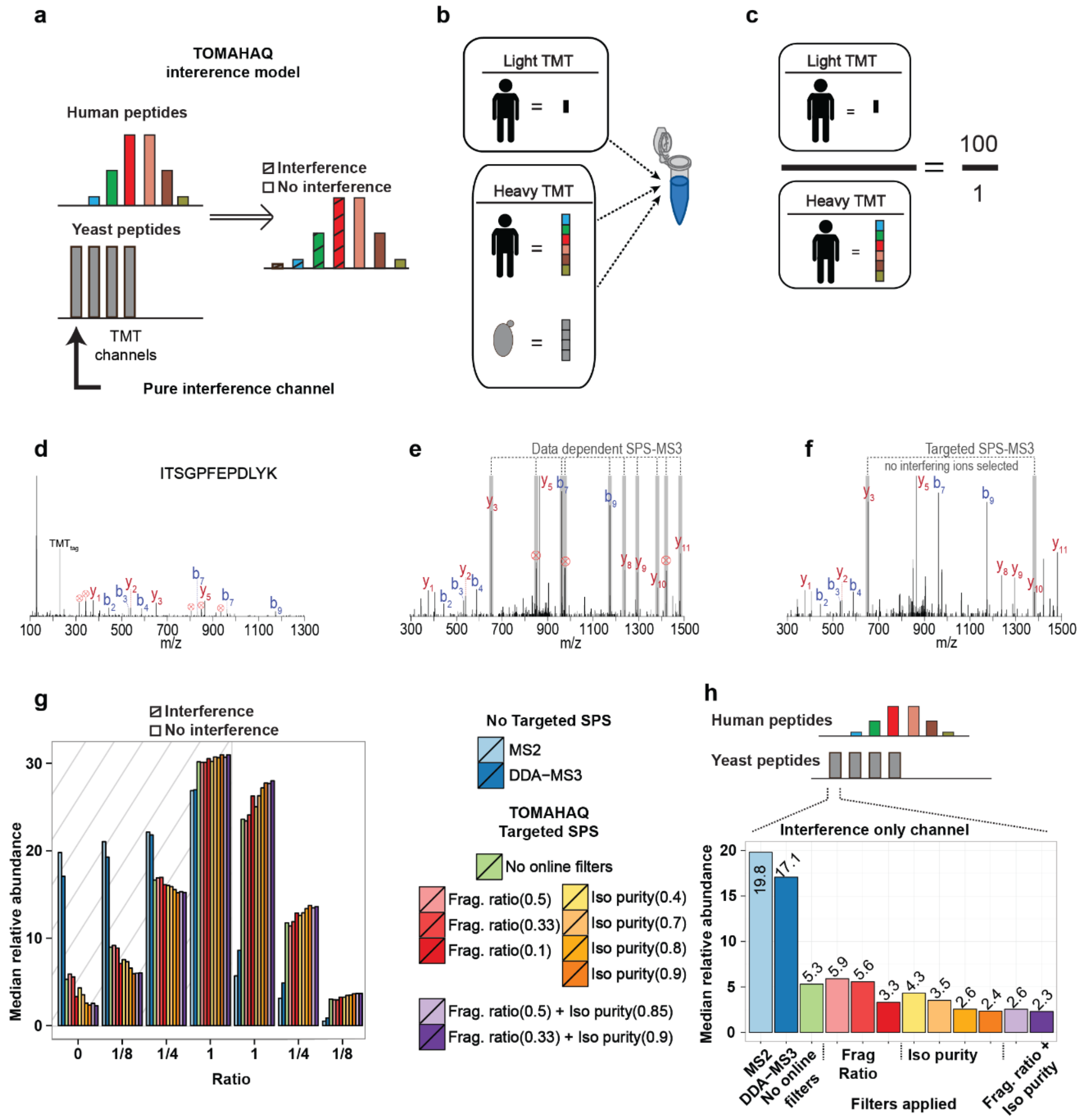
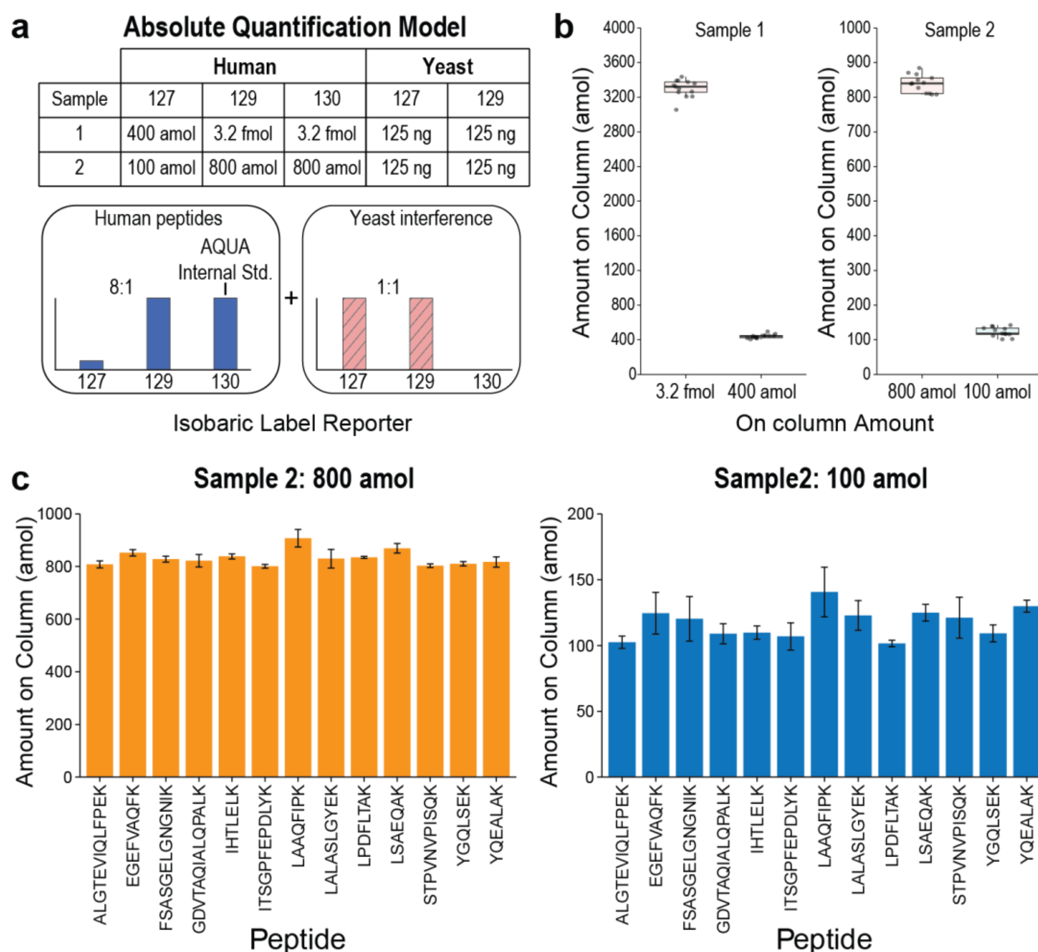
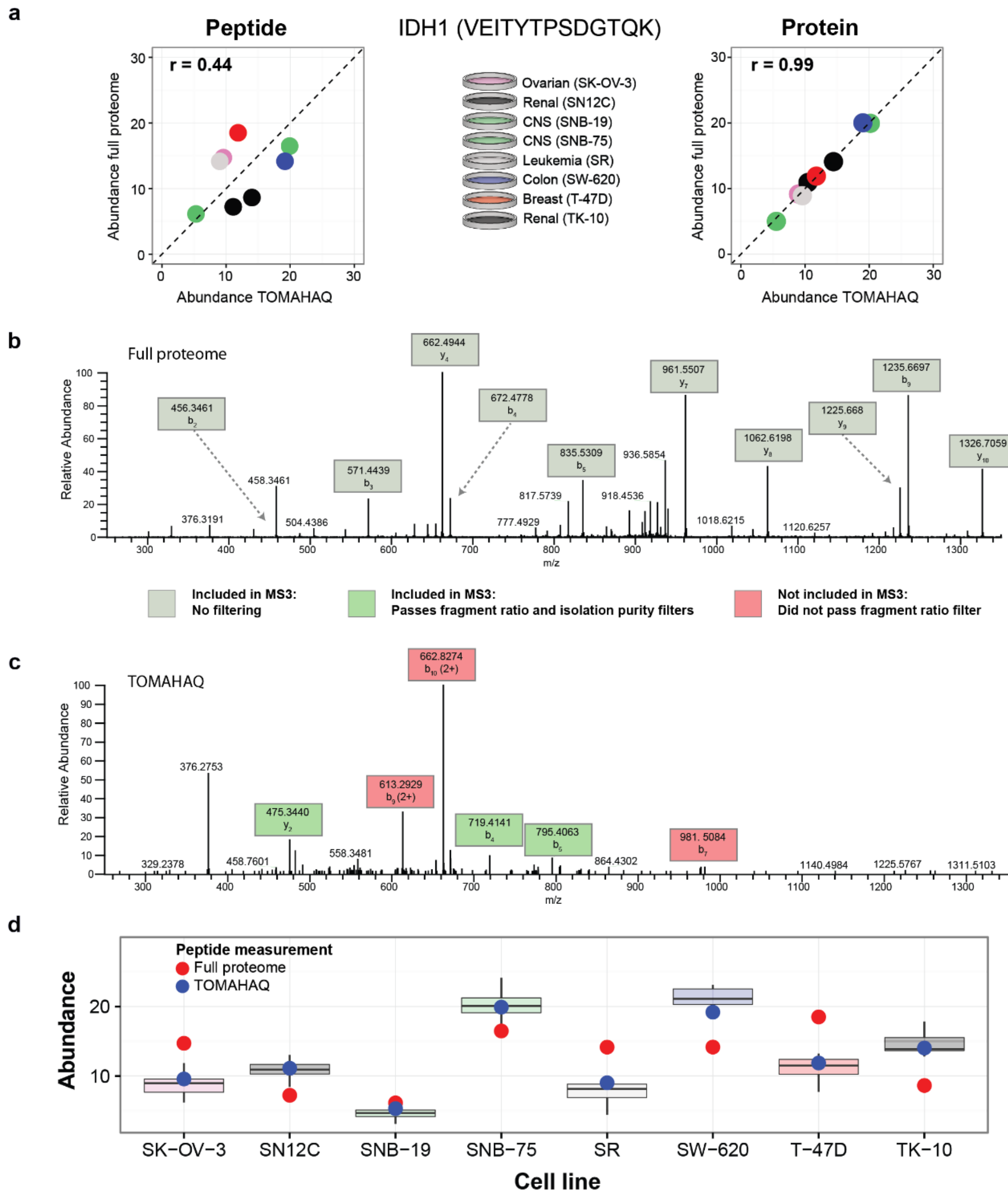
Supplementary Figures



Supplemental Figure 1. Assessing the utility of online SPS filtering, related to Figure 2. (a) An expanded two-proteome model consisting of a mixture of target human peptides at known ratios (1, ¼, and ⅛) and inteferring yeast whole cell lystate was prepared. Three of the six human peptide channels contained yeast interference, leaving three channels free from interference as a control. A channel containing only yeast peptides provided an interference only control. (b) The sample contained TMT0 labeled human trigger peptdies, TMT10 labeled human peptides at known mixtures and TMT10 labeled yeast whole cell lysate. (c) The trigger peptides were mixed at a 100:1 ratio with the target peptides. The sample was analyzed by three different approaches: (d) MS2 (e) data-dependent (DDA) MS3 and (f) TOMAHAQ. An example spectrum for peptide 'ITSGPEFEPDLYK' is displayed. For the MS2 approach (d), all product ions from precursors within the precursor isolation window can potentially contribute TMT reporter