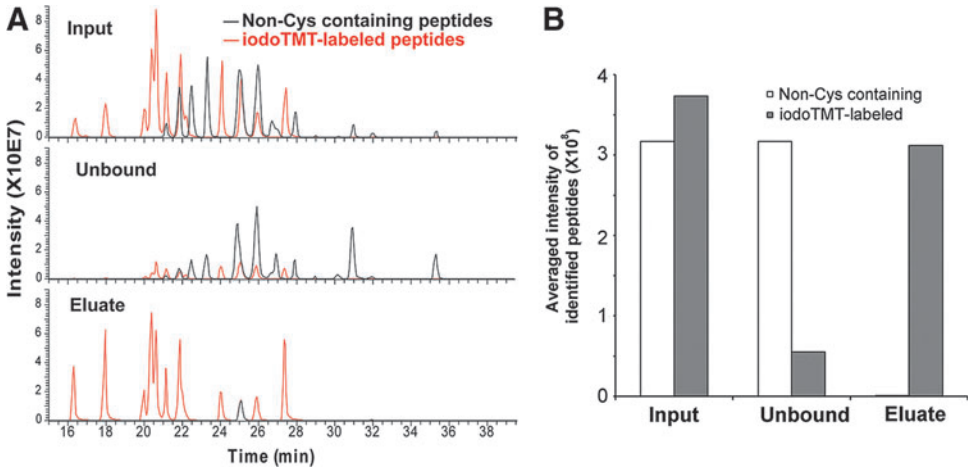
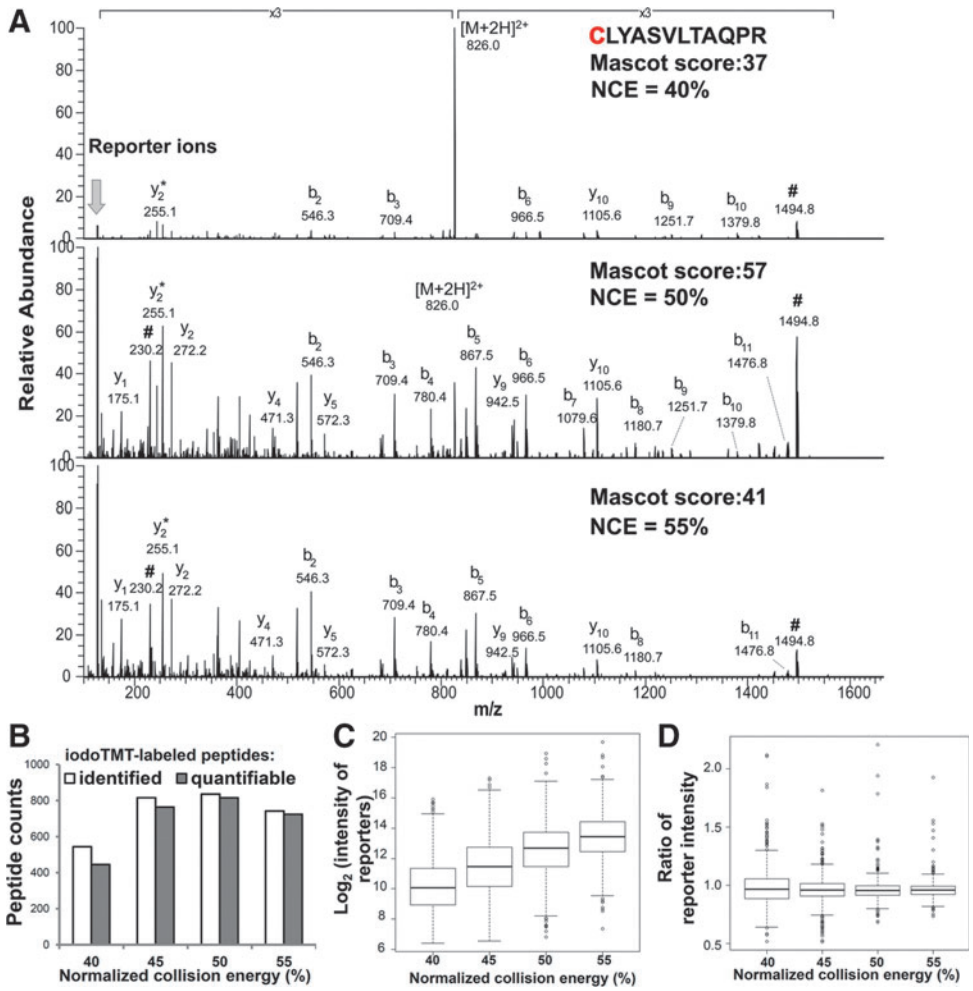
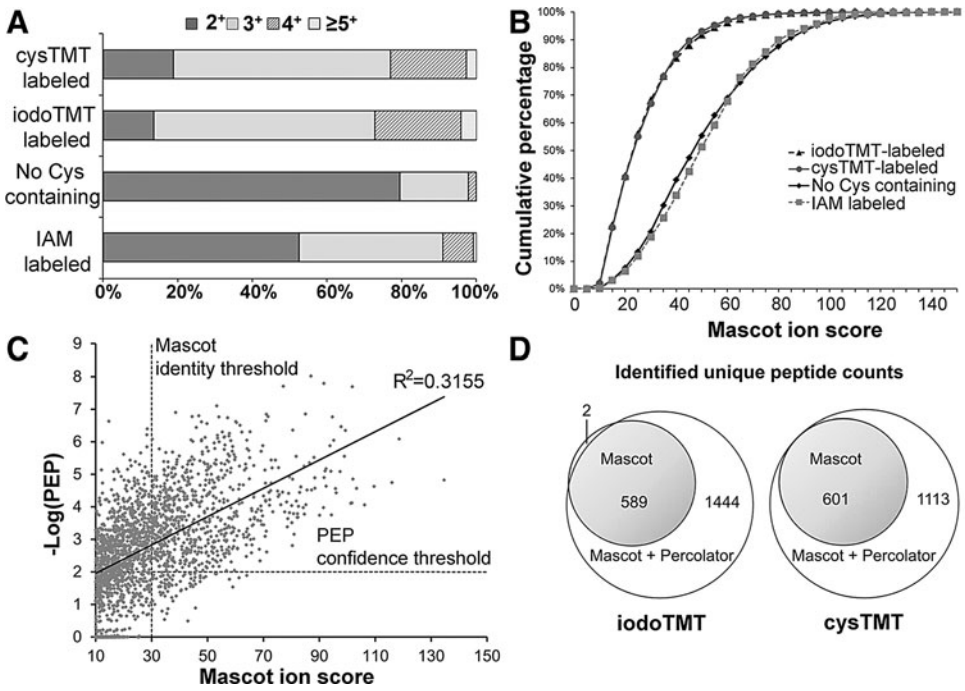
Supplementary Data



