Supplementary Information For

Generation of Multiple Reporter Ions From a Single Isobaric Reagent Increases Multiplexing Capacity for Quantitative Proteomics

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Supplemental Methods

Fmoc Deprotection: 5 mL of a 30% (v/v) solution of piperidine in dimethylformamide (DMF) was added to the reaction vessel, and the resin was agitated for 10 minutes with nitrogen bubbling. Resin was subsequently washed 3 times with 5mL of DMF.

Amide coupling reactions: All reagents were dissolved in N-methyl-2-pyrolidone (NMP). 1.5 mL of a 0.2M solution of carboxylic acid and 1.5 mL of a .195M solution of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) were added to the preactivation vessel, followed by 1.5 mL of a 0.4M solution of Diisopropylethylamine (DIPEA). After mixing via nitrogen bubbling for 30 seconds, the coupling solution was transferred to the reaction vessel containing 150 µmoles of free amine. Resin was agitated for 20 minutes with nitrogen bubbling, washed once with 5mL of DMF, and the coupling cycling was repeated 1 additional time.

Aloc Deprotection: 0.2 equivalents of Tetrakis(triphenylphosphine)palladium(0) were suspended in 5mL of a 37:2:1 mixture of chloroform, acetic acid, and N-methylmorpholine, added to the reaction vessel, and agitated with nitrogen bubbling for 1 hour, followed by a DCM wash. This deprotection reaction was repeated an additional 2 times, and finally the resin was rinsed 5 times each with 5mL of 0.5% DIPEA in DMF and 0.5% sodium diethylthiocarbonate in DMF.

NBS Protection: NBS protection and methylation were done in accordance with the methods published by Biron[29] and Miller[28]. Briefly, 4 eq. of 2-nitrobenzenesulfonyl chloride was dissolved in 5mL of NMP, and 10 eq of 2,4,6-trimethylpyrisine were added. The protection mixture was added to the reaction vessel with nitrogen agitation for 15 minutes, followed by washing with DMF 3 times.

Monomethylation: 4 eq. of methyl 4-nitrobenzenesulfonate in 4mL of DMF were added to the reaction vessel, followed by 4 equivalents of neat 1-methyl-1,3,4,6,7,8-hexahydro-2H-pyrimido[1,2-a]pyrimidine. The mixture was agitated for 30 minutes with nitrogen bubbling, and washed three times with DMF. This reaction was repeated 1 additional time.

NBS Deprotection: A solution of 2-mercaptoethanol (10 eq.) and 1,8-Diazabicycloundec-7-ene (5 eq.) in NMP (5mL) was mixed in the reaction vessel for 5 minutes, drained and rinsed with NMP. The deprotection was repeated 1 additional time.

Deconoic Acid Capping

Following any stage involving acylation of an amino group, any unreacted amine was capped by coupling with 10 molar equivalents of decanoic acid using the standard amino acid coupling protocol.

Resin Cleavage: Resins were agitated for 3 hours in trifluoroacetic acid (TFA) containing 2.5% each of water and triisopropylsilane. Cleavage reactions were filtered into glass vials, and resin was washed 2 times with 2 mL of cleavage buffer. Cleaved solutions were evaporated to dryness under a stream of nitrogen gas, resuspended in a 1 :1 mixture of acetonitrile and water, frozen and lyophilized overnight.

Reductive Methylation: Compounds were dissolved in citrate buffer (pH 5.5), and formaldehyde (4 eq.) was added, followed by dropwise addition of 10eq of sodium cyanoborohydride (5M in 1M NaOH). The reaction was stirred for 2 hours, quenched with 1% TFA to a final pH of 2.5, and stirred for an additional 2 hours. Methylated compounds were purified by solid phase extraction on SEP-PAK C18 cartridges, with washes of 0.1% TFA in water and elution in 0.1% TFA/ 5% Acetonitrile. Eluted compounds were frozen and lyophilized overnight.

NHS-Activation: 10 eq of disuccinimidyl carbonate was dissolved in anhydrous acetonitrile and added to 1 eq of tag free acid. 4 eq of DIPEA was added, and the reaction was allowed to proceed overnight. Crude reaction mixture was directly purified via HPLC-MS (Agilent 12 Series) on a semi-preparative C18 column (Agilent) using mass-triggered fractionation over a linear gradient from 100% buffer A (1% Acetonitrile, 0.1% TFA) to 10% Buffer B (0.1% TFA in Acetonitrile).

Yeast Whole Cell Lysate Digest Preparation: The yeast strain was BY4742 MAT α, derived from S288c. The yeast minimal media was comprised of yeast nitrogenous base with amino acids, ammonium sulfate, and 2% glucose. Three starter cultures were grown in raffinose-containing minimal media overnight from individual colonies. Cultures were grown to reach an optical density (OD) of 0.6 and then harvested.

Yeast cultures were harvested by centrifugation, washed two times with ice cold deionized water, and resuspended at 4°C in a buffer containing 50 mM HEPES pH 8.5, 8 M urea, 75 mM NaCl, protease (complete mini, EDTA-free), and phosphatase (PhosphoStop) inhibitors (Roche). Cells were lysed using the MiniBeadbeater (Biospec) in microcentrifuge tubes at maximum speed for three cycles of 60 sec each, with 3 min pauses between cycles to avoid overheating of the lysates. After centrifugation, lysates were transferred to new tubes. We determined the protein concentration in the lysate using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific).

Proteins were subjected to disulfide reduction with 5 mM tris (2-carboxyethyl)phosphine (TCEP), (room temperature, 25 min) and alkylation with 10 mM iodoacetamide (room temperature, 30 min in the dark). Excess iodoacetamide was quenched with 15 mM dithiotreitol (room temperature, 15 min in the dark). Methanol-chloroform precipitation was performed prior to protease digestion. In brief, four parts neat methanol was added to each sample and vortexed, one part chloroform was added to the sample and vortexed, and three parts water was added to the sample and vortexed. The sample was centrifuged at 4000 RPM for 15 min at room temperature and subsequently washed twice with 100% acetone, prior to air-drying.

Samples were resuspended in 8 M urea, 50 mM HEPES, pH 8.5. The protein extract was then diluted to 1 M urea with 50 mM HEPES pH 8.5 and digested at room temperature for 16 hrs with LysC protease at a 100:1 protein-to-protease ratio. Trypsin was then added at a 100:1 protein-to-protease ratio and the reaction was incubated 6 hrs at 37°C.

Mouse Liver Extract Digest Preparation: Liver tissue was homogenized in 1 ml of lysis buffer (1% SDS, 50 mM Tris (pH 8.8) and Roche complete protease inhibitors). Samples were reduced with 5 mM dithiothreitol for 30 minutes at 37°C followed by alkylation with 15 mM for 30 minutes at room temperature in the dark. The alkylation reaction was quenched by adding 5 mM dithiothreitol for 15 minutes at room temperature in the dark. A 500 uL aliquot was then methanol/chloroform precipitated. Firstly, 2 ml of ice cold methanol was added to the sample and vortexed. Next, 500 uL ice cold chloroform was added and then vortexed. Lastly, 1.5 ml of cold water was added and vortexed. The samples were then centrifuged at 4000 rpm for 20 minutes. The top layer above the protein pellet was removed and additional methanol was added before overtaxing. Samples were centrifuged at 4000 rpm for 10 minutes and the supernatant was removed. The protein pellets were washed a further two times with cold methanol. The samples were allowed to air dry before resuspending the samples in 1 ml of 8 M urea and 50 mM Tris (pH 8.8) before diluting the urea concentration down to ~1.5 M urea with 50 mM Tris. Proteins were quantified using a BCA assay. Protein was then digested using a combination of Lys-C/trypsin at an enzyme-to-protein ratio of 1:100. Firstly, protein was digested overnight with Lys-C followed by 6 hour digestion with trypsin all at 37°C. Samples were then acidified using formic acid to approximately pH 3. Samples were then desalted using a SepPak column. Elutes were then dried using a vacuum centrifuge.

