Supporting Information

Materials and Methods

TMTc+, TMT-MS2, and TMT-MS3 sample preparation:

Samples were essentially prepared as previously described.[1] HeLa S3 cells were grown in suspension to $1x10^6$ cells/mL and yeast cells were grown to an OD of 1.0. Cells were pelleted and lysed in 2% SDS, 50 mM Tris.HCl pH = 7.4. Disulfide bonds of ~500 µg of protein were reduced with 5 mM DTT (500 mM stock, water) at 60 C for 20'. Samples were cooled to room temperature and cysteines were alkylated by the addition of 15 mM N-ethyl maleimide (1 M stock, acetonitrile) at 23 C for 20'. 5 mM DTT (500 mM stock, water) was added at 23 C for 10' to quench any remaining NEM. Salts, small molecules and lipids were removed by a methanol-chloroform precipitation and the protein disc was washed with 50/50 methanol/chloroform one additional time and the protein was allowed to air dry.[2] Protein samples were dissolved in 6 M guanidine hydrolchloride, 10 mM EPPS pH = 8.5 to ~2.5 µg/µL. Samples were heated at 60 C for up to 30 minutes to help resolubilization. Next, samples were dilluted with 10 mM EPPS pH = 8.5 to 2 M Guanidine hydrochloride. Lysates were digested overnight at 23 C with LysC (Wako, 2 µg/µL stock in HPLC water) at a concentration of 10 ng/µL LysC. Samples were further dilluted to 0.5 M guanidine hydrolchloride with 10 mM EPPS pH = 8.5 and an additional 10 ng/µL LysC was added as well as 20 ng/µL of sequencing grade Trypsin (Promega). Samples were mixed by pipetting and incubated at 37 C for 12-16 hours. All solvent was removed in vacuo and each protein sample was re-suspended in 300 mM EPPS pH = 8.5 at a concentration of ~1 µg/µL. 20 µL of the appropriate TMT-reagent (Pierce, 5 mg/250 µL in dry acetonitrile stored at -80) was added to ~100 µg of peptides, mixed, and incubated at 23 C for 2 hours. The reaction was quenched by addition of 10 µL of 5% hydroxyl amine (Sigma, 50% HPLC grade, diluted with HPLC water) at 23 C for 15 minutes.

For reverse phase fractionated samples, ~100 µg of peptides from each condition were combined to create a mixture of ~500 ug of peptides at 1.0 µg/µL. This mixture was acified to pH < 2 with phosphoric acid (HPLC grade, sigma) and cleared by ultracentrifugation at 200,000g at 4 C for 1 hour in polycarbonate tubes (beckman coulter, 343775) in a TLA-100 rotor. The supernatant was sonicated for 10 minutes and then fractionated by medium pH reverse-phase HPLC (Zorbax 300Extend C18, 4.6 x 250 mm column, Agilent) using 5% acetonitrile for 18 minutes followed by an acetonitrile gradient from 5% to 30%. Fractions were collected starting at minute 18 with a flow rate of ~0.8 mL/min into a 96 well-plate every 38 seconds. These fractions were pooled into 24 fractions by alternating the wells in the plate as in.[3] Each fraction was dried and resuspended in 100 µL of HPLC water. Fractions were acidified to pH <2 with HPLC triflouroacetic acid and a stage-tip was performed to desalt the samples.[4] Approximately ~4 µg of each sample was analyzed by LC-MS.

Label free sample preparation:

Label free samples were prepared as above except only a single sample was digested (not tagged with any TMT) and then five separate stage-tips and LC-MS experiments were performed.

LC-MS:

LC-MS experiments were performed on a Thermo Fusion Lumos equiped with an EASY-nLC 1200 System HPLC and autosampler (Thermo). During each individual run, peptides were separated on a 100-360 µm inner-outer diameter microcapillary column, which was manually packed in house first with ~0.5 cm of magic C4 resin (5 micron, 200 angstrom, Michrom Bioresources) followed by ~30 cm of 1.7 µm diameter, 130 Å pore size, Bridged Ethylene Hybrid C18 particles (Waters). The column was kept at 60 C with an in house fabricated column heater.[5] Separatation was achieved by applying a 6 – 30% gradient of acetonitrile in 0.125% formic acid and 2% DMSO at a flow rate of ~300 µL/min over 90 minutes for reverse phase fractionated samples. A voltage of 2.6 kV was applied through a PEEK microtee at the inlet of the column to achieve electrospray ionization.

