Published in final edited form as:

Anal Chem. 2020 June 16; 92(12): 8228–8234. doi:10.1021/acs.analchem.0c00473.

21-plex DiLeu Isobaric Tags for High-throughput Quantitative Proteomics

Dustin C. Frost[†], Yu Feng[†], Lingjun Li^{†,‡,*}

[†] School of Pharmacy, University of Wisconsin–Madison, Madison, Wisconsin 53705, United States

[‡] Department of Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, United States

Abstract

Isobaric tags enable multiplexed quantitative analysis of many biological samples in a single LC-MS/MS experiment. As a cost-effective alternative to expensive commercial isobaric tagging reagents, we developed our own custom *N,N*-dimethyl leucine ‘DiLeu’ isobaric tags for quantitative proteomics. Here, we present a new generation of DiLeu tags that achieves 21-plex quantification in high-resolution HCD MS/MS spectra via distinct reporter ions that differ in mass from each other by a minimum of 3 mDa. The 21-plex set retains the compact tag structure and existing isotopologues of the 12-plex set but includes nine new reporter variants formulated with unique configurations of ¹³C, ¹⁵N, and ²H stable isotopes, each synthesized in-house via a stepwise *N*-monomethylation synthesis strategy using readily available reagents. Thus, multiplexing capacity is expanded significantly while preserving the performance and low cost of the previous implementation. We show that 21-plex DiLeu tags generate strong reporter ions following HCD fragmentation of labeled peptides acquired on Orbitrap platforms at a minimum of 60,000 resolving power (at 400 *m/z*), and we demonstrate accurate 21-plex quantification of labeled K562 human cell line protein digests via single-shot nanoLC-MS/MS analysis on a Q Exactive HF system.

Graphical Abstract

*Corresponding Author: lingjun.li@wisc.edu. Phone: (608) 265-8491. Fax: (608) 262-5345.

Author Contributions

D.F. conceived the research, designed and performed experiments, analyzed data, wrote the manuscript with input from all authors, and prepared figures. Y.F. designed and performed experiments. L.L. conceived, designed and supervised the research.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Labeling efficiency calculation; 21-plex DiLeu reporter ion heavy isotope configurations; Structures of new DiLeu isotopologues and isotopic reagents; Chemical purity verification; Isotopic purity verification; Measured isotopic purities; Isotopic peak interferences to neighboring primary reporter ion signals; Channel mixing ratios for 15:10:5:1 samples.

The authors declare no competing financial interest.

Discovery-based proteomics studies rely on quantifying differences in protein expression across biological states to characterize complex mechanisms and recognize potential diagnostic biomarkers of disease and therapeutic targets. Stable isotope labeling paired with liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis is a well-established approach for simultaneous identification and quantification of multiple samples in parallel. Mass difference methods (e.g. SILAC,¹⁻³ stable isotope dimethyl labeling⁴⁻⁶) impart nominal isotopic mass differences between light- and heavy-labeled peptides, giving rise to unique precursor peaks in MS¹ spectra that allow relative abundances to be determined based on extracted ion chromatogram peak areas. However, mass spectral complexity increases in proportion to the number of quantitative channels, resulting in redundant MS/MS sampling and precursor interferences that reduce proteomic depth and convolute peptide sequence identification,⁷ so these approaches tend to be limited to triplex comparisons. Much greater multiplexing and analytical throughput can be achieved without impacting spectral complexity by employing isobaric labeling (e.g. TMT,⁸⁻¹¹ iTRAQ^{12,13}) that instead bears quantitative information in MS/MS spectra. Isobaric chemical tags are designed such that each variant in the multiplexed set has a unique configuration of isotopes incorporated between their reporter and balance groups but all impart the same nominal mass addition to differentially labeled peptides, yielding single precursor peaks in MS¹ spectra. Upon MS/MS acquisition, fragmentation produces discrete reporter ions in the low m/z region of MS/MS spectra at intensities that reflect peptide abundance in each sample. The high level of multiplexing afforded by isobaric tags is advantageous for the reasons that it facilitates high-throughput analyses of large sample sets with many biological and technical replicates by enabling greater statistical power, decreasing sample preparation and instrument analysis time, and increasing data overlap between samples. By combining many isobaric tag-labeled samples into one sample, the amount of material needed from each individual sample is substantially reduced, and the pooled sample can be analyzed at greater concentration to obtain much higher peptide signal intensities and, consequently, greater proteomic depth. As discovery-based quantitative proteomics research objectives in translational medicine become progressively more ambitious, technologies that facilitate extremely high multiplexing are essential to making these endeavors feasible.

The multiplexing capacity of an isobaric tag is dependent on the number of isotopic permutations allowed by its reporter group and cleavable balance group structures. iTRAQ was initially formulated as a 4-plex set with a compact carbonyl balance group; to support

more isotopic positions and allow formulation as an 8-plex set, a larger balance group linker was added. Likewise, 6-plex TMT features a β -alanine linker as part of its balance group.

The multiplexing capability of isobaric tag strategies can be enhanced by combining isobaric tagging with isotopic mass difference labeling. Such hybrid approaches, previously demonstrated with metabolic SILAC¹⁴ and chemical stable isotope dimethyl labeling approaches,^{15–17} can double or triple the number of samples analyzed in parallel and quantified via reporter ions in a single run, with the tradeoff that mass spectral complexity, redundant sampling, and co-isolation effects¹⁸ are multiplied as well, exacerbating their consequences to proteomic depth and reporter ion quantitative accuracy.