General Formulae For the Maximum Multiplicity of a Given Reagent Architecture

In order to deconvolute reporter ion signal algebraically, the number of resolvable isobaric channels (uniquely resolvable tags per isobaric set) is equal to one less than the sum of the amount of unique primary and secondary reporter ions that can be distinguished. For the tag architecture presented in this manuscript, the number of unique (primary and secondary)

reporter ions that can be distinguished can be calculated algebraically. The generalized formula can be written for any system of primary and secondary reporter ions (not limited to the structures presented in this manuscript). Assuming all 13C and 15N isotopes can be differentially positioned on either of the primary and secondary reporter ions series, where C and N define the maximum number of 13C and 15N that can be labeled (in both ion series), the number of distinguishable reporter ions (Z) is described as

$$Z = (N + 1)(C + 1)*2$$

For isobaric reagents such as TMT, which only generate a single reporter ion series, the maximal number of distinguishable channels Z can be written as:

$$Z = (N + 1)(C + 1)$$

The equations listed in **Figure 2C** assume a limitation to the number of solvable isobaric tags of one less than the number of unique reporter ions that can be generated. This limitation arises from the fact that we consider each reporter ion series in isolation for these calculations, resulting in an underdetermined system when the number of tags is equal to the number of unique reporter ions. Theoretically, one could generate an additional equation relating primary to secondary reporter ion intensity based on an IF statement to separate those peptides with and without highly mobile protons (**Figure 3C**), which would enable a number of tags equal to the number unique reporter. Since the reporter ion splitting ratio, while highly correlated to the presence or absence of highly mobile protons, is a less precise measurement that those between reporter ions within a series (compare R^2 values observed in **Figure 3C**), we did not choose to take advantage of this effect to increase the multiplicity of our reagents. More generally, in cases where the number of equations exceeds the number of encoded isobaric

reagents, we can also minimize the square difference between observed ion pattern c and predicted ion pattern $\hat{\mathbf{c}}$[33]. We vary the ratio of the channel mixing r and minimize ***Diff***.

$$\boldsymbol{min_{\hat{r}}}\ \boldsymbol{Diff}\left(\mathbf{c}, \hat{\mathbf{c}}(\hat{\mathbf{r}})\right) = \boldsymbol{min_{\hat{r}}} \sum_{\mathbf{i}} (\hat{\mathbf{c}}_{\mathbf{i}}(\hat{\mathbf{r}}) - \mathbf{c}_{\mathbf{i}})^{\mathbf{2}} \text{ with } \sum_{\boldsymbol{i}} \hat{\boldsymbol{c}}_{\boldsymbol{i}} = \mathbf{1} \text{ and } \sum_{\boldsymbol{i}} \boldsymbol{c}_{\boldsymbol{i}} = \mathbf{1}$$

Searching for the mixing proportions which minimize the ion envelop similarity function is a standard multivariate optimization problem. ***Diff*** is defined as quadratic similarity function. We therefore obtain an instance of convex optimization and can solve the optimization problem with a simple local search solver as implemented by the fmincon function in MATLAB.

CMT Vs TMT Measurement Precision

Contribution to reporter ion intensity of unrelated peptide side-chain fragments that may be coincidentally isobaric with any of the 7 CMT sixplex reporter ions can be estimated by searching for these ions in TMT labeled samples (**Figure S8**). While we do in fact observe CMT RI signal in such samples, this is predominantly limited to the CMT secondary RI region (**Figure S10**). Furthermore, the two most frequently interfered with signals (nominal mass 126 + 128) correspond to the secondary RIs used to calculate YWCL mixing ratios in **Figure 3A**. While primary RI based measurement precision is indeed slightly reduced compared to values obtained from secondary RI, this difference in precision is small when compared to the difference in precision observed between CMT and TMT measurements using the even the most interfered with reporter ions.

To evaluate reporter ion fragmentation variability in a controlled environment, a 1:1 mixture of synthetic peptides was labelled with either CMT or TMT, and peptides were repeatedly fragmented throughout their elution profiles. When considering only those measurements made under similar signal/noise distributions, we found no significant decrease in CMT measurement precision when compared to TMT (**Figure S11**). This result suggests that reporter ion fragmentation does not introduce inherent additional variability to CMT quantitative measurements. We postulate therefore that increased CMT measurement

variability likely arises from minor impurities insufficiently removed by our synthesis and purification scheme. While LC-MS analysis of our purified reagents does not indicate a significant level of impurities (**Figure S12**), we do occasionally observe spectra corresponding to contaminating NHS-activated compounds when performing unbiased searches[1] for modified peptides (**Figure S13**).

1) Chick, J. M.; Kolippakkam, D.; Nusinow, D. P.; Zhai, B.; Rad, R.; Huttlin, E. L.; Gygi, S. P. *Nat. Biotech.* **2015**, *3* (12), 1154–1169.

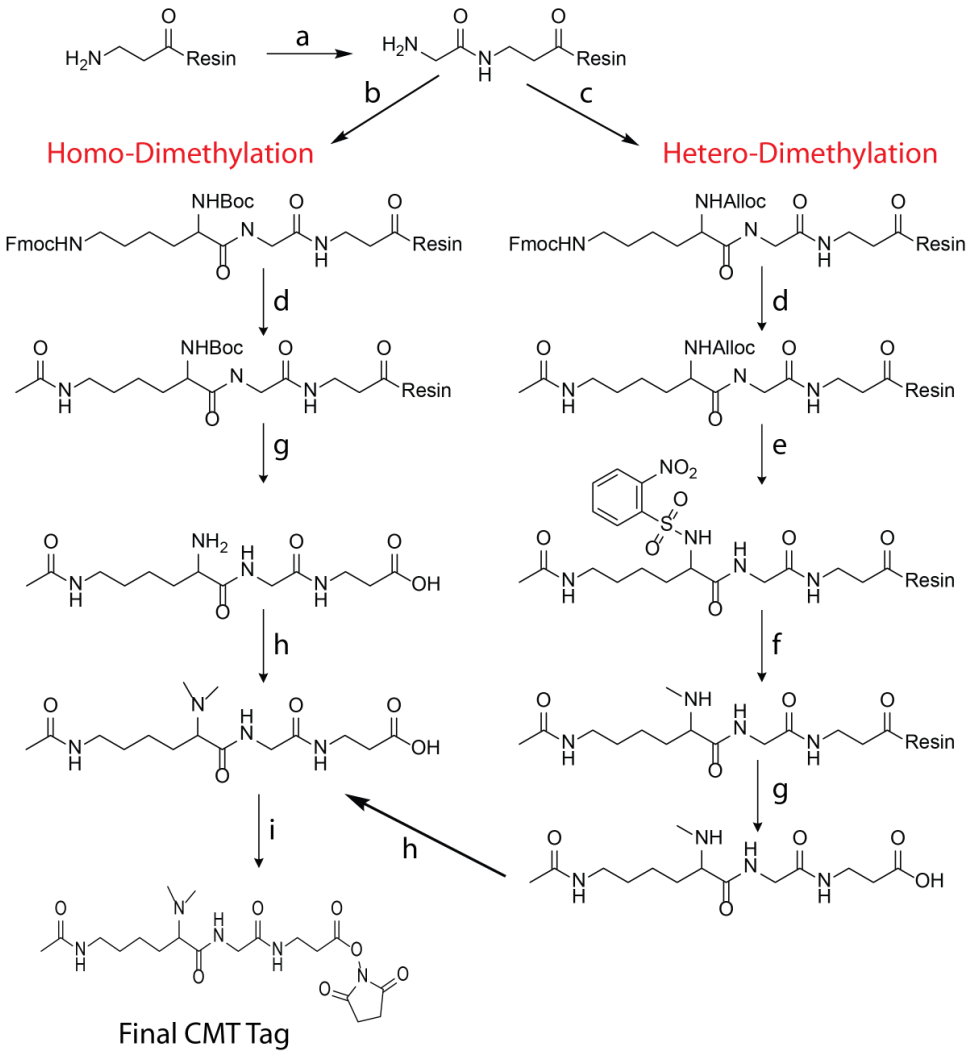


Figure S1: *Synthesis of CMT Isobaric Tags*

A) (i) Fmoc-bAla-OH (2 eq), HATU (2 eq), DIPEA (4eq) (ii) Piperidine:DMF 3:7 B) (i) Boc-Lys(Fmoc)-OH (2 eq), HATU (2 eq), DIPEA (4eq) (2) Piperidine:DMF 3:7 C) (i) Alloc-Lys(Fmoc)-OH (2 eq), HATU (2 eq), DIPEA (4eq) (2) Piperidine:DMF 3:7 D) (i) AcOH (2 eq), HATU (2 eq), DIPEA (4eq) E) (i) tetrakis(thriphenylphosphine)palladium(0) (0.5 eq), CHCl3:AcOH:NMM 37:2:1 (ii) 2-nitrophenylsulfonyl chloride (4 Eq), 2,4,6-trimethylpyridine (10 Eq) F) (i) 1-methyl-1,3,4,6,7,8-hexahydro-2H-pyramido[1,2-a]pyrimidine (4 Eq), methyl-4-nitrobenzoate (4 Eq) (iii) 2-mercaptoethanol (5 Eq), 1,8-diazabicycloundec-7-ene (10 Eq) G) Trifluoroacetic acid:water:triisopropylsilane 95:2.5:2.5 H) Formaldehyde (4 Eq), NaCNBH3 (10 Eq) i) N,N'-Disuccinimyidyl carbonate, DIPEA, ACN

