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High-Resolution Enabled 5-plex Mass Defect-based *N,N*-Dimethyl Leucine Tags for Quantitative Proteomics

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Abstract

Mass defect-based labeling strategy provides high accuracy as an MS¹-centric quantification method, avoiding the ratio compression that affects isobaric label-based reporter ion quantification. We have developed cost-effective 5-plex mass defect *N,N*-dimethyl leucine (mdDiLeu) tags for quantification of various biological samples with increased multiplexing at a given resolving power afforded by the addition of mass difference isotopologues. The combination of mass difference and mass defect produces two labeled peak clusters separated by 5 Da in MS¹ spectra that are detected as five isotopic peaks at high resolution with mass difference of 15, 17, and 18 mDa per tag. Synthesis of each of the 5-plex mdDiLeu tags is accomplished by a single straightforward reaction step, making it accessible to any lab. To demonstrate 5-plex mdDiLeu for quantitative proteomics, we perform proof-of-principle experiments of mdDiLeu-labeled *Saccharomyces cerevisiae* lysate digest on an Orbitrap Fusion Lumos mass spectrometer.

Graphical Abstract

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Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Supplemental methods; 5-plex mdDiLeu reagent syntheses; Example MS spectra of duplex mdDiLeu-labeled peptides; Distribution of detected triplets at RP 240K and 500K; Quantitative performance of new mdDiLeu isotopologues; Identification rate comparison.

The authors declare no competing financial interest.

INTRODUCTION

Stable-isotope labeling coupled with mass spectrometry (MS) is a powerful technique for protein quantification. Chemical stable isotope labels can be categorized as mass-difference or isobaric, where the former is full MS (MS^1)-centric and the latter is tandem MS (MS^2)-centric quantification. Mass-difference labeling approaches, such as SILAC (stable isotope labeling by amino acids in cell culture) and mTRAQ (mass differential tags for relative and absolute quantification) introduce increasing mass additions to precursor ions for each channel, permitting quantification based on the signal intensity or peak area under curve in MS^1 spectra.¹⁻³ Isobaric tags, such as iTRAQ (isobaric tags for relative and absolute quantification) and TMT (tandem mass tags) impart a single mass shift at the MS^1 level but yield discrete reporter ions in MS^2 spectra for relative quantification.⁴⁻⁶ The multi-Dalton spacing of mass-difference labels restricts quantitative capacity due to the increased spectral complexity resulting from the multiple isotopic clusters. Isobaric tags provide up to 12-plex analysis, but the reduced quantitative accuracy from ratio distortion caused by precursor co-isolation confines its utility.^{7,8} Additional MS^3 acquisition can mitigate ratio distortion of reporter ions at the expense of a reduction of identified and quantified peptides.^{9,10} The combination of mass-difference and isobaric labels, demonstrated in HOTMAQ and cPILOT strategies, can increase multiplexing capability in quantitative proteomics.¹¹⁻¹⁴

Coon and coworkers proposed the NeuCode (neutron encoding) method to combine the accuracy of mass difference labeling with the higher multiplexing capacity of isobaric tags by embedding mDa mass defect signatures into isotopologues of lysine.¹⁵ The NeuCode SILAC strategy introduces minimal mass differences to precursor ions that are distinguishable in MS^1 spectra by high-resolution, accurate-mass analysis, permitting multiplexed quantification without increased spectral complexity or ratio distortion from precursor co-isolation. High analytical throughput is then achieved by reducing the mDa mass differences between isotopologues in the multiplex set and increasing MS resolving power (RP) to sufficiently separate multiplex NeuCode-labeled peptide precursor in Orbitrap MS^1 spectra. To maximize acquisition efficiency, a low-resolution (RP 30K) survey scan dictates data-dependent MS^2 sampling followed by a high-resolution MS^1 scan (RP >200K),

acquired in the Orbitrap in parallel with MS² spectra in the ion trap, to discern the embedded NeuCode tags. However, the nature of metabolic labeling limits NeuCode SILAC primarily to cell culture, whereas a chemical tag format offers wider sample flexibility. Additionally, NeuCode SILAC imparts isotopic mass defects onto lysine residues, requiring Lys-C for protein digestion to generate solely quantifiable peptides. In contrast, an amine-reactive chemical tag labels both peptide N-termini and lysine side chains, permitting the use of trypsin, which outperforms Lys-C in terms of the number of identified proteins, or any other enzyme of choice.^{16,17} This offers the added benefit of yielding a greater mDa mass difference between channels for peptides containing lysine that reduces the resolving power necessary for quantitative analysis.