Alternatively, the number of quantitative channels can be increased without affecting spectral complexity by employing high-resolution, accurate-mass MS/MS acquisition and reporter ion isotopologues that differ in mass by just millidaltons. By substituting a ¹³C isotope for a ¹⁵N isotope in the reporter group structure, rather than changing the overall number of isotopes, a relative mass difference of 6.32 mDa is imparted between reporter ions which can be distinguished using an Orbitrap mass analyzer operating at an MS/MS resolving power (RP) of 30K (at 400 *m/z*). This approach was used to generate additional TMT variants to increase multiplexing capacity.^{10,11} The recently redesigned TMTpro tags feature an updated reporter group structure with ¹³C and ¹⁵N variants and a larger tag size (+304 Da), with a double linker balance group to further increase multiplexing over the previous design, and now permit up to 16-plex quantification on Orbitrap platforms.^{19,20} Additionally, MS¹-based quantitative approaches such as NeuCode SILAC^{21–24} and amine-reactive tags,²⁵ mass defect-based pseudoisobaric dimethyl labeling,²⁶ and mass defect-based DiPyrO^{27,28} tags rely on this isotope substitution and high-resolution acquisition of mDa mass differences to achieve multiplexing without the drawbacks of traditional MS¹ mass difference methods.

Employing commercial isobaric tags in large-scale, discovery-based proteomics studies is often a prohibitively expensive endeavor due to the high price of reagent kits. For example, a 16-plex TMTpro kit containing one 5 mg vial of each reagent, suitable for ten experiments at full multiplexing, costs \$8,000 (USD). As a cost-effective alternative, we have developed custom amine-reactive *N,N*-dimethyl leucine ‘DiLeu’ isobaric tags that can be synthesized in-house at high yield, in just three steps using commercially available reagents, at a fraction of the cost and in large bulk amounts required for experiments of any size. DiLeu isobaric tags were initially demonstrated as a 4-plex set utilizing reporter ions at 115, 116, 117, and 118 *m/z*²⁹ and then expanded to an 8-plex set utilizing reporter ions at 115–121 *m/z* enabled by an extended balance group linker to support a greater number of heavy isotopes in the reporter group.³⁰ The multiplexing capacity of the original structure was then increased three-fold from 4-plex to 12-plex by taking advantage of millidalton mass differences resulting from unique configurations of ¹³C, ²H, and ¹⁵N isotopes incorporated into the reporter group to produce eight additional isotopologues that differ in mass by a minimum of ~6 mDa. The resulting set of twelve reporter ions spanning from 115–118 *m/z* can be distinguished in Orbitrap HCD MS/MS spectra acquired at RP 30K. During the development of the 12-plex reagents, it became apparent that it would be possible to further expand the multiplexing capability using reporter variants with even smaller mass differences and

acquisition at an incrementally higher resolving power attainable by existing Orbitrap platforms.

Herein, we describe the design, synthesis, and application of a novel 21-plex set of DiLeu isobaric tags. Building upon the previous implementation, we formulate additional isotopologues with unique configurations of isotopes to yield reporter ions that differ in mass by 3 mDa from the existing variants, and we conceive a new synthetic route that enables their preparation using available isotopic reagents. We determine the Orbitrap resolving power suitable for distinguishing the 21-plex DiLeu reporter ions in HCD MS/MS spectra, and we perform proof-of-principle quantitative proteomics experiments using labeled K562 protein digest samples and nanoLC-MS² analysis on an Orbitrap Q Exactive HF system.

MATERIALS AND METHODS

Chemicals.

Heavy isotopic reagents used for the synthesis of 21-plex DiLeu tags were purchased from Isotec (Miamisburg, OH). ACS grade and Optima LC/MS grade solvents were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Mass spectrometry grade trypsin/Lys C mix and K562 cell line protein extracts were purchased from Promega (Madison, WI).

Protein extract digestion.

K562 human cell line protein extracts (Promega) were digested by trypsin/Lys C mix (Promega). Proteins were reduced in a solution of 5 mM DTT with 7 M urea in 80 mM ammonium bicarbonate pH 8 at 37 °C for 1 hr followed by alkylation of free thiols by addition of 15 mM acrylamide and incubation in the dark for 30 min. The solution was diluted to 1 M urea with 50 mM Tris-HCl pH 8. Proteins were proteolytically digested by addition of trypsin/Lys C mix at a 1:50 enzyme to protein ratio and incubation at 37 °C for 16 hr. The digestion was quenched with TFA to pH < 3, and peptides were desalted using C₁₈ SPE cartridges.

DiLeu synthesis.

DiLeu isobaric tags were synthesized inhouse using commercially available isotopic reagents. The existing isotopologues of the 12-plex were synthesized by a reductive dimethylation scheme as described previously.³¹ The nine additional isotopologues that constitute the 21-plex set were synthesized by a stepwise *N*-monomethylation scheme. Briefly, L-leucine or heavy isotopic L-leucine was protected at the primary amine with benzyl chloroformate (CbzCl) under basic conditions to yield a secondary amine, derivatized with a single methyl group by reaction with heavy isotopic iodomethane and sodium hydride, then concurrently deprotected and derivatized with the second methyl group by reaction with formaldehyde or heavy isotopic formaldehyde under catalytic hydrogenation conditions with Pd/C in H₂ atmosphere.³² The three dimethyl leucine 115 and 116 variants were then subjected to ¹⁸O exchange by incubation with acidified H₂¹⁸O solution (HCl, pH 1) at elevated temperature. Each dimethyl leucine product was purified by flash column

chromatography, dried *in vacuo*, and stored at ambient temperature for later use. Immediately prior to peptide labeling, the active dimethyl leucine triazine esters were produced by reaction with DMTMM and NMM in dry DMF for 30 min at ambient temperature.