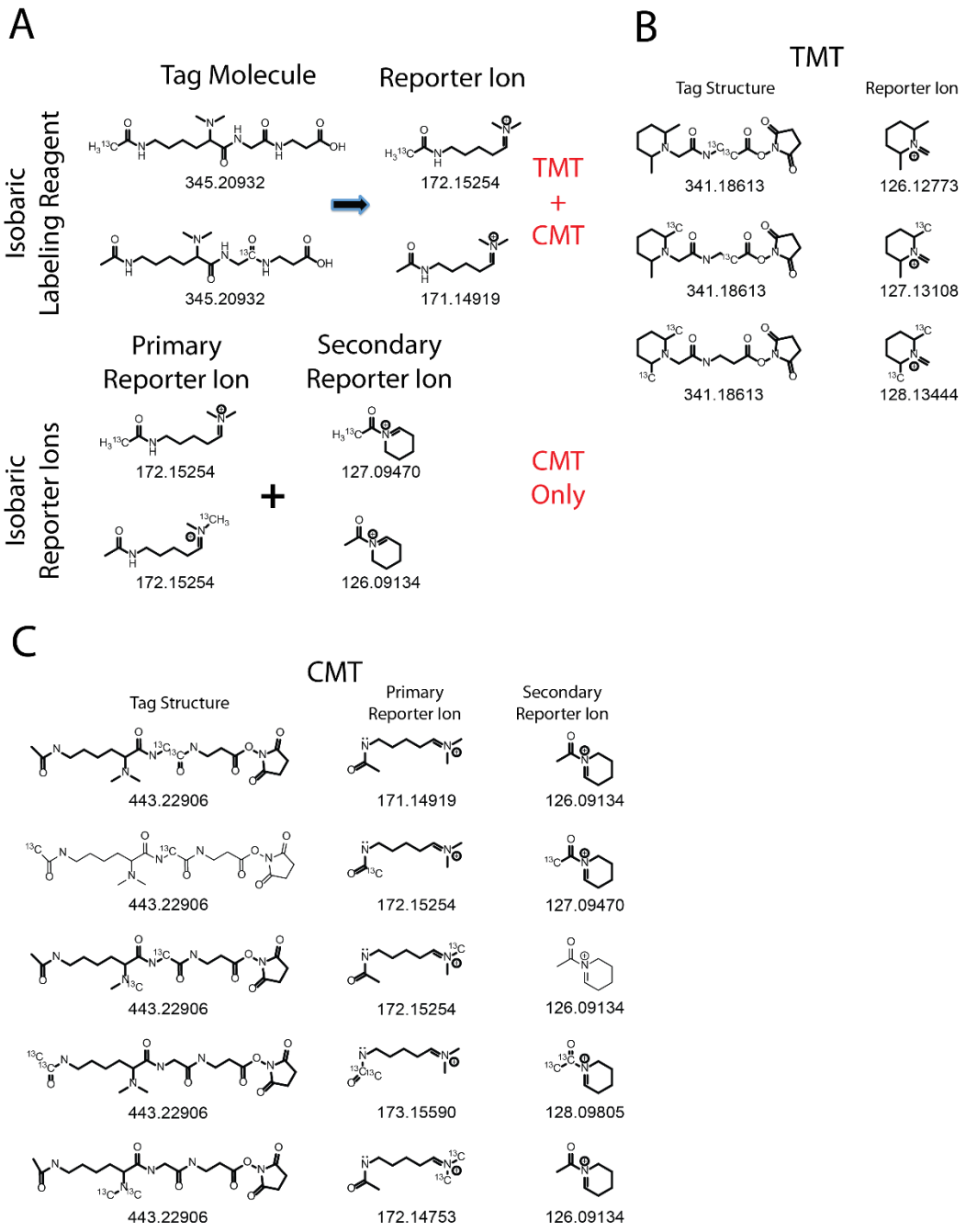


Figure S2: *Unique Characteristics of CMT*

A) The CMT strategy reported here is unique in that both the labeling reagent, and the primary reporter ion can exist in multiple isobaric forms. This allows for multiple distinct labeling reagents to be made with the same number of isotopes on the mass balance region of the tag.

B) Using a total of 2 heavy isotopes per tag, 3 unique isobaric labels are possible with the TMT approach.

C) Also using just 2 heavy isotopes per reagent, the CMT architecture allows for 5 distinct isobaric labels to be designed.

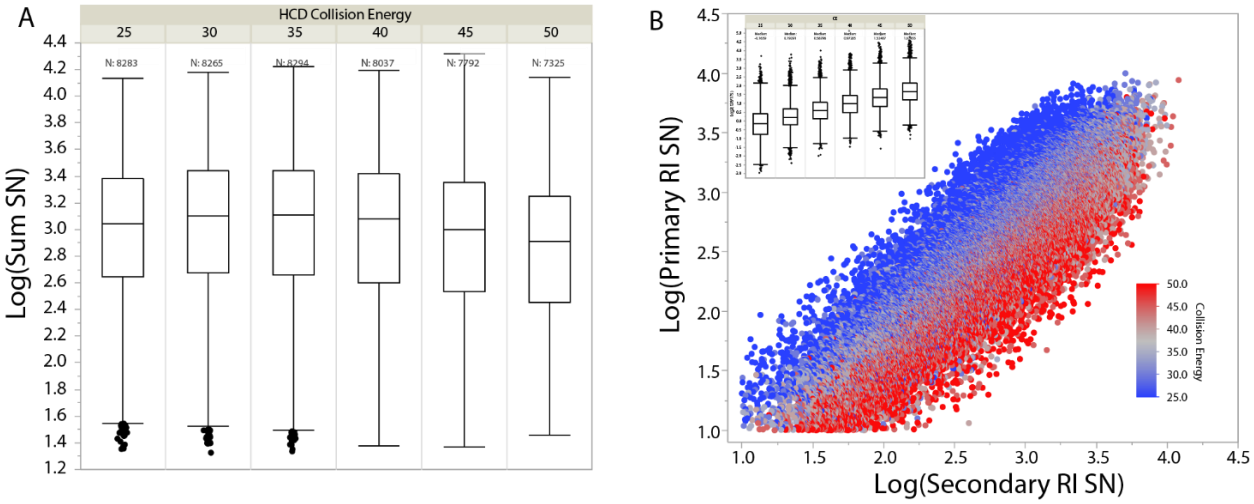


Figure S3: *CMT Fragmentation Energy Optimization*

A) Both Median sum CMT reporter ion intensity (sum of primary and secondary reporter ions), and number of spectra containing a combined signal/noise greater than 20, peak at an HCD collision energy of 35.

B) Secondary reporter ion signal intensity increases with increasing HCD collision energy. Inset: Log2 ratio of secondary/primary reporter ion intensity. Approximately equal proportions of the two ion series are obtained at a collision energy of 30.

Table S1

	TMT (Thermo)						*CMT*					
	126	**127**	**128**	**129**	**130**	**131**	**A**	**B**	**C**	**D**	**E**	**F**
Total Peptides	5559	5114	5422	5419	5248	5328	5156	5380	5977	5540	5413	5521
Unique Peptides	3992	3725	3867	3932	3804	3885	3536	3632	4046	3685	3645	3628

Peptide identification rates obtained by nano-lc-ms on a q-Exactive (Thermo) instrument of a yeast whole cell lysate (YWCL) tryptic digest. Following a search using the SEQUEST algorithm with a database including labeled and unlabeled peptides, both systems were found to essentially quantitatively label all peptides for each of the reagents comprising their respective sixplex tag sets.