We recently demonstrated cost-effective chemical labeling approaches for NeuCode analysis via custom duplex mass defect-based *N,N*-dimethyl leucine (mdDiLeu) and triplex dimethyl pyrimidinyl ornithine (DiPyrO) tags for quantification of peptides and metabolites on Orbitrap platforms.^{18,19} In this work, we combine mass difference and mass defect approaches to increase mdDiLeu multiplexing to 5-plex using two isotopic clusters of mass defect doublet and triplet precursor peaks for relative quantification at resolving powers available to modern Orbitrap platforms. To accomplish this, the 5-plex mdDiLeu tags were formulated with either four or nine heavy isotopes (¹³C, ²H, ¹⁵N) incorporated into their chemical structures in unique configurations to impart a minimum mass difference of 15 mDa between neighboring channels. Synthesis of each of the 5-plex mdDiLeu tags is achieved affordably in high yield via a single reaction step using commercially available starting materials. To demonstrate the feasibility of this hybrid design, we synthesize new mdDiLeu tag isotopologues, label yeast protein digests, and perform proof-of-principle quantitative proteomic experiments on the Orbitrap Fusion Lumos.

MATERIALS AND METHODS

Chemicals.

Heavy isotopic reagents used for the synthesis of 5-plex mdDiLeu labels 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 spec grade trypsin/Lys C mix and yeast protein extract were purchased from Promega (Madison, WI).

mdDiLeu Tag Synthesis.

Detailed synthesis of 5-plex mdDiLeu is reported in the Supporting Information (Figure S1).²⁰ Briefly, L-Leucine or isotopic L-Leucine and sodium cyanoborohydride (NaBH₃CN) or sodium cyanoborodeuteride (NaBD₃CN) were combined in H₂O or D₂O in an ice-water bath. Formaldehyde or isotopic formaldehyde was added dropwise, and the mixture was stirred for 30 min. The target product was purified by flash column chromatography, dried *in vacuo*, and stored in a desiccator at ambient temperature.

Yeast Protein Extract Digestion.

S. cerevisiae protein extracts were digested by trypsin/Lys C mix according to the manufacturer's protocol and desalted using SepPak C₁₈ cartridges (Waters, Milford, MA). Digested peptides were divided into equal aliquots and dried *in vacuo* prior to mdDiLeu labeling.

Protein Digest Labeling.

mdDiLeu tags were activated by reaction with 0.7x molar ratio amounts of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetra-fluoroborate (DMTMM) and *N*-methylmorpholine (NMM) in anhydrous *N,N*-dimethylformamide (DMF) at room temperature for 45 min. Immediately following activation, yeast protein digest labeling was performed in 60:40 acetonitrile (ACN)/0.5M triethylammonium bicarbonate buffer pH 8.5 by addition of activated mdDiLeu tags in dry DMF at a label to protein digest ratio of 20:1 (w/w). The reaction was vortexed for 1 hr at room temperature and quenched by addition of hydroxylamine to a concentration of 0.25%. 3-plex mdDiLeu-labeled samples were combined in known ratios of 1:1:1 and 1:5:10, and 5-plex mdDiLeu-labeled peptide samples were combined in ratios of 1:1:1:1:1 and 1:5:10:5:1. Pooled samples were cleaned by SCX SPE and desalted by C₁₈ SPE.

NanoLC-MS².

Samples were analyzed by nanoLC-MS/MS using a Dionex Ultimate 3000 UPLC system coupled to a Thermo Scientific Orbitrap Fusion Lumos mass spectrometer. Labeled peptide samples were dried *in vacuo* and dissolved in 3% ACN, 0.1% formic acid in water. Peptides were 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 3% to 35% mobile phase B over 100 min at a flow rate of 300 nL/min. FTMS survey scans of peptide precursors from 350-1500 *m/z* were performed in the Orbitrap at RP 500K for quantification and RP 30K (at 200 *m/z*) for data-dependent acquisition (DDA) with an AGC target of 4×10^5 and maximum injection time of 100 ms. Using a DDA cycle time of 3 s, the most intense precursors were selected by quadrupole isolation for rapid scan high-energy C-trap dissociation (HCD) ITMS² analysis in the LTQ with an isolation width of 1.0 Da, a normalized collision energy (NCE) of 30, an AGC target of 2×10^4 , a maximum injection time of 20 ms, and a scan range of 200-1200 *m/z*. Selected precursors were subject to dynamic exclusion for 45 s with a mass tolerance of 70 mDa to avoid redundant sampling of differentially-labeled peptides.