21-plex DiLeu labeling.

Peptide samples were dissolved in 60:40 ACN:0.5M triethylammonium bicarbonate buffer pH 8.5, and labeling was performed by addition of activated label in dry DMF at a label:protein digest ratio of 20:1 (w/w) and incubation with vortexing at ambient temperature for 1 hr. The reaction was quenched by addition of hydroxylamine to 0.25% v/v, and labeled peptide samples were combined in known ratios of 1:1 or 15:10:5:1 between DiLeu channels. Pooled samples were cleaned via SCX SPE, desalted via C18 SPE, and dried *in vacuo*.

NanoLC-MS².

Labeled peptide samples were analyzed by nanoLC-MS² using either a Thermo Scientific Orbitrap Elite mass spectrometer interfaced with a Waters nanoAcquity UPLC system or a Thermo Scientific Q Exactive HF mass spectrometer interfaced with a Dionex Ultimate 3000 UPLC system. Samples were dissolved in 3% ACN, 0.1% formic acid in water, and loaded onto a 75 μ m inner diameter microcapillary column fabricated with an integrated emitter tip and packed with 15 cm of BEH C18 particles (1.7 μ m, 130Å, Waters). Mobile phase A was composed of water and 0.1% formic acid. Mobile phase B was composed of ACN and 0.1% formic acid. Separation was performed using a gradient elution of 5% to 35% mobile phase B over 150 min at a flow rate of 300 nL/min. On the Orbitrap Elite, FT-MS survey scans of peptide precursors from 350–1600 m/z were performed in the Orbitrap at RP 120K (at 400 m/z) with an AGC target of 1×10^6 and maximum injection time of 100 ms. The top 15 precursors were then selected for data-dependent HCD FT-MS² analysis at RP 60K with an isolation width of 2.5 Da, a normalized collision energy (NCE) of 27, an AGC target of 5×10^4 , a maximum injection time of 250 ms, and a lower mass limit of 100 m/z . Precursors were subject to dynamic exclusion for 20 s with a 10 ppm tolerance. On the Q Exactive HF, FT-MS survey scans of peptide precursors from 350–1600 m/z were performed in the Orbitrap at RP 120K (at 200 m/z) with an AGC target of 3×10^6 and maximum injection time of 20 ms. The top 12 precursors were then selected for data-dependent HCD FT-MS² analysis at RP 120K with an isolation width of 2.5 Da, a normalized collision energy (NCE) of 27, an AGC target of 5×10^5 , a maximum injection time of 240 ms, and a lower mass limit of 100 m/z . Precursors were subject to dynamic exclusion for 15 s with a 10 ppm tolerance.

Data analysis.

Mass spectra were processed using Proteome Discoverer (PD; version 2.1, Thermo Scientific) to identify and quantify proteins and peptides. Raw files were searched against the UniProt human protein database using Sequest HT. Searches were performed with a precursor mass tolerance of 25 ppm and a fragment mass tolerance of 0.03 Da. Static modifications consisted of propionamide derivatization of cysteine (+71.0359 Da) and DiLeu (+145.12801 Da) on peptide N-termini and lysine. Dynamic modifications consisted

of oxidation of methionine (+15.99492 Da), deamidation of asparagine and glutamine (+0.98402 Da), and acetylation (+42.01057 Da) of protein N-termini. Peptide spectral matches (PSMs) were validated based on q-values to 1% FDR using percolator. Quantification of reporter ions in MS² spectra was performed in PD using an integration tolerance of 20 ppm for the most confident centroid. Only the PSMs that contained all 21 reporter ions were considered. Reporter ion intensities for PSMs were exported to Excel, and isotopic interference correction factors, determined using the measured isotopic abundances for each channel and with respect to the mixing ratios, were applied, and relative abundances were calculated by dividing each channel's intensity by the sum of intensities.

RESULTS AND DISCUSSION

The DiLeu isobaric labeling reagent structure is consistent with the general structure of other isobaric reagents in that it is composed of a reporter group, a balance group, and an amine-reactive group. *N,N*-dimethyl leucine comprises the reporter and balance groups of the isobaric tag in which heavy stable isotopes are incorporated, and a triazine ester aminereactive moiety permits highly selective and efficient modification of peptide N-termini and lysine side chains (Figure 1A). To further expand the multiplexing capacity of DiLeu beyond the previous 12-plex generation, we retained the compact structure and designed nine new isotopologues with unique masses that each differ from the existing variants by 2.92 mDa. Augmenting the 12-plex set with these additional isotopologues affords a 21-plex set comprised of three 115 variants, five 116 variants, six 117 variants, and seven 118 variants (Figure 1B).