A

	Mixing Ratio 1:1			Mixing Ratio 10:1		
	CMT Secondary	CMT Primary	TMT	CMT Secondary	CMT Primary	TMT
Mean	1.16	1.16	1.00	10.99	10.95	10.55
Median	1.16	1.16	1.00	11.00	10.97	10.49
CV	10.21	9.83	4.87	12.43	11.38	6.87

B

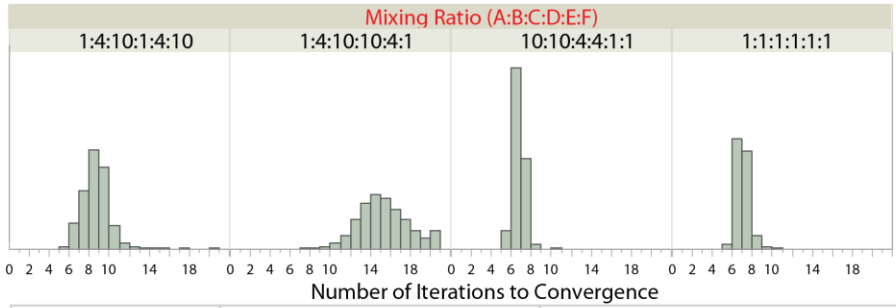
CMT

Reagent	Statistics	Mixing Ratio 1:4:10:1:4:10	1:4:10:10:4:1	10:10:4:4:1:1	1:1:1:1:1:1
A	*Mean*	1.05	1.16	9.60	0.90
	CV	16.03	21.25	7.64	6.90
B	*Mean*	4.03	3.78	9.93	1.02
	CV	10.85	7.70	5.81	9.24
C	*Mean*	9.68	9.71	4.11	1.04
	CV	4.72	4.66	7.02	9.16
D	*Mean*	1.20	10.02	4.14	1.03
	CV	36.55	5.41	10.61	10.17
E	*Mean*	4.00	4.10	1.14	0.98
	CV	6.94	6.16	31.51	7.01
F	*Mean*	10.03	1.26	1.08	1.03
	CV	4.02	23.64	12.35	6.81

TMT

Reagent	Statistics	Mixing Ratio 1:4:10:1:4:10	1:1:1:1:1:1
126	*Mean*	0.83	0.92
	CV	15.05	5.75
127	*Mean*	3.56	0.96
	CV	5.19	4.61
128	*Mean*	10.73	0.95
	CV	2.89	4.86
129	*Mean*	0.74	1.03
	CV	17.17	4.67
130	*Mean*	3.76	1.07
	CV	8.10	6.36
131	*Mean*	10.39	1.07
	CV	3.40	5.91

C



D

Mixing Ratio	*Secondary Reporter Ion*			*Primary Reporter Ion*			
	126	**127**	**128**	**172**	**173**	**174**	**175**
1:4:10:1:4:10	4	14	12	10	14	5	1
1:4:10:10:4:1	4	5	21	1	14	14	1
10:10:4:4:1:1	1	11	18	1	5	14	10
1:1:1:1:1:1	1	2	3	1	2	2	1

Figure S4: *Summary Statistics For CMT and TMT Duplex and Sixplex YWCL Mixing Experiments*

A) Summary table reporting the mean, median and coefficient of variation of CMT and TMT mixing ratios determined from CMT and TMT duplex experiments (**Figure 3A**) with input mixing ratio of either 1:1 or 10:1.

B) Summary table reporting the mean, median and coefficient of variation of CMT and TMT mixing ratios determined from CMT and TMT sixplex experiments (**Figure 3B**) with input mixing ratios indicated in the order A:B:C:D:E:F (CMT) and 126:127:128:129:130:131

C) Histograms of iteration count to isotopic envelope correction convergence for the experiments in **Figure 3B**

D) Theoretical (de-isotoped) reporter ion distributions for the mixing ratios used in the CMT experiments of **Figure 3B**

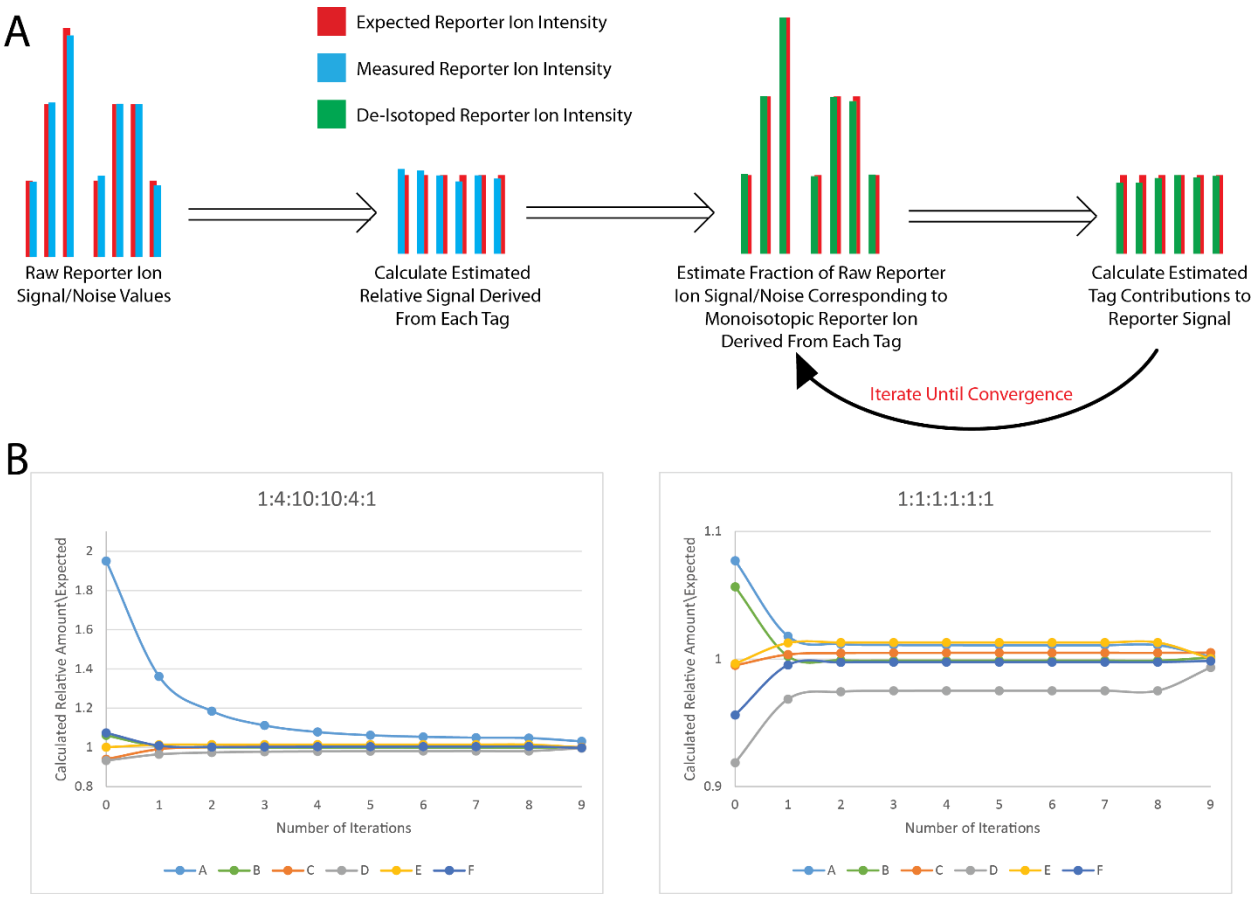


Figure S5: *Iterative process for CMT isotopic envelope correction*

A) Isotopic envelopes correction is achieved with CMT reporter ion signals in a two-step process. When computing fractional tag contribution in a sample of unknown mixing ratio, approximate relative tag contributions to the signal are computed as in **Figure 2C** based on measured raw reporter ion intensities. From these computed CMT tag contributions, isotopic envelope distortion is estimated based on previously measured values for individual tags, and the raw reporter ion intensities are corrected based on this estimated distortion. This process is iterated until the calculations converge. Converged values are then normalized based on the fraction of the isotopic envelope contributed by the monoisotopic peak.