TMTc+ experiments:

The instrument was operated in data dependent mode with a survey scan performed at a resolution setting of 120k (200 m/z) with a scan range of 500 – 1400 m/z with an RF Lens (%) of 60, AGC target of 1.0e6 and a maximum injection time of 100 ms. Only charges states of 2+ were included. A dynamic exclusion window of 60 seconds with a mass tolerance of +/- 10 ppm was used. Peptides with a minimum intensity of 3e6 or higher were subjected to an MS2 scan using an isolation window of 0.4 Th (or of different size if indicated) using the quadrupole. Peptides were fragmented using an HCD collision energy of 32% and a mass spectrum was acquired using the Orbitrap with a resolution of 60k with an AGC target of 5.0e5 and a maximum injection time of 120 ms. The scan range mode of the Orbitrap was Auto: m/z High.

TMT-MS3 experiments:

These were performed as recommended.[6] 10 SPS precursors from the MS2 were used for the MS3 using MS1 isolation window sizes of 0.5 for the MS2 spectrum and isolation windows of 1.2, 1.0, and 0.8 m/z for 2+, 3+ and 4-6+ peptides respectively. An orbitrap resolution of 50k was used in the MS3 with an AGC target 1.0e5 and a maximum injection time of 150 ms.

TMT-MS2 experiments:

The instrument was operated essentially as previously described for the TMTc+ experiments except all charge states between 2+ and 6+ were included with an MS1 scan range of 350 – 1400 m/z and an AGC target of 5.0e4 and a maximum injection time of 120 ms was used. The Orbitrap for the MS2 was operated at a resolution of 50k (200 m/z) and a scan range mode of Auto: m/z normal. There was no minimum precursor intensity threshold set.

The TMT-MS2 experiments were performed using the narrowest isolation window possible (0.4 Th) to acquire the best data possible. Note that often in the literature wider isolation windows are used, which would result in more interference.

Label free experiments:

The instrument was operated in data dependent mode with a survey scan performed at a resolution setting of 120k (200 m/z) with a scan range of 350 – 1500 m/z with an RF Lens (%) of 60, AGC target of 1.0e6 and a maximum injection time of 100 ms. Charge states between 2+ and 6+ were included. A dynamic exclusion window of 60 seconds with a mass tolerance of +/-

10 ppm was used. Petides were fragmented using an HCD collision energy of 30% and a mass spectrum was acquired using the Orbitrap with a resolution of 15k with an AGC target of 5.0e4 and a maximum injection time of 22 ms.

TMTc, TMTc+, TMT-MS2 and TMT-MS3 Data analysis:

The data was analyzed using the Gygi Lab GFY software licenced from Harvard.[7] To convert mass spectrometry data from the Thermo RAW file format to the mzXML format using ReAdW.exe (http://svn.code.sf.net/p/sashimi/code/), as well as correct incorrect peptide charge state and monoisotopic m/z assignments. Assignment of MS2 spectra was performed using the SEQUEST algorithm[8] by searching the data against the approriate proteome reference set acquired from Uniprot (SwissProt + Trembl) along with common contaminants such as human keratins and trypsin on 08/07/2016. The target-decoy strategy[9] was used to construct a second database of reversed sequences that were used to estimate the false discovery rate on the peptide level. SEQUEST searches were performed using a 20 ppm precursor ion tolerance with the requirement that both n and c-terminal peptide ends are consistent with the protease specificites of LysC and Trypsin. For high-resolution MS2 data (TMT-MS2, TMTc+) the fragment ion tolerance of the MS2 spectrum was set to 0.02 Th, whereas this value was set to 1 Th for low-resolution MS2 spectra acquired with TMT-MS3. TMT (+229.162932 Da) was set as a static modification on n-termini and lysines peptides, and N-ethyl maleimide (+125.047679 Da) was set as a static modification on cysteine residues. Oxidation of methionine (+15.99492 Da) was set as a variable modifciation. A peptide level MS2 spectral assignment false discovery rate of 1% was obtained by applying the target decoy strategy with linear discriminant analysis.[10] The linear discriminant analysis used the following features: XCorr and delta XCorr from sequest, charge state, peptide length and, absolute peptide mass accuracy. The positive training set was constructed using forward peptides that are within three standard deviations of the theoretical m/z of the precursor. The negative training set consisted of all reverse peptides. Peptides of length seven amino acids or longer were rank ordered by linear discrimnant analysis score and were filtered to a ratio of 1% reverses / forwards + reverses. Peptides were assigned to proteins and a second filtering step to obtain a 1% FDR on the protein level was applied as in.[11] Peptides that matched multiple proteins were assigned to the proteins with the most unique peptides.[12] TMT-MS2 and TMT-MS3 experiments were only used for quantitative information if at least 75% of the signal in the MS1 within the range of the isolation window was from the precursor (Isolation Specificity > 0.75). No Isolation specificity fitlers were applied to the TMTc+ data. For TMT-MS2, TMT-MS3 and TMTc+, peptides were only considered to be quantified if the sum sn across all five channels was > 100.