Key to the formulation of the novel DiLeu isotopologues with 2.92 mDa mass differences is the incorporation of an odd number of ²H isotopes onto the reporter (Figure S1). Previously, ²H isotopes were added in pairs via reductive dimethylation of leucine; to instead selectively integrate one or three ²H isotopes onto a single methyl group, we devised a stepwise *N*-monomethylation synthesis strategy by which leucine is protected at the primary amine with benzyl chloroformate to form a secondary amine, derivatized with a single *N*-methyl group using heavy isotopic iodomethane, and concurrently deprotected and derivatized with the second *N*-methyl group by reaction with formaldehyde under catalytic hydrogenation conditions (Scheme 1). This approach deliberately preserves the strategic positioning of deuterium atoms proximal to the amine, providing the benefit of minimizing their interaction with reversed-phase stationary phase during chromatographic separation³³ and mitigating significant retention time shifts between deuterated and nondeuterated channels. The new 115 and 116 variants subsequently undergo acid-catalyzed ¹⁸O exchange of the carboxyl group that contains the balance group. The stepwise *N*-monomethylation synthesis strategy allows us to produce each of the new isotopologues in-house using readily available heavy isotopic reagents (L-leucine, formaldehyde, iodomethane, and water-¹⁸O) in just three or four steps (Figure S2), thus retaining the cost advantage of DiLeu with the 21-plex set—the overall cost of labeling for a 21-plex DiLeu quantitative proteomics experiment, given 100 µg of protein digest per channel, is estimated to be approximately \$60. To ensure stability during long-term storage, DiLeu reagents are stored dry in the inactive carboxylic acid form and are activated to the amine-reactive triazine ester form immediately prior to peptide labeling for optimal labeling efficiency.

Resolving 21-plex DiLeu reporter ions in MS/MS spectra requires high-resolution acquisition. Upon HCD fragmentation, DiLeu tags 115a, 115b, and 115c, for example, each incorporating a single ^{15}N , ^{13}C , or ^2H isotope, respectively, generate reporter ions differing in mass by 6.32 mDa ($^{15}\text{N} \rightarrow ^{13}\text{C}$) or 2.92 mDa ($^{13}\text{C} \rightarrow ^2\text{H}$) at 115.12476 m/z , 115.13108 m/z , and 115.13400 m/z (Figure 3A). To differentiate ~6 mDa mass differences between DiLeu reporters, an Orbitrap resolving power of 30K (at 400 m/z , 96 ms transient time) is sufficient.³¹ To empirically demonstrate the resolving power required to differentiate reporter ions with a 2.92 mDa mass difference, we produced reporter analogs of 115b and 115c, mixed them at equal concentration, and acquired HCD FT-MS² spectra on the Orbitrap Elite at RP 15K-240K (Figure 3B). At RP 60K (192 ms transient time), baseline separation of the reporter ion peaks is observed. This resolving power is attainable on all current and previous generation Orbitrap platforms as well as high-resolution quadrupole time-of-flight (QTOF) and Fourier Transform Ion Cyclotron Resonance (FTICR) platforms. For modern Orbitrap platforms that measure resolving power to a 200 m/z reference (e.g. Eclipse, Fusion Lumos, Exploris, Q Exactive HF), RP 90K (192 ms transient time) or greater is suitable for resolving the 2.92 mDa mass difference between DiLeu reporter ions. The increase in resolving power necessary for 21-plex DiLeu, compared to 12-plex DiLeu or TMT, results in longer MS/MS scan times, with the consequence of fewer scans acquired and thus fewer peptide spectral matches.

The order of isotopic incorporation and choice of isotopic reagents used for synthesis were optimized for maximum isotopic purity. Specifically, we found that nearly complete ^2H incorporation (99%) could be more conveniently achieved via *N*-methylation using heavy isotopic iodomethane, whereas *N*-methylation by heavy isotopic formaldehyde under catalytic hydrogenation conditions was susceptible to less complete ^2H incorporation due to the combined isotopic impurities of the solvent ($\text{MeO}^2\text{H}:\text{H}_2\text{O}$) and $^2\text{H}_2$ gas that also participate in the reaction. ^{18}O exchange was performed as the final step to avoid potential back-exchange during other reaction steps.

Following synthesis of each new variant, we verified by direct infusion MS analysis that the stepwise *N*-monomethylation synthesis scheme yielded acceptable chemical purity (Figure S3) and isotopic purity (Figure S4). The isotopic purities of the new isotopologues are congruent with the existing twelve isotopologues; primary reporter ion abundances for all variants measured at 90–94% of the total relative isotopic abundance, with the –1 and +1 Da complement ions accounting for the remaining isotopic abundance (Figure S5). The isotopic interferences between all channels of the complete 21-plex set were also determined (Figure S6 and Table S1). The 21-plex DiLeu reagents were then mixed at 2:1 ratios and acquired by HCD FT-MS² at RP 60K to confirm that all channels were baseline resolved as anticipated (Figure 3C).

21-plex DiLeu reagents were activated and used to label K562 cell line protein extract tryptic digest samples, and the pooled sample was acquired by high-resolution nanoLC-MS² on the Orbitrap Elite using an optimized HCD NCE of 27 (determined previously³⁴). An example HCD FT-MS² spectrum of a peptide identified with high coverage of b- and y-type ions alongside intense reporter ion signals is shown (Figure 2).

