum has six reporter ion peaks (114.14, 115.14, 116.15, 117.15, 118.17, 119.17). $I_r$ = relative intensity. $m/z$ = mass-to-charge ratio.

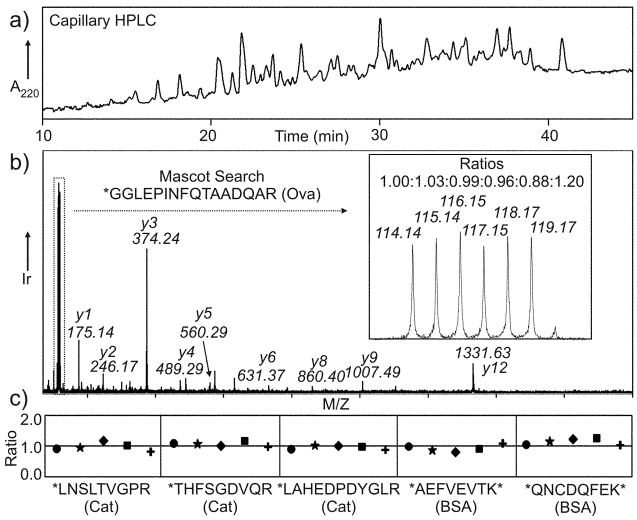



**Fig. 3** (a) Capillary HPLC chromatogram of the tryptic peptide mixture after DiART-labeling. $A_{220}$ = absorbance at 220 nm. (b) MS/MS spectrum of one peptide chosen from chicken ovalbumin (Ova). The asterisk on the N-terminus of the peptide sequence (GGLEPINFQTAADQAR) indicates that it is labeled with DiART tags. This peptide was identified by Mascot with a high-confidence score. Those *b* and *y* fragments are indicated. The inset is the expanded spectrum in the range of 113–121. Relative ratios of six reporter ions were also obtained from Mascot. $I_r$ = relative intensity. $m/z$ = mass-to-charge ratio. (c) Relative ratios (115 : 114 circle, 116 : 114 star, 117 : 114 diamond, 118 : 114 square, 119 : 114 plus) of reporter ions from five other peptides whose sequences and origins are shown. BSA = bovine serum albumin. Cat = bovine catalase.

peptide labeled with different DiART reagents was obtained at a 1.00 : 1.03 : 0.99 : 0.96 : 0.88 : 1.20 ratio after the isotope impurity of DiART reagents was corrected, showing only a small deviation from the known 1 : 1 : 1 : 1 : 1 : 1 ratio.[10] After five other peptides were quantified similarly to obtain enough data that are statistically meaningful, the average coefficient of variation was calculated as 0.11 (Fig. 3c), which was comparable to TMT and iTRAQ tags.[11] In addition, we observed that the cleavable linker in the DiART reagents was exceptionally easy to fragment and the reporter ions were usually predominating peaks in most MS/MS spectra, greatly contributing to the accuracy of quantitation because these signature ion peaks have high signal-to-noise ratios.[12]

In summary, DiART reagents confirm that $^{2}$H-associated chromatographic isotope effects can be eliminated if $^{2}$H-containing molecules are properly designed. The protocol for protein identification and quantitation based on these reagents can be easily incorporated into Mascot, one of the most widely used software packages for MS/MS data analysis. Therefore, DiART reagents offer a cost-effective approach for protein quantitation. Currently, we are testing the performance of DiART-labeled peptides in other types of mass spectrometers, such as ESI-based LTQ-Orbitrap, and will report our results in due course.

## Notes and references

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