B) Graphical representation of iterative deisotoping on idealized mixtures. Based on measured isotopic impurities for each tag, expected raw relative reporter ion intensities were calculated for theoretical sixplex mixing ratios of 1:1:1:1:1:1 (left), and 1:4:10:1:4:10 (right). Iterative deiosotoping of the theoretical signal intensities and the results after each iteration of the process are shown, as well as the final normalization step.

A

Experiment 1 (2 Samples, Technical Triplicates)						
TMT Label	126	127	128	129	130	131
CMT Label	A	B	C	D	E	F
Sample	AJ (Male)	AJ (Male)	AJ (Male)	Cast (Male)	Cast (Male)	Cast (Male)
Experiment 2 (6 Unique Samples)						
TMT Label	126	127	128	129	130	131
CMT Label	A	B	C	D	E	F
Sample	B6 (Male)	Cast (Male)	PWK (Male)	B6 (Female)	Cast (Female)	PWK (Female)

B

Tag	Forward hits	Reverse hits	Forward + Reverse	Total MS/MS	Total MS3	Validated Forward hits	Validated Reverse hits	Total	Unique	Total Quantified	Total Protein	Protein FDR	Peptide FDR	Success Rate
TMT (1)	23794	4845	28639	28835	28835	17371	28	17399	15675	12922	2644	1.97	0.32	60.34
TMT (2)	23514	4720	28234	28430	28432	16469	28	16497	15149	12186	2360	1.95	0.34	58.03
CMT (1)	22817	5274	28091	28320	28332	15759	27	15786	13712	9491	2536	1.97	0.34	55.74
CMT (2)	23049	5155	28204	28421	28421	15876	25	15901	13939	9729	2408	1.99	0.31	55.95

Figure S6: *Summary of Peptide and Protein Identification Results For CMT and TMT Mouse Liver Extract Labeling Experiments*

A) Experimental design for mouse liver homogenate comparison.

B) Summary table reporting the overall number of peptides and proteins from mouse liver extract identified with either CMT or TMT reagents in each of the mouse liver extract experiments.

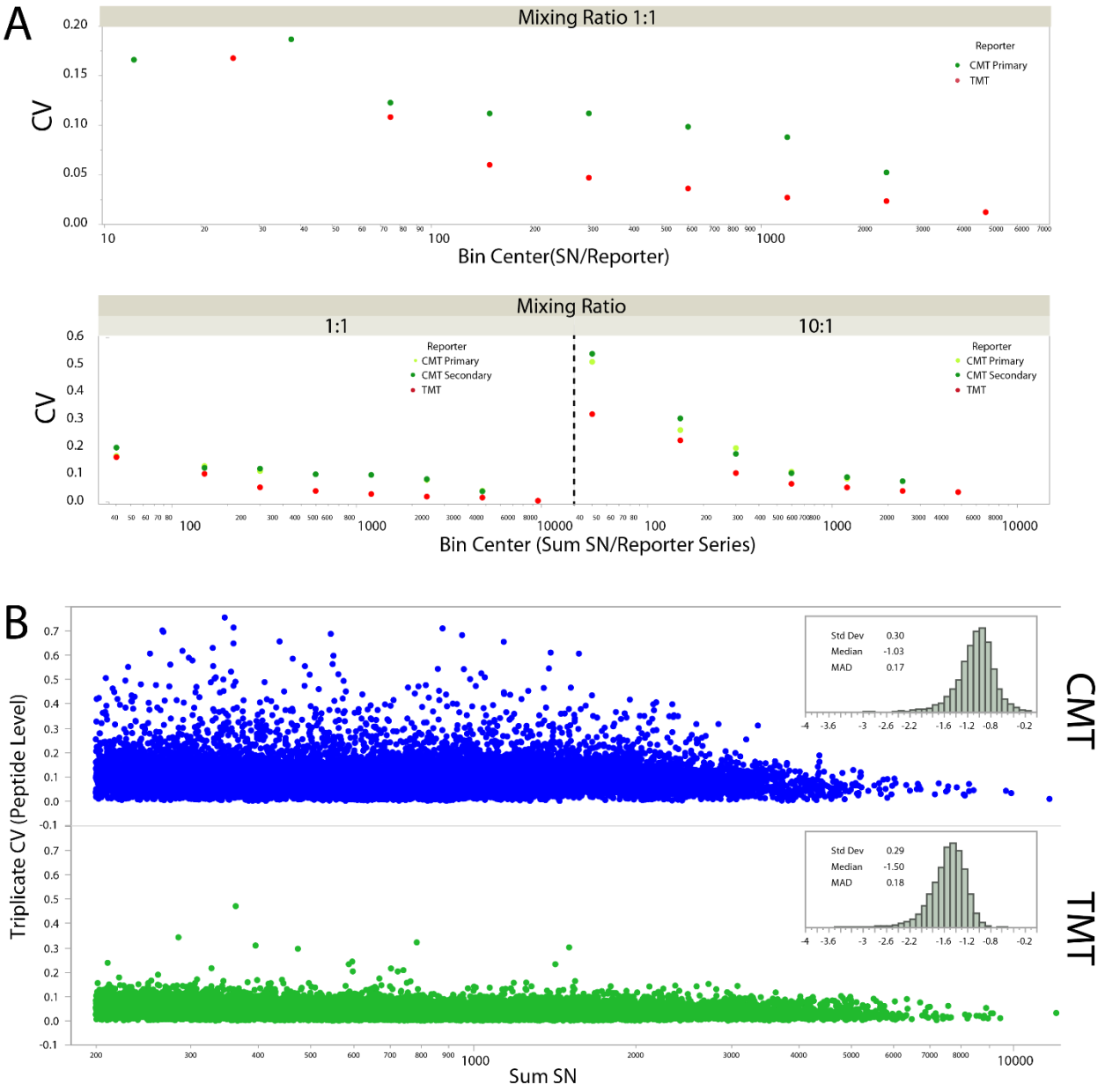


Figure S7: *Quantitative measurements made with CMT reagents are systematically slightly less precise than measurements made with TMT*

A) Coefficient of variation (CV) across all measurements from duplex quantitation experiments described in **Figure 3A** at specified signal/noise thresholds for CMT and TMT reporter ions demonstrates that CMT measurements are systematically less precise than those of TMT

B) CV for triplicate peptide measurements of CMT and TMT labelled mouse liver homogenate tryptic digests (**Figure 4**) indicates an approximately 2-fold reduction in

precision of CMT measurements when compared to those made with TMT. Inset: Distribution of the logarithm of the triplicate CV for all measurements.