We then performed proof-of-principle quantitative proteomics experiments by analyzing 21-plex DiLeu-labeled K562 digest samples via high-resolution nanoLC-MS² acquisition on the Q Exactive HF. The charge state, sequence length, and XCorr value distributions of identified PSMs were compared between an unlabeled sample and a 21-plex DiLeu-labeled sample combined in unity (Figures 4A–C). Notably, identified DiLeu-labeled peptides are more confidently sequenced compared to unlabeled peptides, as the distribution of XCorr values extends substantially further towards higher values. Out of all PSMs, 63.7% were identified with an XCorr ≥ 3.0 in the labeled sample versus 25% in the unlabeled sample, and 37.8% of labeled PSMs had an XCorr ≥ 4.0 versus just 4.5% of unlabeled PSMs. Shorter peptides (6–10 amino acids) were identified in greater numbers owing to the tag itself being a dimethylated amino acid that supplements the length of labeled peptides. A moderate charge state enhancement was observed for labeled peptides compared to unlabeled peptides, as the numbers of labeled peptides identified with charge states 2+ and 3+ were equivalent at 46% and 47%, respectively.

To assess quantitative performance, we analyzed labeled samples mixed at unity and at 15:10:5:1 ratios between channels according to Table S2. Isotopic interference correction factors were applied to the reporter ion intensities and the relative abundances for each channel were plotted for all quantified PSMs (Figures 4D–E). Measured median abundances agreed well with the mixing ratios, with average coefficients of variations of 20.6% and 20.2% for the unity sample and 15:10:5:1 sample, respectively. Greater deviation in precision was observed for a few of the new channels compared to the rest, possibly due to slightly lower chemical purities of the particular heavy isotopic reagents used for syntheses that impact chemical purity of some of the resulting tags.

The overall reporter ion population generated by fragmentation of isobaric tag-labeled peptides remains static at a given collision energy, regardless of multiplexing. As a result, dividing the bulk reporter ion population across a greater number of channels proportionally reduces each channel's abundance.¹⁰ Accordingly, increasing the number of channels from 12 to 21 reduces the individual channels' intensities by 43%. Because the DiLeu tag intrinsically generates particularly intense reporter ions upon collision induced fragmentation at modest collision energies,³⁴ we predicted that the plentiful ion population could be amply divided across more channels without significant risk to the reliability of quantification, making DiLeu especially well-suited for even greater levels of multiplexing. Out of 1,813 proteins and 10,593 PSMs identified in single LC-MS/MS analysis of a 21-plex DiLeu-labeled K562 digest sample (unity mixture), 1,797 proteins (99%) and 10,041 PSMs (95%) were quantified across all channels (Figure 4F). The average reporter ion S/N for PSMs was plotted (Figure 4G) with a median of 527. Though it has been reported that ions differing analyzers at high MS/MS signal intensities,³⁵ the high rate of quantification observed indicates this to be a scarce occurrence in our analyses with moderate sample loads and typical AGC settings specified in the acquisition method. Upon inspection of the data, we did not observe any coalescence of 21-plex DiLeu reporter ion peaks in MS² spectra, even at reporter ion peak intensities exceeding 4×10^7 on the Q Exactive HF and 2×10^6 on the Orbitrap Elite, confirming our notion that the DiLeu tag's high reporter ion intensity would facilitate an increase in the number of quantitative channels.

Distortion of reporter ion abundances due to precursor co-isolation effects in complex samples has been well-characterized for isobaric tag quantification approaches.^{18,36,37} To mitigate this, MS³ acquisition of labeled fragments by synchronous precursor selection (SPS) MS³ on the Orbitrap Fusion and Eclipse platforms is recommended to improve quantitative accuracy with the tradeoff of reduced duty cycle efficiency and decreased proteomic depth.^{38,39} In this work, the known mixing ratio samples contain consistent channel abundances across all precursors, so MS² is sufficient to demonstrate quantitative potential. For unknown complex samples, CID IT-MS² followed by HCD SPS FT-MS³ should be employed for 21-plex DiLeu in a similar manner that we reported previously for 12-plex DiLeu,¹⁶ with consideration that the increase in the number of channels may warrant an increase in the number of MS³ notches or a greater MS³ injection time to yield equivalent reporter ion S/N in MS³ spectra.

CONCLUSION

In summary, we report a third generation of our custom DiLeu isobaric tags that achieves for the first time 21-plex quantification via discrete reporter ions measured in MS/MS spectra. Isotopologues differing in mass by 3 mDa were designed, synthesized in-house by a stepwise *N*-monomethylation synthesis strategy using accessible heavy isotopic reagents, characterized by MS analysis, and supplemented with the existing 6 mDa variants to create the 21-plex DiLeu isobaric reagent set. DiLeu reporter ions were shown to be fully resolved in HCD FT-MS² spectra at RP 60K (at 400 *m/z*), and proof-of-principle nanoLC-MS² analyses of labeled protein digest samples were performed to demonstrate the suitability of the 21-plex DiLeu tags for routine quantitative proteomics experiments on mainstream Orbitrap platforms. Compared to commercially available isobaric tags, DiLeu tags offer much greater multiplexing capacity but at considerably lower cost. As such, we believe that 21-plex DiLeu isobaric tags offer the potential to substantially facilitate the high-throughput analyses and large-scale experiments that are increasingly being conducted in discovery-based quantitative proteomics workflows.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This research was supported in part by NIH grants P41GM108538, R01DK071801, RF1AG052324, S10RR029531, and funding provided by the Office of the Vice Chancellor for Research and Graduate Education at UW-Madison. L.L. acknowledges a Vilas Distinguished Achievement Professorship and Charles Melbourne Johnson Distinguished Chair Professorship with funding provided by the Wisconsin Alumni Research Foundation (WARF) & UW-Madison School of Pharmacy.