TMTc+ deconvolution

The isotopic impurities of each TMT tag used were measured as was done previously.[13] Shown below (Fig S5) are the structures and isotopic impurity matrices for the TMT reagents used in this work.

For TMTc+, the complement reporter ion cluster was located and the observed ratios were extracted as in.[13] Using the measured shape of the isolation window (see table S1), the relative abundance of each peak that was isolated from the precursor peak was determined and used in the deconvolution algorithm. The actual deconvolution is performed exactly the same as in.[13]

A standalone program that requires only the raw file, scan number and peptide sequences for each spectral match is available upon request to deconvolve any data acquired with TMTc+.

Label free data analysis and quantification:

Label free raw files were processed with MaxQuant version 1.5.7.4 as described in. For label-free quantification, LFQ was selected. Peptides were searched with a FASTA file that contained both the human and yeast proteomes with the human proteome at the top of the file. We used the default parameters under the instrument setting. For match between runs we used the match type “Match from and to” between the raw file containing only HeLa peptides and the second raw file containing HeLa and yeast. We used a custom python script to select the subset of peptides that had unique yeast sequences, and from these we select those unique yeast peptides that were had the “Identification type” of “By matching”. We used the “Intensity” column to produce the quantification measurements we refer to as “Intensity in the main text” and the “LFQ Intensity” column to produce the corresponding LFQ measurements.