Data Analysis.

Mass spectra were processed using Proteome Discoverer (PD; version 2.1, Thermo Scientific) to identify proteins and peptides. Raw files were searched against the UniProt *Saccharomyces cerevisiae* complete database using Sequest HT. Searches were performed with a precursor mass tolerance of 45 mDa (mdDL₃₀₁ & mdDL₀₄₀) & 70 mDa (mdDL₈₀₁, mdDL₂₆₁, & mdDL₀₉₀) and a fragment mass tolerance of 0.6 Da. Static modifications

consisted of carbamidomethylation of cysteine residues (+57.02146 Da) and mdDiLeu tags (+145.14047 Da [mdDL₀₄₀] and +150.17186 [mdDL₀₉₀]) on peptide N-termini and lysine (K) residues. Dynamic modifications consisted of oxidation of methionine (+15.995 Da) and acetylation (+42.011 Da) of protein N-termini. Peptide spectral matches (PSMs) were validated based on q-values to 1% FDR using percolator.

Quantification of peptides identified by PD was performed using PyQuant.²¹ Raw files were converted to mzML format using ProteoWizard msconvert for PyQuant processing with the corresponding MSF files from PD containing unfiltered identifications for proteins, peptide sequences, and peptide spectral matches. The label scheme specified mdDL₃₀₁, mdDL₀₄₀, mdDL₈₀₁, mdDL₂₆₁, and mdDL₀₉₀ as tags on peptide N-termini and K residues, and quantification was performed with a precursor mass tolerance of 5 ppm using the sum of intensities of the first two isotopic peaks in the isotopic cluster. PyQuant script arguments can be found in supplemental methods (Supporting Information).

RESULTS AND DISCUSSION

Rationale for 5-plex mdDiLeu.

The general structure of the mdDiLeu tag is composed of an *N,N*-dimethyl leucine mass defect tag and an amine-reactive triazine ester group for selective modification of peptide N-termini and lysine side chains (Figure 1A). The 5-plex mdDiLeu set is comprised of two isotopologues with four heavy isotopes (mdDL₃₀₁ & mdDL₀₄₀; +145 Da) and three new isotopologues with nine heavy isotopes (mdDL₈₀₁, mdDL₂₆₁, & mdDL₀₉₀; +150 Da) incorporated into the tag structure to impart a minimum mass difference of 15.1 mDa per tag between neighboring channels (Figure 1B). Synthesis of each mdDiLeu tag is achieved with just a single, high-yield reductive dimethylation reaction of L-leucine, making it accessible to any lab with minimal chemical synthesis expertise, and all isotopic starting materials are commercially available. The use of ¹⁸O isotopes in the formulation of our previously reported mass defect-based tags can affect quantification accuracy slightly due to a small percentage of ¹⁶O present as an isotopic impurity following activation of ¹⁸O exchanged L-leucine to the amine-reactive triazine ester form. To improve quantitative performance, while also simplifying synthesis and reducing reagent costs, current and future iterations are formulated without ¹⁸O isotopes.

The 5-plex mdDiLeu set employs a hybrid approach that combines mass difference with mass defect labeling to produce a pair of isotopic clusters for each peptide ($m = 5$ Da per tag) that are detected as doublet and triplet mass defect precursor peaks ($m = 15.1$ mDa per tag) upon high-resolution, accurate-mass MS¹ analysis. Alternatively, the new light and heavy mdDL₈₀₁ & mdDL₀₉₀ tags may be used on their own to yield a greater mDa difference ($m = 32.6$ mDa per tag) for duplex analysis at lower resolving power. Using formulas previously described by Coon and co-workers,²² we calculated the theoretical MS¹ resolving powers (at m/z 200) suitable for resolving duplex and 5-plex mdDiLeu-labeled tryptic peptides given their respective minimum mDa mass differences between channels per incorporated tag (Figure 1C). Because the mdDiLeu tag efficiently labels both peptide N-termini and lysine side chains, the measured mass difference between labeled tryptic peptides that contain a C-terminal lysine residue is doubled with the benefit that lower