REFERENCES

- (1). Chen X; Smith LM; Bradbury EM Site-Specific Mass Tagging with Stable Isotopes in Proteins for Accurate and Efficient Protein Identification. *Anal. Chem* 2000, 72 (6), 1134–1143. 10.1021/ac9911600. [PubMed: 10740850]
- (2). Ong S-E; Blagoev B; Kratchmarova I; Kristensen DB; Steen H; Pandey A; Mann M Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to

- Expression Proteomics. *Mol. Cell. Proteomics* 2002, 1 (5), 376–386. 10.1074/mcp.M200025-MCP200. [PubMed: 12118079]
- (3). Zhu H; Pan S; Gu S; Morton Bradbury E; Chen X Amino Acid Residue Specific Stable Isotope Labeling for Quantitative Proteomics. *Rapid Commun. Mass Spectrom* 2002, 16 (22), 2115–2123. 10.1002/rcm.831. [PubMed: 12415544]
- (4). Hsu JL; Huang SY; Chow NH; Chen SH Stable-Isotope Dimethyl Labeling for Quantitative Proteomics. *Anal. Chem* 2003, 75 (24), 6843–6852. 10.1021/ac0348625. [PubMed: 14670044]
- (5). Ji C; Guo N; Li L Differential Dimethyl Labeling of N-Termini of Peptides after Guanidination for Proteome Analysis. *J. Proteome Res* 2005, 4 (6), 2099–2108. 10.1021/pr050215d. [PubMed: 16335955]
- (6). Boersema PJ; Raijmakers R; Lemeer S; Mohammed S; Heck AJR Multiplex Peptide Stable Isotope Dimethyl Labeling for Quantitative Proteomics. *Nat. Protoc* 2009, 4 (4), 484–494. 10.1038/nprot.2009.21. [PubMed: 19300442]
- (7). Mertins P; Udeshi ND; Clauser KR; Mani D; Patel J; Ong S; Jaffe JD; Carr SA ITRAQ Labeling Is Superior to MTRAQ for Quantitative Global Proteomics and Phosphoproteomics. *Mol. Cell. Proteomics* 2011, 11 (6), M111.014423. 10.1074/mcp.m111.014423.
- (8). Thompson A; Schäfer J; Kuhn K; Kienle S; Schwarz J; Schmidt G; Neumann T; Johnstone R; Mohammed AKA; Hamon C Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by MS/MS. *Anal. Chem* 2003, 75 (8), 1895–1904. 10.1021/ac0262560. [PubMed: 12713048]
- (9). Dayon L; Hainard A; Licker V; Turck N; Kuhn K; Hochstrasser DF; Burkhard PR; Sanchez JC Relative Quantification of Proteins in Human Cerebrospinal Fluids by MS/MS Using 6-Plex Isobaric Tags. *Anal. Chem* 2008, 80 (8), 2921–2931. 10.1021/ac702422x. [PubMed: 18312001]
- (10). McAlister GC; Huttlin EL; Haas W; Ting L; Jedrychowski MP; Rogers JC; Kuhn K; Pike I; Grothe RA; Blethrow JD; et al. Increasing the Multiplexing Capacity of TMTs Using Reporter Ion Isotopologues with Isobaric Masses. *Anal. Chem* 2012, 84 (17), 7469–7478. 10.1021/ac301572t. [PubMed: 22880955]
- (11). Werner T; Becher I; Sweetman G; Doce C; Savitski MM; Bantscheff M High-Resolution Enabled TMT 8-Plexing. *Anal. Chem* 2012, 84 (16), 7188–7194. 10.1021/ac301553x. [PubMed: 22881393]
- (12). Ross PL; Huang YN; Marchese JN; Williamson B; Parker K; Hattan S; Khainovski N; Pillai S; Dey S; Daniels S; et al. Multiplexed Protein Quantitation in *Saccharomyces Cerevisiae* Using Amine-Reactive Isobaric Tagging Reagents. *Mol. Cell. Proteomics* 2004, 3 (12), 1154–1169. 10.1074/mcp.M400129MCP200. [PubMed: 15385600]
- (13). Choe L; D'Ascenzo M; Relkin NR; Pappin D; Ross P; Williamson B; Guertin S; Pribil P; Lee KH 8-Plex Quantitation of Changes in Cerebrospinal Fluid Protein Expression in Subjects Undergoing Intravenous Immunoglobulin Treatment for Alzheimer's Disease. *Proteomics* 2007, 7 (20), 3651–3660. 10.1002/pmic.200700316. [PubMed: 17880003]
- (14). Dephoure N; Gygi SP Hyperplexing: A Method for Higher-Order Multiplexed Quantitative Proteomics Provides a Map of the Dynamic Response to Rapamycin in Yeast. *Sci. Signal* 2012, 5 (217), rs2 10.1126/scisignal.2002548. [PubMed: 22457332]
- (15). Evans AR; Robinson RAS Global Combined Precursor Isotopic Labeling and Isobaric Tagging (CPILLOT) Approach with Selective MS3 Acquisition. *Proteomics* 2013, 13 (22), 3267–3272. 10.1002/pmic.201300198. [PubMed: 24124127]
- (16). Frost DC; Rust CJ; Robinson RAS; Li L Increased N,N-Dimethyl Leucine Isobaric Tag Multiplexing by a Combined Precursor Isotopic Labeling and Isobaric Tagging Approach. *Anal. Chem* 2018, 90 (18), 10664–10669. 10.1021/acs.analchem.8b01301. [PubMed: 30095893]
- (17). Jiang H; Zhang L; Zhang Y; Xie L; Wang Y; Lu H HST-MRM-MS: A Novel High-Sample-Throughput Multiple Reaction Monitoring Mass Spectrometric Method for Multiplex Absolute Quantitation of Hepatocellular Carcinoma Serum Biomarker. *J. Proteome Res* 2019, 18 (1), 469–477. 10.1021/acs.jproteome.8b00790. [PubMed: 30346787]
- (18). Saw YO; Salim M; Noirel J; Evans C; Rehman I; Wright PC ITRAQ Underestimation in Simple and Complex Mixtures: “The Good, the Bad and the Ugly.” *J. Proteome Res* 2009, 8 (11), 5347–5355. 10.1021/pr900634c. [PubMed: 19754192]

