Supporting Information

High-resolution Enabled 12-plex DiLeu Isobaric Tags for Quantitative Proteomics

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Supplemental Methods

Yeast Lysate Enzymatic Digestion. *Saccharomyces cerevisiae* lysate was provided by Promega (Madison, WI). 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 IAA and incubation in the dark for 30 min. The alkylation reaction was quenched with 5 mM DTT, and 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 (Promega, Madison, WI) at a 1:25 enzyme to protein ratio and incubation at 37 °C for 16 hr. The digestion was quenched with TFA to pH < 3, and the peptides were desalted using SepPak C_{18} SPE cartridge (Waters, Milford, MA). Digested peptides were divided into twelve equal aliquots in triplicate, dried *in vacuo*, and dissolved in 60:40 ACN:0.5M TEAB pH 8.5 prior to labeling.

Peptide modification search. To assist in determining appropriate database search parameters in Proteome Discover, preliminary mass spectra from a 12-plex DiLeu labeled yeast lysate sample were processed using PEAKS 7 (Bioinformatics Solutions Inc, Waterloo, ON, Canada). Raw files were searched against UniProt *Saccharomyces cerevisiae* complete database (September, 2013) using PEAKS algorithm with trypsin selected as the enzyme and two missed cleavages allowed. Searches were performed with a precursor mass tolerance of 25 ppm and a fragment mass tolerance of 0.03 Da. PEAKS PTM was selected in order to find peptide modifications.

Isotopic Interference Correction. Twelve BSA tryptic digest samples were labeled with each of the 12-plex DiLeu labels and analyzed independently via LC-MS^2 at a resolving power of 60k. The highest intensity MS^2 spectrum from each of nine equal time windows

across the chromatogram was exported, and the normalized reporter ion intensities of the +1 and -1 isotopic peaks were averaged. Isotopic interference correction factors were determined based on the fractional interfering contributions of +1 and -1 isotopic peaks to the primary reporter ion peak of neighboring channels. Using the method introduced by Shadforth *et al*, intensities corresponding to the interfering +1 and -1 isotopic peaks are added to each raw reporter ion signal while interfering isotopic contributions from neighboring reporter ions are subtracted.[1] For example, the signal observed in the MS^2 spectrum for 116a is: $S_{116a} = y_{116a}I_{116a} + z_{115a}I_{115a} + x_{117a}I_{117a}$. Variables y_n, z_n, and x_n represent the percentages of the true reporter ion intensities, I_n, from the actual reporter ion, y_nI_n and the +1 and -1 interferences, $z_{115a}I_{115a}$ and $x_{117a}I_{117a}$, that contribute to S_n. An equation for each DiLeu label was constructed, and I_n for all 12 labels was determined in terms of S_n using a MathCad solve block (**Figure S-1**; supplemental file "*12-plex DiLeu Isotopic Correction Formulae.pdf*").[2]