resolving power is sufficient for quantification compared to peptides labeled with a single tag. The resulting maximum mass difference of 65.2 mDa between channels can be baseline resolved for most labeled peptides at RP 240K (Figure S2). This marks a decrease in the resolving power requirement, compared to the previous iteration of mdDiLeu,¹⁹ such that Orbitrap platforms other than the Fusion are suitable for analysis. A resolving power of 240K is sufficient for quantification of 95% of tryptic peptides labeled with duplex mdDiLeu tags, while a resolving power of 500K is sufficient for quantification of 94% of tryptic peptides labeled with 5-plex mdDiLeu tags. Using MaxQuant to determine detected multiplets of a yeast tryptic digest sample labeled with mdDL₈₀₁, mdDL₂₆₁, and mdDL₀₉₀ tags ($m = 15.1$ mDa per tag), we found that LC-MS/MS acquisition at RP 500K on the Orbitrap Fusion Lumos resulted in 20% more resolved multiplets compared to RP 240K (Figure S3).

To maximize acquisition efficiency and better assign precursor charge states in FTMS¹ spectra,¹⁵ an initial FTMS¹ scan at RP 30K was used to trigger data-dependent ITMS² scans. The mass defect peaks within each isotopic cluster are isolated together for MS² acquisition within a 1.0 Da window. To reveal the mass defect signatures in the MS¹ precursor scan for quantification, a subsequent FTMS¹ scan at RP 500K is utilized (Figure 1D). Using the hybrid Orbitrap Fusion Lumos, rapid scan ITMS² spectra are acquired in the LTQ in parallel with the high-resolution FTMS¹ spectra acquired in the Orbitrap to minimize the impact of the high-resolution scan on the quantity of collected MS² spectra.
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Multiplex mdDiLeu Quantification.

To evaluate the quantitative performance of the three new mdDiLeu isotopologues, we performed proof-of-principle experiments by labeling yeast protein digests with mdDL₈₀₁, mdDL₂₆₁, and mdDL₀₉₀ tags and combining in 1:1:1, 1:5:10, and 10:5:1 ratios between channels. Out of 28300, 24650, and 26770 peptide spectral matches (PSMs) identified in duplicate acquisitions for the 1:1:1, 1:5:10, and 10:5:1 samples, respectively, 90.1%, 68.5%, and 59.8% were quantified. The median ratios of quantified PSMs match the expected mixing ratios within 10%, 6.7%, and 29%, respectively (Figure S4). We observed a trend in which the mdDL₀₉₀ channel is reported at depressed abundance, and this is particularly consequential to quantitative ratios when the mdDL₀₉₀ channel is mixed at low concentration with respect to the other channels. This may be a result of PyQuant's algorithm calculating discrete MS¹ extracted ion chromatogram (XIC) quantification data for every MS² scan and averaging for each peptide. Consequently, quantification results are influenced by retention time shifts caused by differences in deuterium incorporation between tags. Thus, more accurate quantification may likely be reported by an algorithm that operates without consideration of MS² scans. Established software with such MS¹ quantification algorithms are not yet compatible or sufficiently optimized for mass defect-based quantification schemes employing custom tags.

5-plex mdDiLeu-labeled yeast digest samples mixed in unity were acquired in duplicate alongside duplicate unity triplex samples to determine the effect of increasing multiplexing with mass difference upon identification rate. Compared to triplex sample, the 5-plex sample

yielded an average of 13.9% fewer proteins, 14.8% fewer unique peptide sequences, and 11.8% more PSMs (Figure S5). This slight reduction in protein and unique peptide sequence identifications is expected due to redundant sampling between the two mass difference clusters, while the greater number of PSMs is due to increased mass spectral complexity. Targeted MS² acquisition of only one of the peak partners, based on the 5 Da mass difference, would reduce redundant sampling and balance identification rates between 5-plex and triplex labeled samples.