- (19). Thompson AH; Woellmer N; Koncarevic S; Selzer S; Böhm G; Legner H; Schmid P; Kienle S; Penning P; Höhle C; et al. TMTpro: Design, Synthesis and Initial Evaluation of a Proline-Based Isobaric 16-Plex Tandem Mass Tag Reagent Set. *Anal. Chem* 2019, *acs.analchem*.9b04474. 10.1021/acs.analchem.9b04474.
- (20). Li J; Vranken J. G. Van; Vaiteš LP; Schweppe DK; Huttlin EL; Etienne C; Nandhikonda P; Viner R; Robitaille AM; Thompson AH; et al. TMTpro Reagents: A Set of Isobaric Labeling Mass Tags Enables Simultaneous Proteome-Wide Measurements across 16 Samples. *Nat. Methods* 2020, 2 (April), 1–6. 10.1038/s41592020-0781-4.
- (21). Hebert AS; Merrill AE; Bailey DJ; Still AJ; Westphall MS; Strieter ER; Pagliarini DJ; Coon JJ Neutron-Encoded Mass Signatures for Multiplexed Proteome Quantification. *Nat. Methods* 2013, 10 (4), 332–334. 10.1038/nmeth.2378. [PubMed: 23435260]
- (22). Rose CM; Merrill AE; Bailey DJ; Hebert AS; Westphall MS; Coon JJ Neutron Encoded Labeling for Peptide Identification. *Anal. Chem* 2013, 85 (10), 5129–5137. 10.1021/ac400476w. [PubMed: 23638792]
- (23). Rhoads TW; Rose CM; Bailey DJ; Riley NM; Molden RC; Nestler AJ; Merrill AE; Smith LM; Hebert AS; Westphall MS; et al. Neutron-Encoded Mass Signatures for Quantitative Top-down Proteomics. *Anal. Chem* 2014, 86 (5), 2314–2319. 10.1021/ac403579s. [PubMed: 24475910]
- (24). Merrill AE; Hebert AS; MacGilvray ME; Rose CM; Bailey DJ; Bradley JC; Wood WW; El Masri M; Westphall MS; Gasch AP; et al. NeuCode Labels for Relative Protein Quantification. *Mol. Cell. Proteomics* 2014, 13 (9), 2503–2512. 10.1074/mcp.M114.040287. [PubMed: 24938287]
- (25). Hebert AS; Merrill AE; Stefely JA; Bailey DJ; Wenger CD; Westphall MS; Pagliarini DJ; Coon JJ Amine-Reactive Neutron-Encoded Labels for Highly Plexed Proteomic Quantitation. *Mol. Cell. Proteomics* 2013, 12 (11), 3360–3369. 10.1074/mcp.m113.032011. [PubMed: 23882030]
- (26). Zhou Y; Shan Y; Wu Q; Zhang S; Zhang L; Zhang Y Mass Defect-Based Pseudo-Isobaric Dimethyl Labeling for Proteome Quantification. *Anal. Chem* 2013, 85 (22), 10658–10663. 10.1021/ac402834w. [PubMed: 24180428]
- (27). Frost DC; Buchberger AR; Li L Mass Defect-Based Dimethyl Pyrimidinyl Ornithine (DiPyrO) Tags for Multiplex Quantitative Proteomics. *Anal. Chem* 2017, 89 (20), 10798–10805. 10.1021/acs.analchem.7b02098. [PubMed: 28795795]
- (28). Chen B; Feng Y; Frost DC; Zhong X; Buchberger AR; Johnson J; Xu M; Kim M; Puccetti D; Diamond C; et al. Quantitative Glycomic Analysis by Mass-Defect-Based Dimethyl Pyrimidinyl Ornithine (DiPyrO) Tags and High-Resolution Mass Spectrometry. *Anal. Chem* 2018, 90 (13), 7817–7823. 10.1021/acs.analchem.8b00548. [PubMed: 29779369]
- (29). Xiang F; Ye H; Chen R; Fu Q; Li LN,N-Dimethyl Leucines as Novel Isobaric Tandem Mass Tags for Quantitative Proteomics and Peptidomics. *Anal. Chem* 2010, 82 (7), 2817–2825. 10.1021/ac902778d. [PubMed: 20218596]
- (30). Frost DC; Greer T; Xiang F; Liang Z; Li L Development and Characterization of Novel 8-Plex DiLeu Isobaric Labels for Quantitative Proteomics and Peptidomics. *Rapid Commun. Mass Spectrom* 2015, 29 (12), 1115–1124. 10.1002/rcm.7201. [PubMed: 25981542]
- (31). Frost DC; Greer T; Li L High-Resolution Enabled 12-Plex DiLeu Isobaric Tags for Quantitative Proteomics. *Anal. Chem* 2015, 87 (3), 1646–1654. 10.1021/ac503276z. [PubMed: 25405479]
- (32). Stodulski M; Mlynarski J Synthesis of N-Alkyl-N-Methyl Amino Acids. Scope and Limitations of Base-Induced N-Alkylation of Cbz-Amino Acids. *Tetrahedron Asymmetry* 2008, 19 (8), 970–975. 10.1016/j.tetasy.2008.03.025.
- (33). Zhang R; Sioma CS; Thompson R. a; Xiong L; Regnier FE. Controlling Deuterium Isotope Effects in Comparative Proteomics. *Anal. Chem* 2002, 74 (15), 3662–3669. [PubMed: 12175151]
- (34). Greer T; Lietz CB; Xiang F; Li L Novel Isotopic N,N-Dimethyl Leucine (IDiLeu) Reagents Enable Absolute Quantification of Peptides and Proteins Using a Standard Curve Approach. *J. Am. Soc. Mass Spectrom* 2014, 26 (1), 107–119. 10.1007/s13361-014-1012-y. [PubMed: 25377360]
- (35). Werner T; Sweetman G; Savitski MF; Mathieson T; Bantscheff M; Savitski MM Ion Coalescence of Neutron Encoded TMT 10-Plex Reporter Ions. *Anal. Chem* 2014, 86 (7), 3594–3601. 10.1021/ac500140s. [PubMed: 24579773]