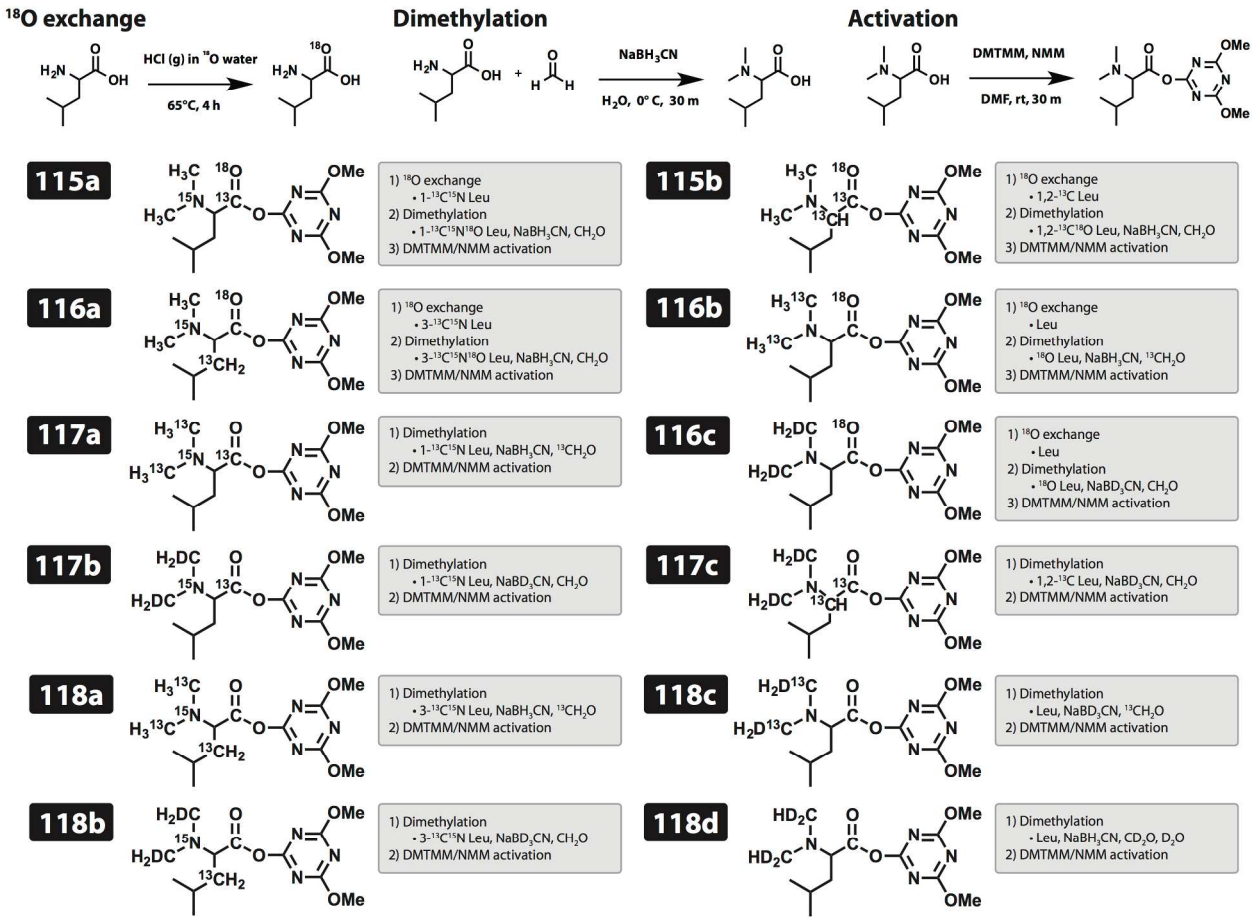
Supplemental Discussion

The triazine ester is more susceptible to hydrolysis than *N*-hydroxysuccinimide (NHS) ester and will lose activity over time in the activation solution due to trace amounts of water produced during the activation reaction, but we find it convenient to store dimethyl leucines in their inactive form and perform a fresh activation prior to labeling. If necessary, active DiLeu can be kept in solution at -20° C with $MgSO_4$ for up to a week.

We used PEAKS 7 to determine modifications for database searching. Using the PEAKS PTM search, we found that approximately 10% of total identified PSMs from DiLeu labeled tryptic digests contained a methylation modification of susceptible residues

(histidine, lysine, arginine, serine, and threonine) or methyl ester modification of C-termini and aspartic acid and glutamic acid residues. We suspect that this is a result of byproducts produced during the DiLeu activation step with DMTMM/NMM. The activation reagent DMTMM Cl can undergo self-immolative degradation in DMF, which leads to the formation of chloromethane (MeCl).[3] While the DMTMM BF_4 form used in this work has been reported to reduce such degradation, it seems to still occur to some degree, and we point to a similar side-product as being the likely cause of the methylation and methyl ester modifications we observe with some peptides. Including methylation and methyl ester modifications (+14.01565 Da) in the database search parameters ensures that these peptides are identified.

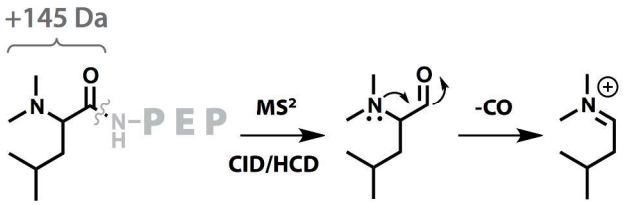
Supplemental Figures



Scheme S-1 • 12-plex DiLeu Reagent Syntheses. DiLeu reagents are synthesized in two or three steps. Labels 115a-b and 116a-c require ^{18}O exchange of leucine or isotopic leucine. Leucine or heavy leucine undergoes reductive dimethylation with formaldehyde or isotopic formaldehyde using sodium cyanoborohydride or sodium cyanoborodeuteride in water or deuterium oxide. Dimethyl leucines require activation with DMTMM and NMM prior to peptide labeling.

Figure S-1 • Isotopic interference correction formulae

See supplemental file *"Figure S-1 - 12-plex DiLeu Isotopic Correction Formulae.pdf"*



Scheme S-2 • DiLeu Reporter Fragmentation. The scheme for generation of DiLeu reporter ion during MS^2 CID/HCD fragmentation is shown. A mass shift of 145 Da is observed for labeled peptides. Upon collision-induced dissociation, the carbonyl balance group is lost as a neutral fragment and the reporter ion is observed in the low mass region of tandem-mass spectra.