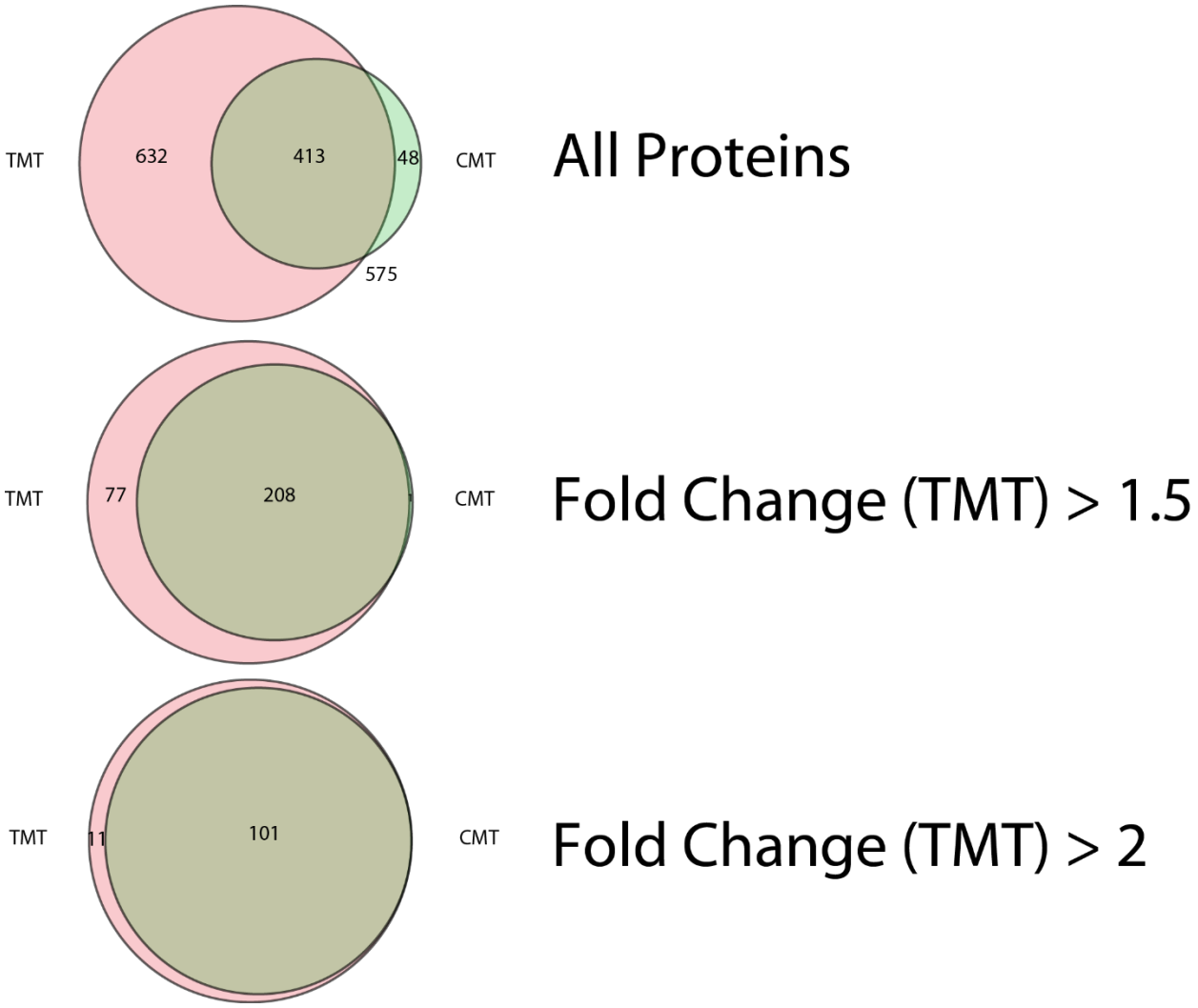
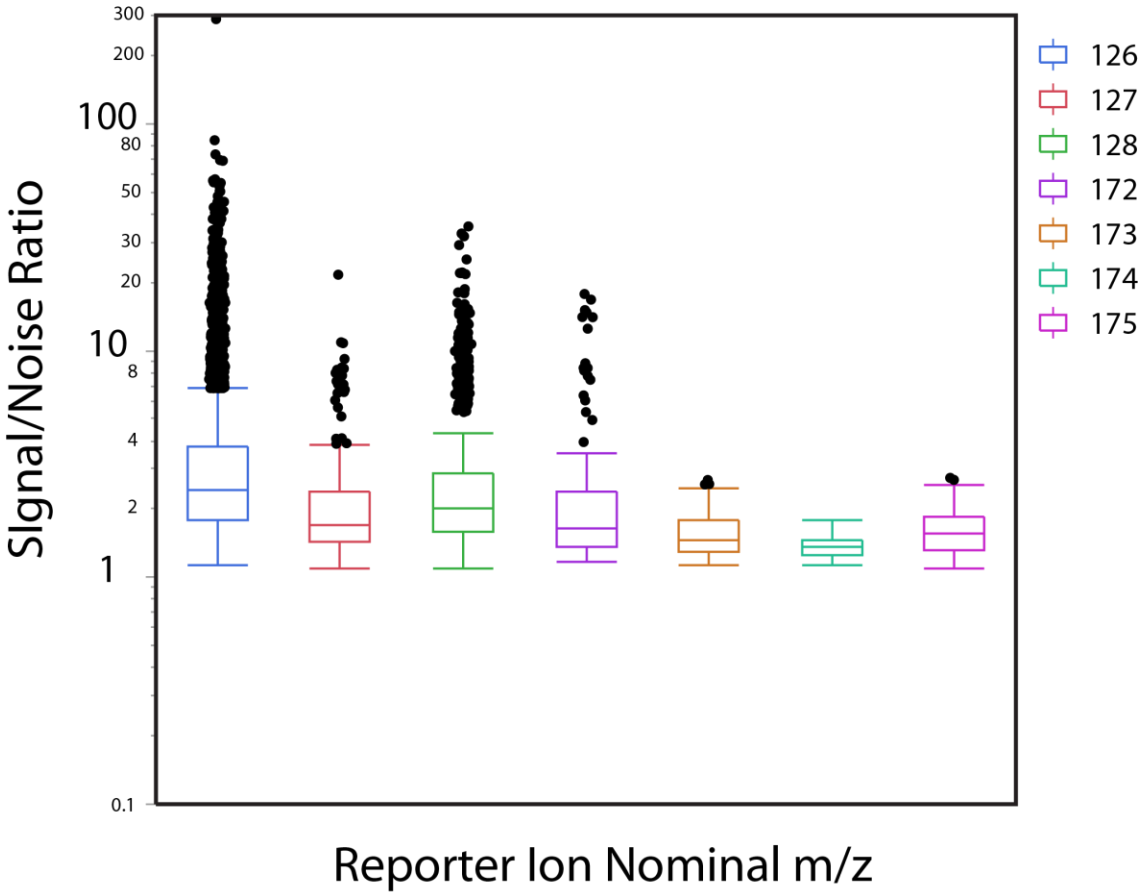


Figure S8: *Overlap of Mouse Liver Proteins Identified With Significantly Differential Expression Between AJ and CAST Strains Using CMT and TMT*

Overlap between proteins identified by CMT and TMT as having significantly different AJ/Cast ratio (**Figure 4**) as determined by having a Benjamini and Hochberg corrected P Value <0.01. TMT is significantly more sensitive to identifying significant fold changes when those fold changes are small.

A



B

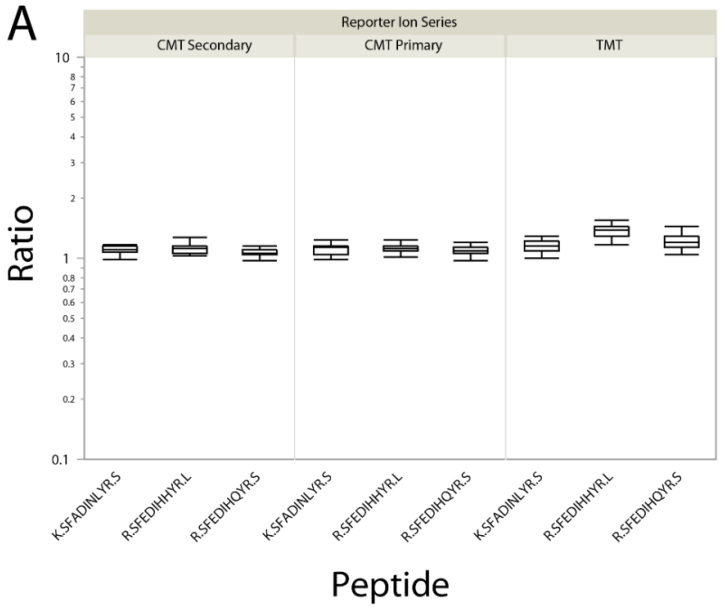
	126	127	128	172	173	174	175
N (%)	6538 (26)	358 (1.4)	2681 (11)	125 (0.5)	88 (0.4)	42 (0.2)	56 0.2)
Mean	4.25	2.27	2.84	2.91	1.61	1.37	1.64
Std Dev	6.29	1.79	2.62	3.38	0.40	0.16	0.41
Max	288.88	21.58	35.24	17.75	2.69	1.79	2.75
Median	2.44	1.69	2.01	1.64	1.46	1.35	1.56

Figure S9: *Coincidentally Isobaric Peptide Fragment Ions Do Not Contribute Appreciable Interference to Quantitative Measurements of CMT Reporter Ion Intensity*

A) CMT reporter ion detection in TMT labeled LC-MS-experiments, presumably from peptide side-chain fragmentation or non-peptidic sample components unrelated to CMT.