- (36). Bantscheff M; Boesche M; Eberhard D; Matthieson T; Sweetman G; Kuster B Robust and Sensitive ITRAQ Quantification on an LTQ Orbitrap Mass Spectrometer. *Mol. Cell. Proteomics* 2008, 7 (9), 1702–1713. 10.1074/mcp.m800029-mcp200. [PubMed: 18511480]
- (37). Karp NA; Huber W; Sadowski PG; Charles PD; Hester SV; Lilley KS Addressing Accuracy and Precision Issues in ITRAQ Quantitation. *Mol. Cell. Proteomics* 2010, 9 (9), 1885–1897. 10.1074/mcp.m900628-mcp200. [PubMed: 20382981]
- (38). Ting L; Rad R; Gygi SP; Haas W MS3 Eliminates Ratio Distortion in Isobaric Multiplexed Quantitative Proteomics. *Nat. Methods* 2011, 8 (11), 937–940. 10.1038/nmeth.1714. [PubMed: 21963607]
- (39). McAlister GC; Nusinow DP; Jedrychowski MP; Wühr M; Huttlin EL; Erickson BK; Rad R; Haas W; Gygi SP MultiNotch MS3 Enables Accurate, Sensitive, and Multiplexed Detection of Differential Expression across Cancer Cell Line Proteomes. *Anal. Chem* 2014, 86 (14), 7150–7158. 10.1021/ac502040v. [PubMed: 24927332]

Figure 1.

21-plex DiLeu isobaric tag structure. (A) The 21-plex DiLeu isobaric labeling reagent consists of a reporter group, balance group, and amine-reactive group. Stars indicate positions of isotopic substitution. (B) Stable isotopes (¹³C, ²H, and ¹⁵N) incorporated into the reporter group are massbalanced by stable isotopes (¹³C, ¹⁸O) in the balance group. Unique combinations of isotopes incorporated into the reporter group yield isotopologues that differ in relative mass by 2.92 mDa.

Figure 2.

Resolving 21-plex DiLeu reporter ions. (A) Reporter ion variants 115a, 115b, and 115c contain a single ^{15}N , ^{13}C , or ^2H isotope, respectively, resulting in distinct masses differing by 6.32 mDa ($^{15}\text{N} \rightarrow ^{13}\text{C}$) or 2.92 mDa ($^{13}\text{C} \rightarrow ^2\text{H}$). (B) The 115b and 115c reporter ions ($m = 2.92\text{ mDa}$) were acquired by HCD MS^2 at RP 15–240K on the Orbitrap Elite. (C) The 21-plex reporters were mixed at 1:2 ratios and acquired by HCD FT- MS^2 at RP 60K on the Orbitrap Elite.

Figure 3.

Example MS² spectrum of a 21-plex DiLeu-labeled K562 tryptic peptide acquired by HCD MS² at RP 60K on the Orbitrap Elite.

Figure 4.

21-plex DiLeu proof-of-principle proteomics identification and quantification results. Labeled and unlabeled K562 tryptic digest samples were analyzed via nanoLC-MS² on the Q Exactive HF. The distribution of (A) peptide XCorr values, (B) peptide length, and (C) charge state of PSMs from the labeled sample were plotted against those from the unlabeled sample. Labeled K562 tryptic digest samples prepared in unity ratios and in 15:10:5:1 ratios were analyzed via nanoLC-MS² on the Q Exactive HF. Measured quantitative ratios of PSMs (box & whiskers) are shown for a replicate of (D) the unity ratio sample and (E) the 15:10:5:1 ratio sample. Box plots demarcate the median (line), the 25th & 75th percentile (box), and the 5th and 95th percentile (whiskers). (F) The percentages of proteins and PSMs quantified out of those identified are shown for one acquisition of the unity ratio sample. (G) The average reporter ion signal to noise ratio histogram for quantified PSMs is shown for one acquisition of the unity ratio sample.

Scheme 1.

Synthesis of new DiLeu isotopologues by stepwise *N*-mono-methylation strategy. Example synthesis route for DiLeu 118f is shown.