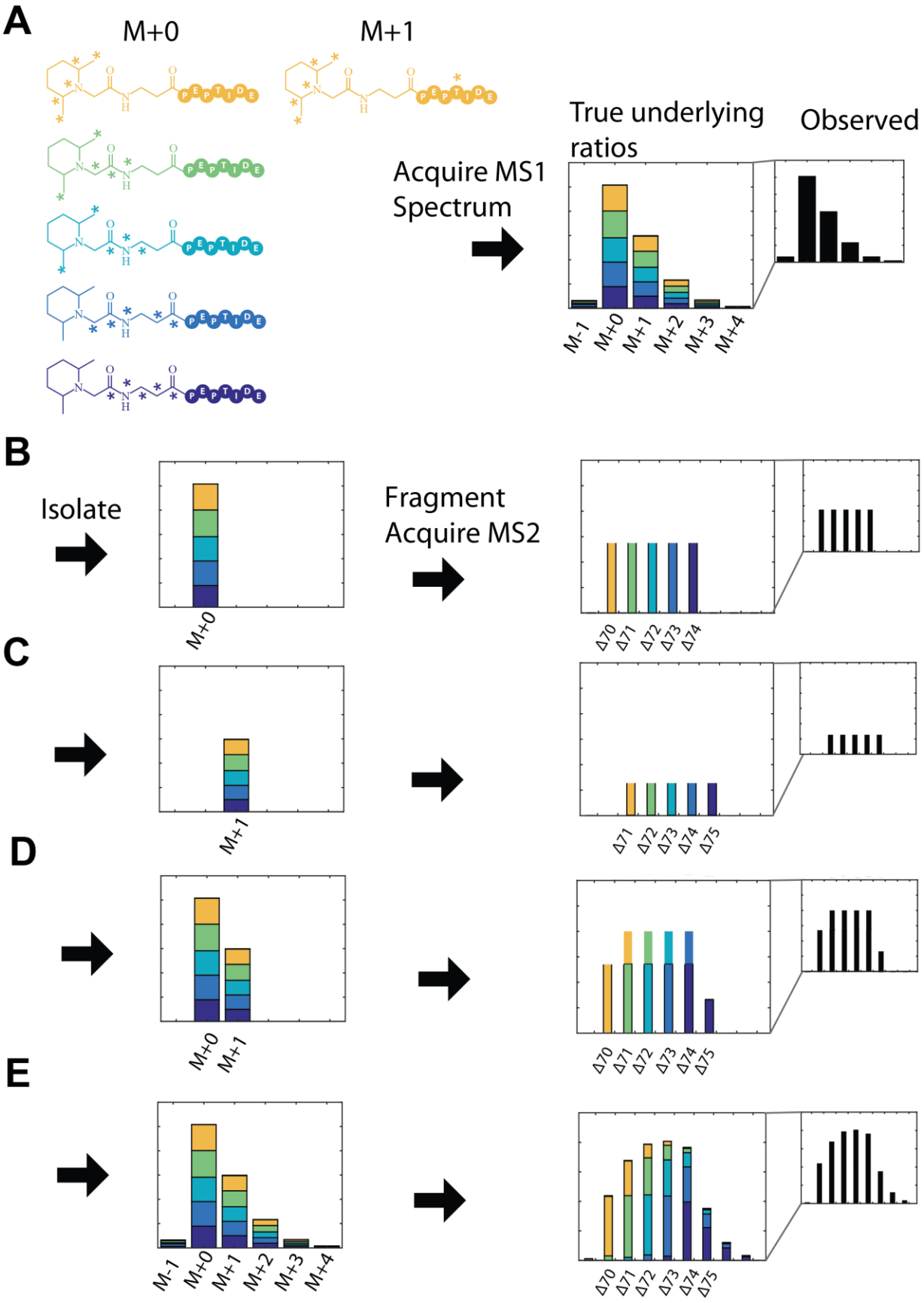

A
M+0
M+1
Acquire MS1
Spectrum
True underlying
ratios
Observed
M-1
M+0
M+1
M+2
M+3
M+4
B
Isolate
Fragment
Acquire MS2
M+0
Δ70
Δ71
Δ72
Δ73
Δ74
C
M+1
Δ71
Δ72
Δ73
Δ74
Δ75
D
M+0
M+1
Δ70
Δ71
Δ72
Δ73
Δ74
Δ75
E
M-1
M+0
M+1
M+2
M+3
M+4
Δ70
Δ71
Δ72
Δ73
Δ74
Δ75

Figure S1. *Intuition for convolution of complement reporter ions as a function of precursor envelope isolation purity.* **A)** A theoretical sample containing many peptides present in an equal 1:1:1:1:1 ratio is tagged with 5 different TMT reagents (positions of heavy isotopes are indicated with stars). Shown are all 5 reagents attached to the peptide. Due to ~1% of all carbon being ^{13}C, a subset of the peptide species will also contain an additional an additional ^{13}C atom resulting in a total mass of M+1 (eg. of 1 condition is shown in yellow). The other tags will be present in the same abundance for the M+1 species but are omitted for clarity. An even smaller fraction of peptide with have an M+2 (two heavy isotopes), or M+3 and higher. After the peptides tagged with 5 different TMT reagents are mixed together and ionized at the same time, an initial mass spectrum can be acquired and this single peptide species will form an isotopic envelope as shown. Note that the true underlying 1:1:1:1:1 ratios are conserved within each peak of the envelope. During an actual experiment, the ratios would be unknown and what is observed is shown in black. **B)** Theoretical isolation and fragmentation of only the M+0 peak from the precursor envelope. After isolation only the M+0 peak is present. Following fragmentation all TMT tags are fragmented in the same place and each condition loses a different mass due to the shuffling of the heavy isotopes within the TMT reagent. The correct ratios can be read out directly from what would be observed in a real experiment (black) **C)** Theoretical isolation and fragmentation of only the M+1 peak from the precursor envelope. The same exact situation as in B) arises except the mass offsets are all increased by one Dalton (relative to the M+0). **D)** Theoretical isolation and fragmentation of both the M+0 and M+1 peaks simultaneously. The M+0 and M+1 peaks are each present in the same relative abundance as B) and C) and thus produce the same offsets with the same mass shifts. However, in this case, the added signals convolve due to the 1 Dalton offset that occurs (The purple in the delta 75 channel is from the M+1 peak). The problem is immediately evident for the observed ratios (black) because there are 6 different reporter channels when there should only be 5 and their ratios are now distorted. **E)** When the entire precursor envelope is isolated and fragmented the convolution becomes more complicated.