B) Table outlining the number of spectra observed (percent of all spectra in parentheses), mean, median, and maximum signal/noise as well as standard deviation observed for

signals corresponding to CMT reporter ion masses. While nominal masses of the CMT reporter ions are listed for convenience, all detection events were within 3 mTh



Peptide	RI Series	N	CV
K.SFADINLYR.S	CMT Secondary	10	5.6
	CMT Primary	10	6.5
	TMT	10	7.7
R.SFEDIHHYR.L	CMT Secondary	71	5.4
	CMT Primary	71	4.1
	TMT	30	7.6
R.SFEDIHQYR.S	CMT Secondary	41	7.6
	CMT Primary	41	9.6
	TMT	30	8.9

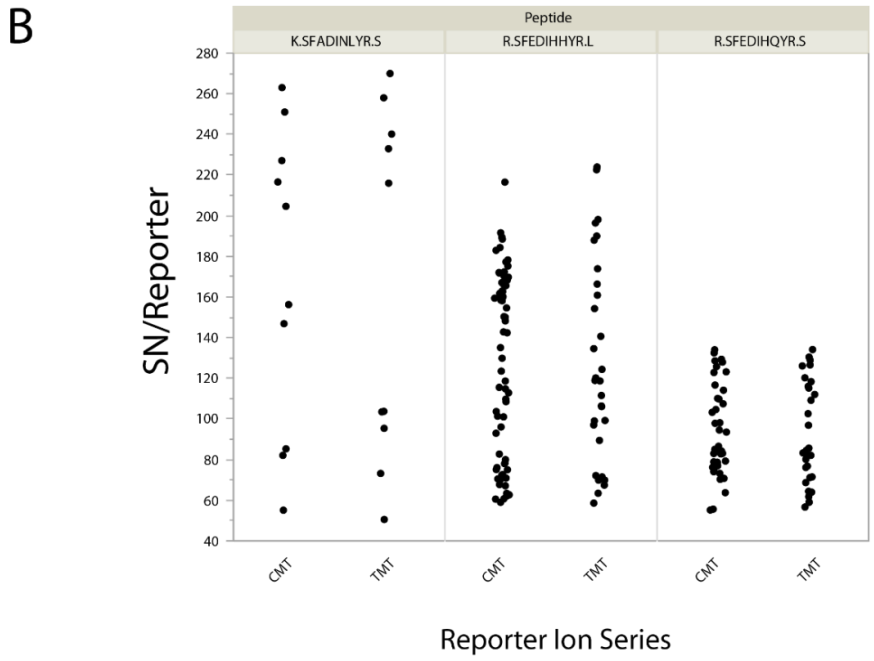


Figure S10: *Multiple Measurements Across A Single Chromatographic Peak Of CMT and TMT Labeled Peptides Have Similar Measurement Precision.*

A) Synthetic peptides with the specified amino acid sequences were labeled with either TMT 129 and TMT 131, or CMT A and CMT E and mixed at a 1:1 ratio. (Left) Box and whisker plots depicting measured ratios. (Right) Table depicting the number of measurements made, as well as the coefficient of variation across those measurements for each peptide and labeling scheme.

B) Since measurement precision is correlated to signal/noise (SN) ratio, only those measurements with SN ratios within overlapping ranges of the SN distributions for both tags were considered for each peptide.

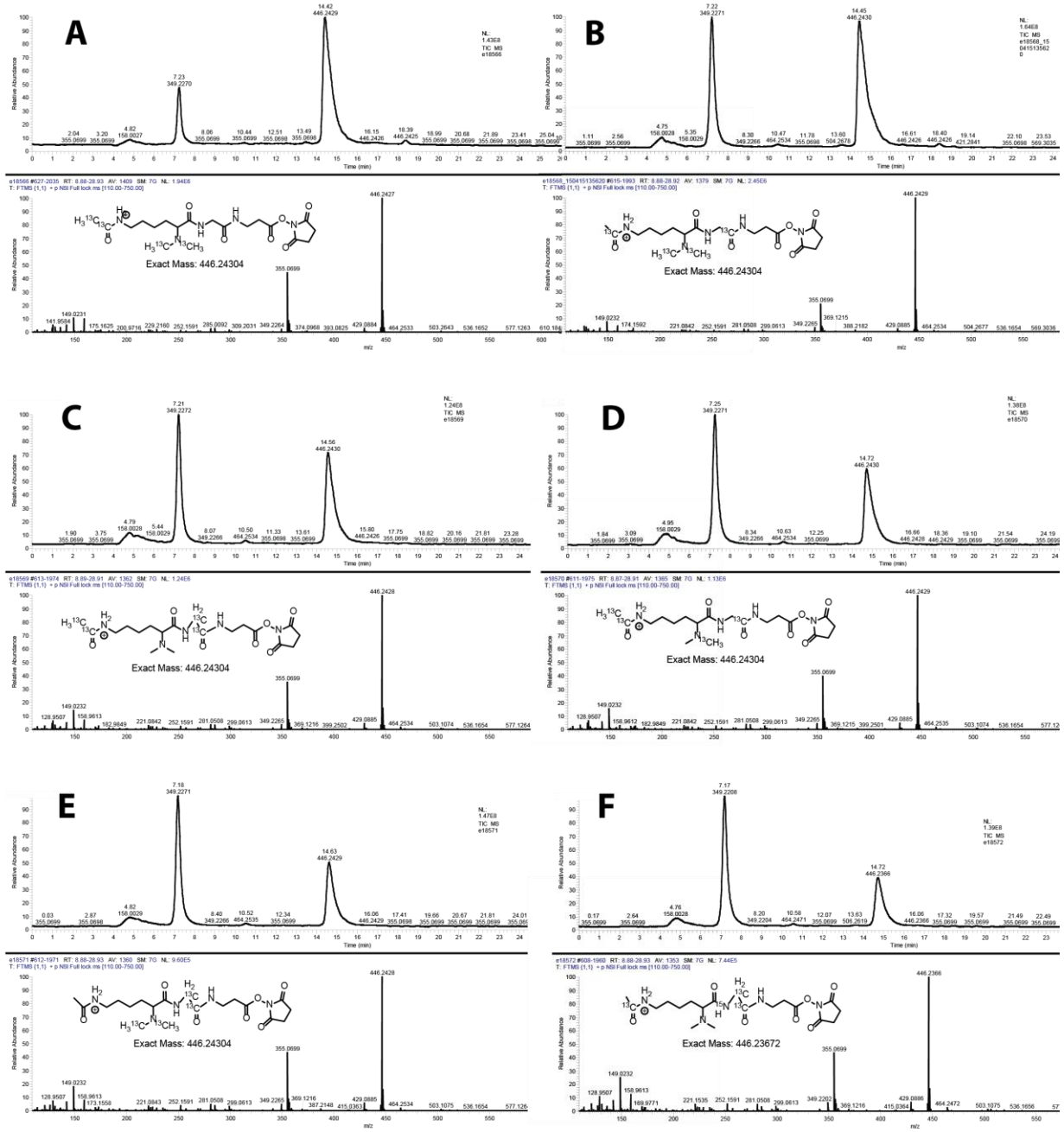


Figure S11: *LC-MS chromatograms and integrated mass spectra of purified CMT NHS esters A-F*