	115a	115b	116a	116b	116c	117a	117b	117c	118a	118b	118c	118d
-1(^{1}H)					13.64% 115.1340		5.56% 116.1310	5.47% 116.1373		6.37% 117.13439	5.04% 117.1407	0.41% 117.1465
-1(^{12}C)		0.33% 114.12773	0.78% 115.12477	0.85% 115.1310		0.74% 116.12812		0.28% 116.14028	1.69% 117.13148	0.57% 117.13732	0.92% 117.14364	
-1(^{14}N)	0.50% 114.12773		0.61% 115.13109			0.79% 116.13444	0.60% 116.14028		0.66% 117.13780	0.50% 117.14364		
0	92.36% 115.12476	93.92% 115.13108	93.73% 116.12812	94.29% 116.13444	81.25% 116.14028	94.03% 117.13147	87.86% 117.13731	89.07% 117.14363	94.12% 118.13483	87.68% 118.14067	89.90% 118.14699	94.05% 118.15283
+1(^{13}C)	7.14% 116.12812	5.75% 116.13444	4.88% 117.13148	4.85% 117.13780	5.11% 117.14364	4.44% 118.13483	5.98% 118.14067	5.21% 118.14699	3.53% 119.13819	4.87% 119.14403	4.13% 119.15035	5.54% 119.15619

Table S-1 • Primary and isotopic peak fractions of total reporter ion signal. The total reporter ion intensity for each channel is distributed across the primary reporter ion peak (0) and isotopic peaks (±1). A discrete -1 isotopic peak is detected for each type of stable isotope incorporated into the reporter group (^{13}C, ^{15}N, ^{2}H). Only peaks accounting for greater than 0.1% of the total signal are reported. Values determined at resolving powers of 30k and 60k were in close agreement, so only those from the 60k experiment are reported.

Primary and isotopic fractional signals (%)*

Primary signal + interferences*	115a	115b	116a	116b	116c	117a	117b	117c	118a	118b	118c	118d
115a	92.36%		0.78%									
115b		93.92%	0.61%	0.85%								
116a	7.14%		93.73%			0.74%						
116b		5.75%		94.29%		0.79%						
116c					81.25%		0.60%	0.28%				
117a			4.88%			94.03%			1.69%			
117b							87.86%			0.57%		
117c					5.11%			89.07%		0.50%	0.92%	
118a						4.44%			94.12%			
118b							5.98%			87.68%		
118c								5.21%			89.90%	
118d												94.05%

Table S-2 • Isotopic peak interferences to neighboring primary reporter ion signals. The fractional intensities of +1 and -1 isotopic peaks of each channel (in columns) interfere with primary reporter ion intensities (in rows) and contribute to the measured primary reporter ion signals. * - Interferences determined at a resolving power of 60k (@ 400 m/z).