Next, we acquired 5-plex mdDiLeu-labeled yeast digest samples combined at 1:1:1:1:1, 10:5:1:5:10, and 1:5:10:5:1 ratios in triplicate. The average numbers of proteins, peptides, and PSMs identified and quantified across the triplicate samples are summarized in Figure 2A. An average of 47740, 46320, and 44240 PSMs were identified across triplicates for the 1:1:1:1:1, 10:5:1:5:10, and 1:5:10:5:1 samples, respectively. The greater numbers of proteins and peptides identified in the dynamic mixing ratio samples compared to the unity mixing ratio sample is possibly due to the high abundance of a single channel, resulting in a more consistent precursor mass across each peptide's elution profile, impacting dynamic exclusion during acquisition and precursor mass matching database search. Quantification rates for PSMs were 90.5%, 67.7%, and 60.7% for the 1:1:1:1:1, 10:5:1:5:10, and 1:5:10:5:1 samples, respectively, corresponding to 85.7%, 73.6%, and 61.7% of proteins quantified. These rates are equivalent to the triplex mixtures, demonstrating that the increase to 5-plex using mass difference is inconsequential to quantitative performance. The lower rate of quantification for the dynamic mixing ratio samples compared to the unity mixing ratio sample may be due to lost low abundance channel peaks at the beginning and end of peptide elution profiles, which would be exacerbated by the quantification algorithm's reliance on the MS² scans. Also, partial coalescence may result in inconsistent recognition of the low abundance channels of peptides measured at high overall signal intensity. Collectively, the resulting median ratios of quantified PSMs (Figure 2B) agree well with the expected mixing ratios, with the same trend observed for the mdDL₀₉₀ channel; the measured median ratios of all channels are within an average of 8.5%, 5.0%, and 8.5% of the mixing ratios. These quantitative performance metrics demonstrate the feasibility of 5-plex mdDiLeu for quantitative proteomics.

CONCLUSION

We described a novel 5-plex mdDiLeu tagging strategy for multiplexed MS¹-centric quantitative proteomics. Three new mdDiLeu isotopologues were designed, and mass difference and mass defect approaches were combined to enhance multiplexing capacity without the need for increased resolving power. Each of the 5-plex mdDiLeu tags is synthesized by a single dimethylation reaction, ensuring high yield, low cost, and accessibility. Through proof-of-principle quantification experiments, we demonstrated the quantitative performance of three new mdDiLeu isotopologues and combined 5-plex mdDiLeu tags. The modern Orbitrap Fusion Lumos platform affords sufficiently high resolving power of 500K to distinguish the mDa mass differences encoded into the 5-plex mdDiLeu tags, while previous Orbitrap instrumentation is capable of resolving the new light and heavy isotopologues at RP 240K. Future work could endeavor to demonstrate 6-plex quantification ($m = 6$ mDa) without mass difference using MS¹ acquisition at RP 1M on

the Fusion Lumos 1M and apply the mdDiLeu labeling strategy to disease-related PTM quantification studies to discover potential biomarkers and therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

General structure of 5-plex mdDiLeu and resolving power required to quantify mdDiLeu-labeled peptides. (A) The mdDiLeu labeling reagent consists of a dimethyl leucine mass defect tag and an amine-reactive triazine ester group. Stars indicate positions of isotopic substitution. (B) Four or nine heavy stable isotopes (^{13}C , ^2H , ^{15}N) are incorporated onto the mass defect tag in differing configurations to create a 5-plex set with a minimum mass defect of 15.1 mDa. Each tag is notated as mdDL# $^{13}\text{C}\#^2\text{H}\#^{15}\text{N}$. (C) Theoretical calculation predicting the percentages of mdDiLeu-labeled tryptic peptides that are resolved and quantifiable at full width at 10% maximum (FWTM) peak height at Orbitrap resolving powers up to one million (at m/z 200). (D) An MS^1 spectra of a 5-plex mdDiLeu-labeled yeast tryptic peptide sample analyzed on the Orbitrap Fusion Lumos is shown. Acquisition at RP 500K (orange) reveals quantifiable peaks of peptide mdDiLeu-ELDTAQK-mdDiLeu that are concealed at RP 30K (black).

Figure 2.

(A) The number of proteins, unique peptides, and PSMs identified (dashed column) and quantified (solid column) in triplicate 5-plex mdDiLeu-labeled yeast digest samples combined at ratios of 1:1:1:1:1 (red), 10:5:1:5:10 (blue), and 1:5:10:5:1 (violet). (B) Measured quantitative ratios of PSMs for 5-plex mdDiLeu-labeled samples combined at ratios of 1:1:1:1:1, 10:5:1:5:10, and 1:5:10:5:1. Boxplots demonstrate the median (line), the 25th and 75th percentile (interquartile range; box), and 1.5 times the interquartile range (whiskers).