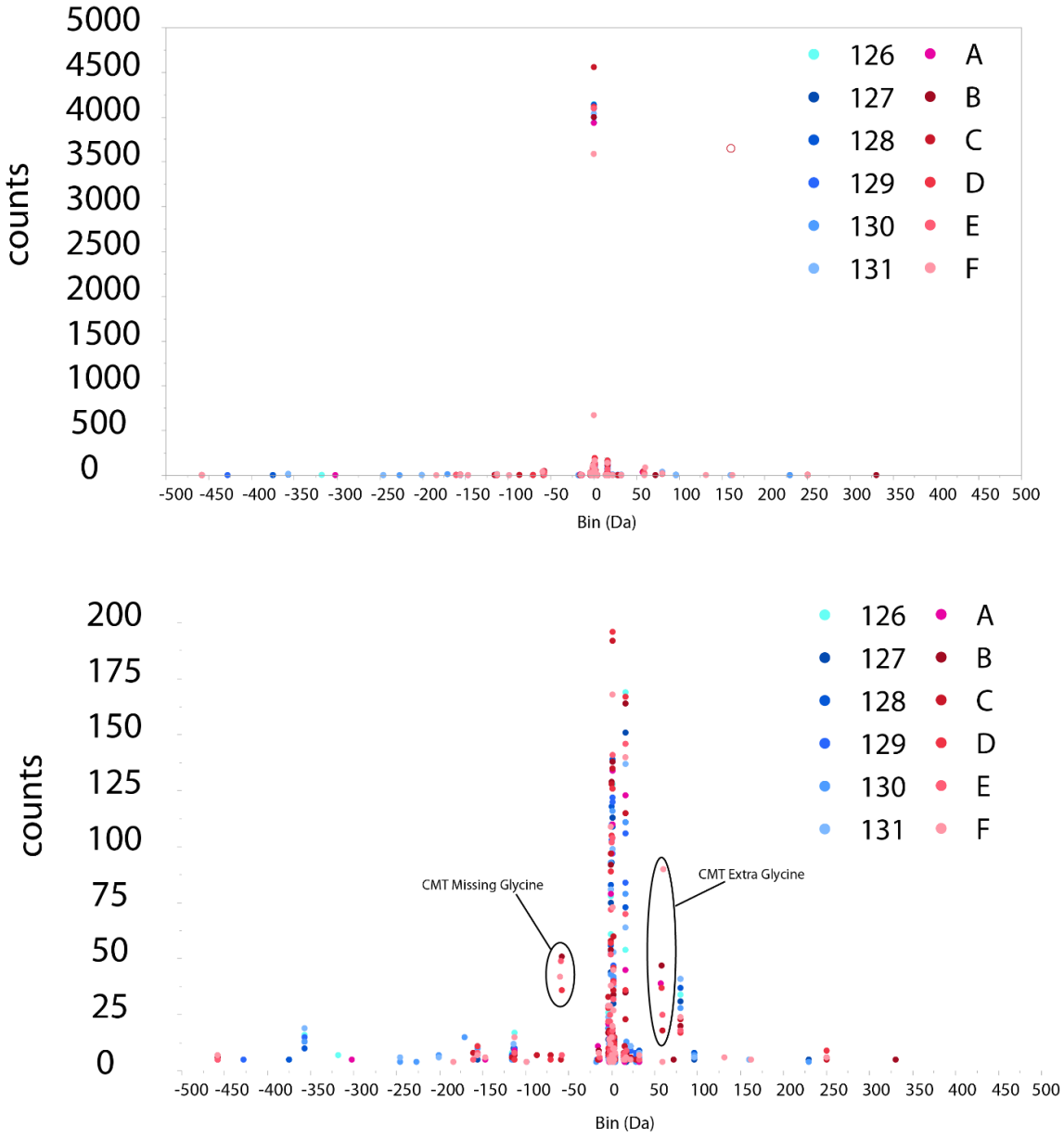


Figure S12: *Ultra Mass Tolerant Searches Identify CMT Labeling Side Products Caused By Minor Impurities In CMT Sixplex Reagents*

Spectral counts (percentage of maximal) of peptides identified with mass shifts in CMT labelled YWCL tryptic digest. Samples were labeled with each of the CMT sixplex reagents, and analyzed individually on a Q-Exactive instrument. Peptides are assumed to be CMT modified at the N-terminus and lysine side chains by default, and the SEQUEST algorithm was used to perform mass-error tolerant database searches (allowable mass deviation was +/- 500 Da). (Top) All peptide IDs: The overwhelmingly most frequent observation is no mass deviation from the

expected CMT modified peptide mass. Blue Shades: TMT labeled samples. Red: CMT Labeled samples. (Bottom) IDs with between 0 and 200 spectral counts: The only appreciable impurity detected in CMT labeled samples corresponds to either missing glycine in the CMT reagent, or an additional glycine.

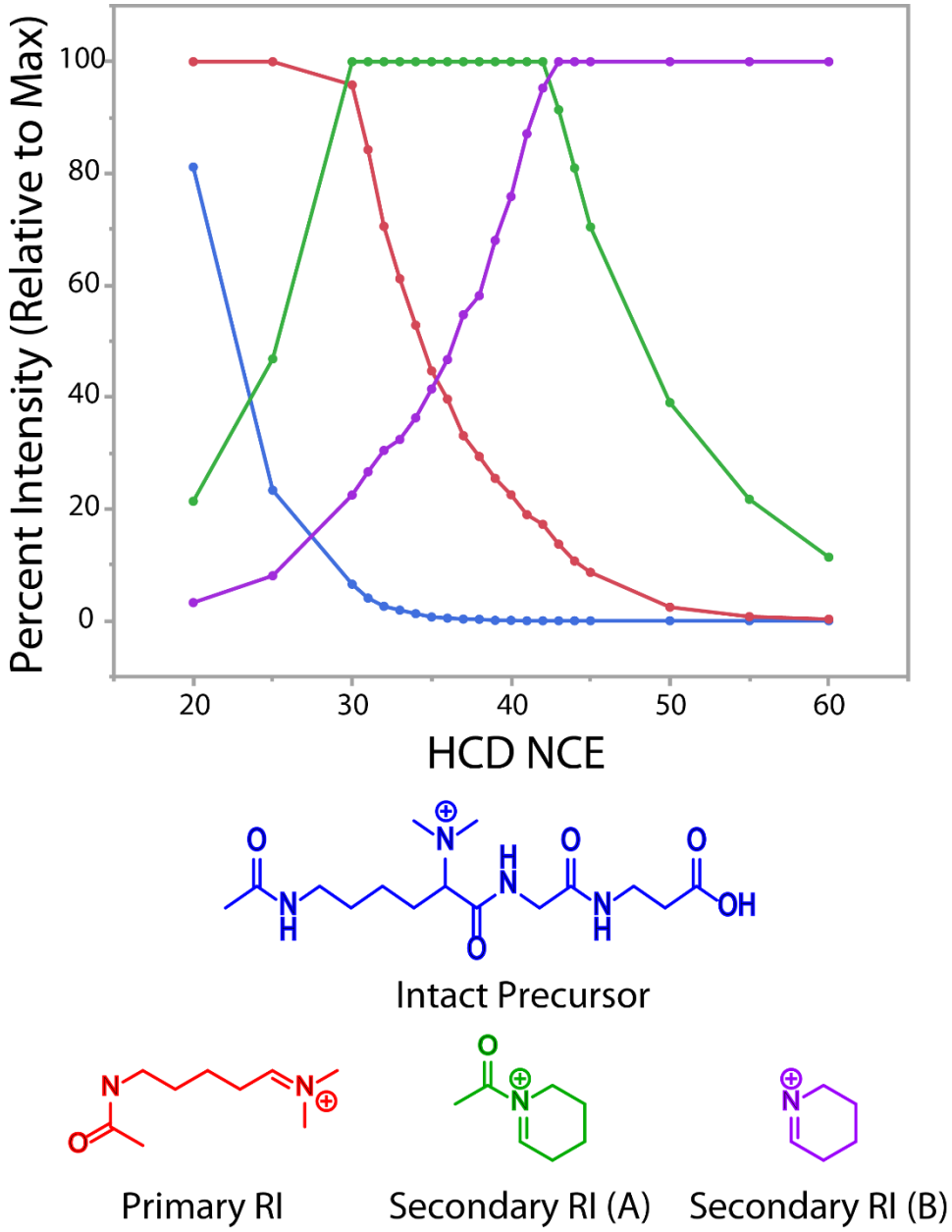


Figure S13: *Energy-dependent fragmentation characteristics of CMT primary and secondary reporter ions.*

Both CID and HCD fragmentation of CMT free acid (blue) produce CMT primary (red) and CMT secondary (A, green)) reporter ions, while a secondary CMT reporter ion (B, purple) is observed under HCD conditions on a Q-Exactive instrument (Thermo). HCD normalized

collision energy (NCE) can be tuned to produce approximately equal amounts of all three reporter ions, which will be essential for simultaneous use of all three ion series for quantitative information. We anticipate being able to further tune these fragmentation ratios by optimization of the chemical structure of CMT reagents.