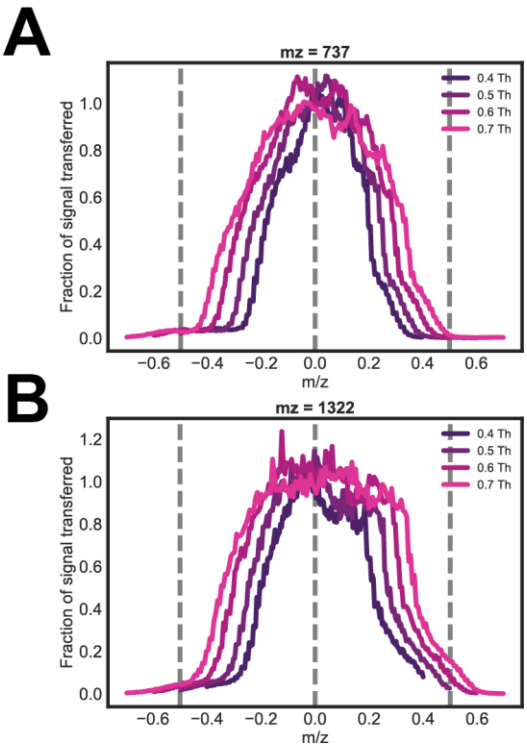


Figure S2. *Measured isolation window shapes from an infused MRFA peptide at m/z = 737 and infused Ultramark 1621 at m/z = 1322* **A)** The fraction of signal transferred was measured as the radio-frequency of the quadrupole was scanned around the m/z of interest, in this case m/z = 737 Th. The plots are normalized so that the peptide of interest is localized at m/z = 0 Th. The grey stripes at -0.5 Th, 0.0 Th, and 0.5 Th indicate the location of different peaks in a precursor envelope for a peptide with a 2+ charge. Shown are isolation windows ranging from 0.4 Th to 0.7 Th in size. **B)** The same as an A but for the peak of Ultramark 1621 with an m/z of 1322. Note that while the general trend of all isolation windows relative to each other is conserved, there is an m/z dependence to their shapes.

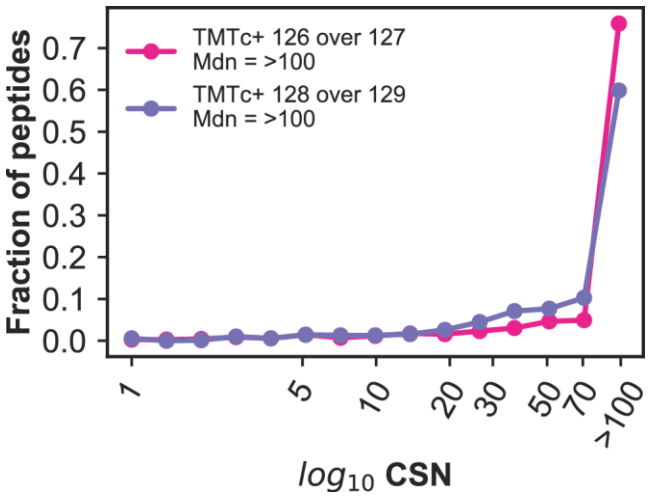


Figure S3. *Comparison of TMTc+ CSN measurements using different TMT reagents*

The CSN for unique yeast peptides as in Fig 3A was measured using the ratio of either 126 over 127 (pink) or 128 over 129 (purple). A perfect measurement for both is infinity.