SUPPLEMENTARY FIG. S1. The immunocapturing efficiency of iodoTMT-labeled peptides by immobilized anti-TMT resin. BSA was reduced by TCEP, alkylated by iodoTMT0, tryptic digested in-solution and the resulting peptides were then incubated with the anti-TMT resin. Aliquots from the unbound fraction and the 0.5% trichloroacetic acid/40% acetonitrile eluate were both subjected to liquid chromatography (LC)-MS/MS analysis, along with the non-fractionated total. The composite extracted ion chromatograms **(A)** and the respective average peak intensities **(B)** of all confidently identified BSA tryptic peptides with and without TMT modifications indicated that a majority of the non-Cys-containing and thus non-TMT-labeled peptides flowed through directly while only a small amount of TMT-labeled peptides failed to be captured and found in the unbound fraction. Conversely, all identified BSA peptides except a low abundant one in the eluate fraction were TMT-labeled, thus signifying a high degree of enrichment. BSA, bovine serum albumin; Cys, cysteine; iodoTMT, irreversible isobaric iodoacetyl Cys-reactive tandem mass tag; MS, mass spectrometry; TCEP, tris(2-carboxyethyl) phosphine.



SUPPLEMENTARY FIG. S2. Optimization of the HCD collision energy setting for the analysis of iodoTMT-labeled peptides. For optimization of collision energy, 300 μg of Rat-1 lysate was reduced by 5 m*M* of TCEP and labeled by iodoTMT tag, followed by tryptic digestion, immunocapture, and LC-MS/MS analysis. The HCD MS/MS spectra of individual iodoTMT-labeled peptides from Rat-l lysate acquired using different NCE were critically evaluated for the intensity of sequence-informative b/y ions *versus* the reporter ions, as exemplified by the spectra shown here for the doubly charged peptide "CLYASVLTAQPR" **(A)**. The highest total number of identified and quantified peptides was obtained with NCE at 45–50% **(B)**, whereas both the intensity of reporter ions **(C)** and the precision of their measured ratios **(D)** increase with higher NCE. HCD, higher-energy C-trap dissociation; NCE, normalized collision energy.



SUPPLEMENTARY FIG. S3. Percolator improves the identification of both iodoTMT- and cysTMT-labeled peptides. **(A)** Mascot score and **(B)** charge state distributions of peptide spectrum matches for iodoTMT-labeled, cysTMT-labeled, IAM-labeled, and non-Cys-containing peptides. More than 80% of iodoTMT-labeled peptides bore 3+ or higher charges, compared to only 20% for peptides without Cys residue or 48% for peptides with IAM alkylated Cys. About 40% of iodoTMT-labeled peptides were identified by a Mascot score <20, compared with only 7.6% of non-labeled peptides identified at such a low score. **(C)** The PEP of each iodoTMT-labeled PSM as calculated by the Mascot Percolator was plotted against its Mascot ion score to demonstrate its correlation and derived orthogonality. Dash lines indicate the threshold based on Mascot scores or PEPs for determining the confidence of peptide identification (FDR < 0.01). **(D)** Venn diagram summary of identified iodoTMT- or cysTMT-labeled peptide hits under 1% FDR, as determined by Mascot score alone or with additional PEP considerations provided by Mascot Percolator. *Note:* The Percolator module uses a semi-supervised method to train a machine-learning algorithm to discriminate between correct and incorrect PSMs (5, 34, 68). A set of features related to the quality of the match (*e.g.*, search engine scores, charge state, precursor mass deviation, and average fragment mass deviation (Supplementary Table S1) were computed through a given FDR to train a robust classifier for re-scoring and reporting associated *q*-value and PEP of each PSM in the data set. Therefore, all PSMs from both forward and decoy databases were sorted by PEP and the identification threshold was set to reach the designed FDR. FDR, false discovery rate; IAM, iodoacetamide; PEP, posterior error probability; PSM, peptide spectrum match.