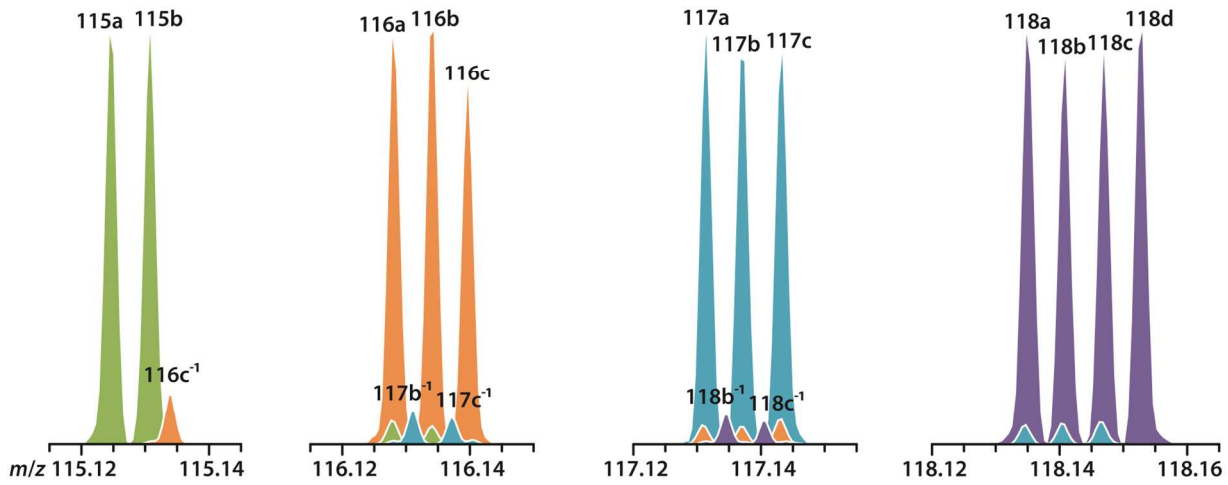


Figure S-2 • Ambiguous isotopic peak interferences at 30k resolving power. At a resolving power of 30k, the -1 isotopic peaks from channels 117b & 117c (blue outline) and 118b & 118c (purple outline) interfere with the primary reporter ion peaks of the surrounding 116 (orange outline) and 117 channels. Because these peaks are unresolved, their ambiguous contributions impact quantitative accuracy to an unknown degree at this resolution.

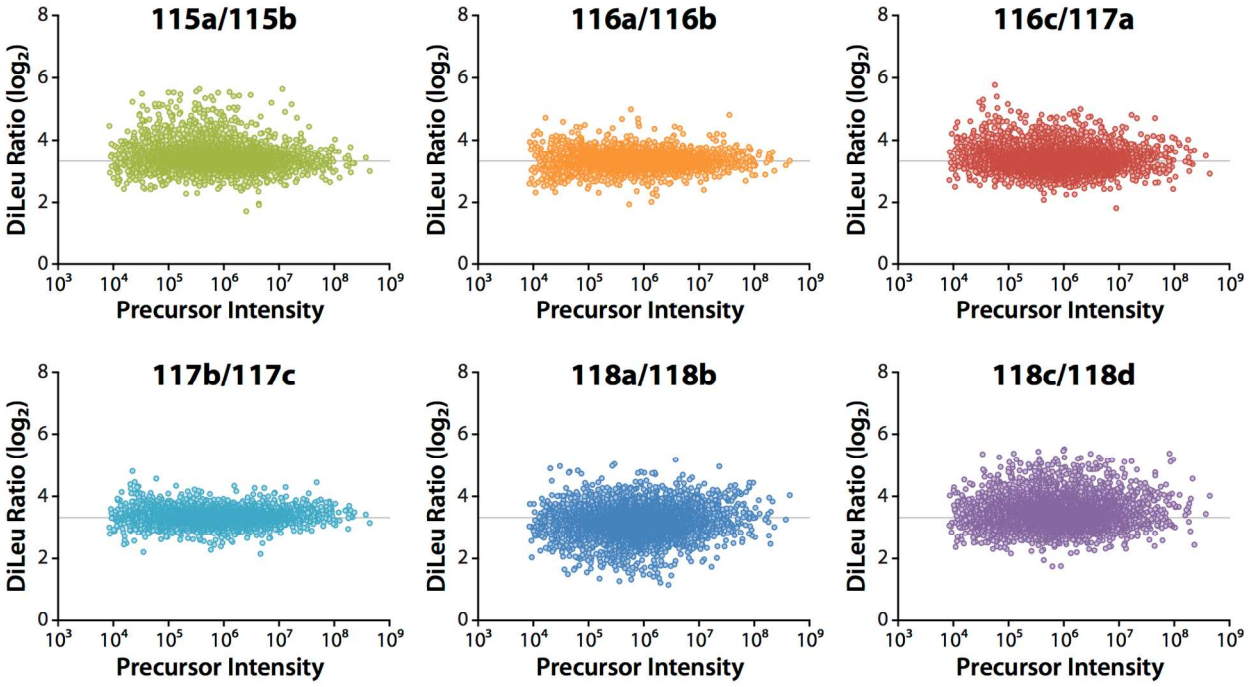


Figure S-3 • Reporter ion ratio variability across internal sample dynamic range. 12-plex DiLeu reporter ion ratios for PSMs (n = 5171) from a labeled yeast lysate digest mixed in 10:1 ratios between neighboring channels were plotted as a function of precursor ion signal intensity. Coefficients of variation across the five orders of magnitude of precursor ion signal intensity were 10.69%, 7.06%, 10.52%, 6.36%, 14.29%, and 13.29% for ratios 115a/115b, 116a/116b, 116c/117a, 117b/117c, 118a/118b, and 118c/118d, respectively.

Supplemental References

(1) Shadforth, I. P.; Dunkley, T. P. J.; Lilley, K. S.; Bessant, C. *BMC Genomics* **2005**, *6*, 145.

(2) Sturm, R. M.; Lietz, C. B.; Li, L. *Rapid Commun. Mass Spectrom.* **2014**, *28*, 1051–1060.

(3) Raw, S. A. *Tetrahedron Lett.* **2009**, *50*, 946–948.