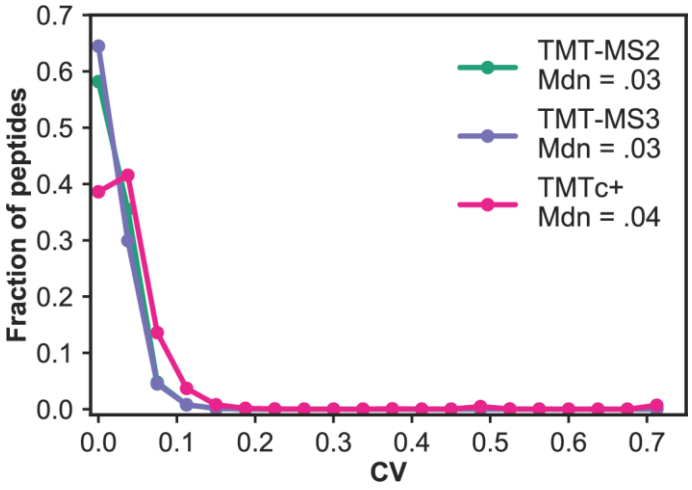
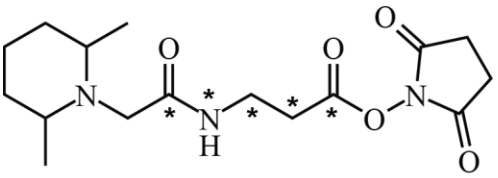


Figure S4. *Comparison of CV measurements for TMT-MS2, TMT-MS3, and TMTc+*

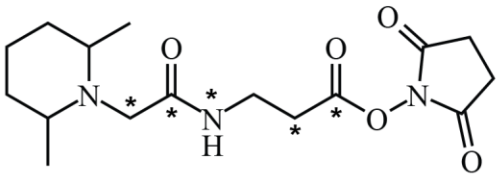
The CVs of unique human peptides were measured (perfect measurement would be 0) for peptides from a reverse-phase fractionated sample. The CVs of TMT-MS3 and TMT-MS2 are essentially identical and slightly superior to that of TMTc+.

TMT 126



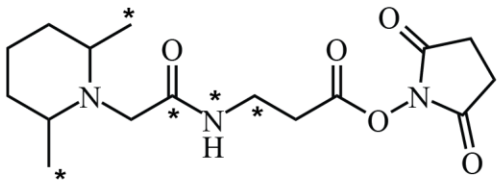
$$I_{126} = \begin{bmatrix} 0.026 & 0.893 & 0.003 \\ 0 & 0 & 0.078 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \end{bmatrix}$$

TMT 127C



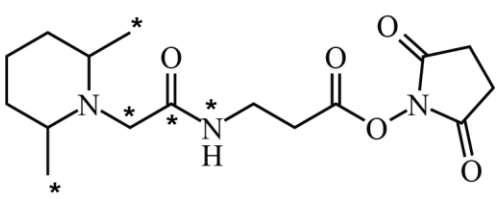
$$I_{127} = \begin{bmatrix} 0.0030 & 0 & 0.001 \\ 0.007 & 0.907 & 0.014 \\ 0 & 0 & 0.068 \\ 0 & 0 & 0.001 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \end{bmatrix}$$

TMT 128C



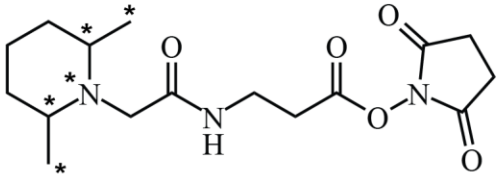
$$I_{128} = \begin{bmatrix} 0.001 & 0 & 0 \\ 0.014 & 0 & 0.002 \\ 0.01 & 0.894 & 0.02 \\ 0 & 0 & 0.058 \\ 0 & 0 & 0.001 \\ 0 & 0 & 0.002 \end{bmatrix}$$

TMT 129C



$$I_{129} = \begin{bmatrix} 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0.014 & 0 & 0 \\ 0.004 & 0.898 & 0.031 \\ 0 & 0.003 & 0.05 \\ 0 & 0 & 0 \end{bmatrix}$$

TMT 131



$$I_{131} = \begin{bmatrix} 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0.022 & 0 & 0.001 \\ 0 & 0.896 & 0.032 \\ 0 & 0 & 0.048 \end{bmatrix}$$

Figure S5. *Structures and isotopic impurity matrices of the TMT 5-plex used.*

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