ions. This can result in reporter ion contamination due to co-isolated and co-fragmented peptides (circles with 'x'). The data-dependent MS3 (e) utilizes a second level of gas-phase purification to select a portion of the product ions for additional isolation and fragmentation. The DDA-MS3 method selects these ions in a data-dependent manner which is based on the intensity of the ion. In circumstances where abundant interferring peptides are co-isolated and co-fragmented, it is likely that the off-target product ions may be more abundant than target product ions (circles with 'x'). As before, this can produce perturbed reporter ion ratios. Finally, TOMAHAQ (f) utilizes targeted SPS ions, thereby encompassing the benefits of the DDA-MS3 method while eliminating the impact of abundant interferring ions. The accuracy and purity of TOMAHAQ is further improved through the use of online filters that assess the isolation purity (proportion of isolation window attributable to the target) and the ratios among target product ions. All three methods were compared using the two-proteome mixture and included the use of including various iterations of isolation purity and fragment ratio tolerances for TOMAHAQ (g). The MS2 and DDA-MS3 methods (blue bars) resulted in highly perturbed ratios at 4-fold (MS2: 1.09, DDA-MS3: 1.19, median) and 8-fold (MS2: 1.27, DDA-MS3: 1.19, median). However, the targeting of SPS ions (green bars) resulted in an improvement in accuracy with ratios of 4-fold: (2.62 and 8-fold: 5.37, median). The inclusion of the online SPS filters, fragment ratio and isolation purity (dark purple bar) resulted in further improved quantitative accuracy (4-fold: 2.97 and 8-fold: 6.45, median). This is evident in the interference-only channel, where the MS2 and MS3-DDA methods contained ~4x the proportion of interference relative to the SPS targeting method (h). The inclusion of the online filters further reduces the proportion of interference and the combination of SPS ion targeting and the online filters resulted in an ~10x decrease in interference. No noticeable effect on duty cycle was observed when the online filters were employed. In fact, with all filters enabled, we routinely collected up to 8 quantitative MS^3 spectra per target peptide, for over 100 peptides in 2 hours of analysis.



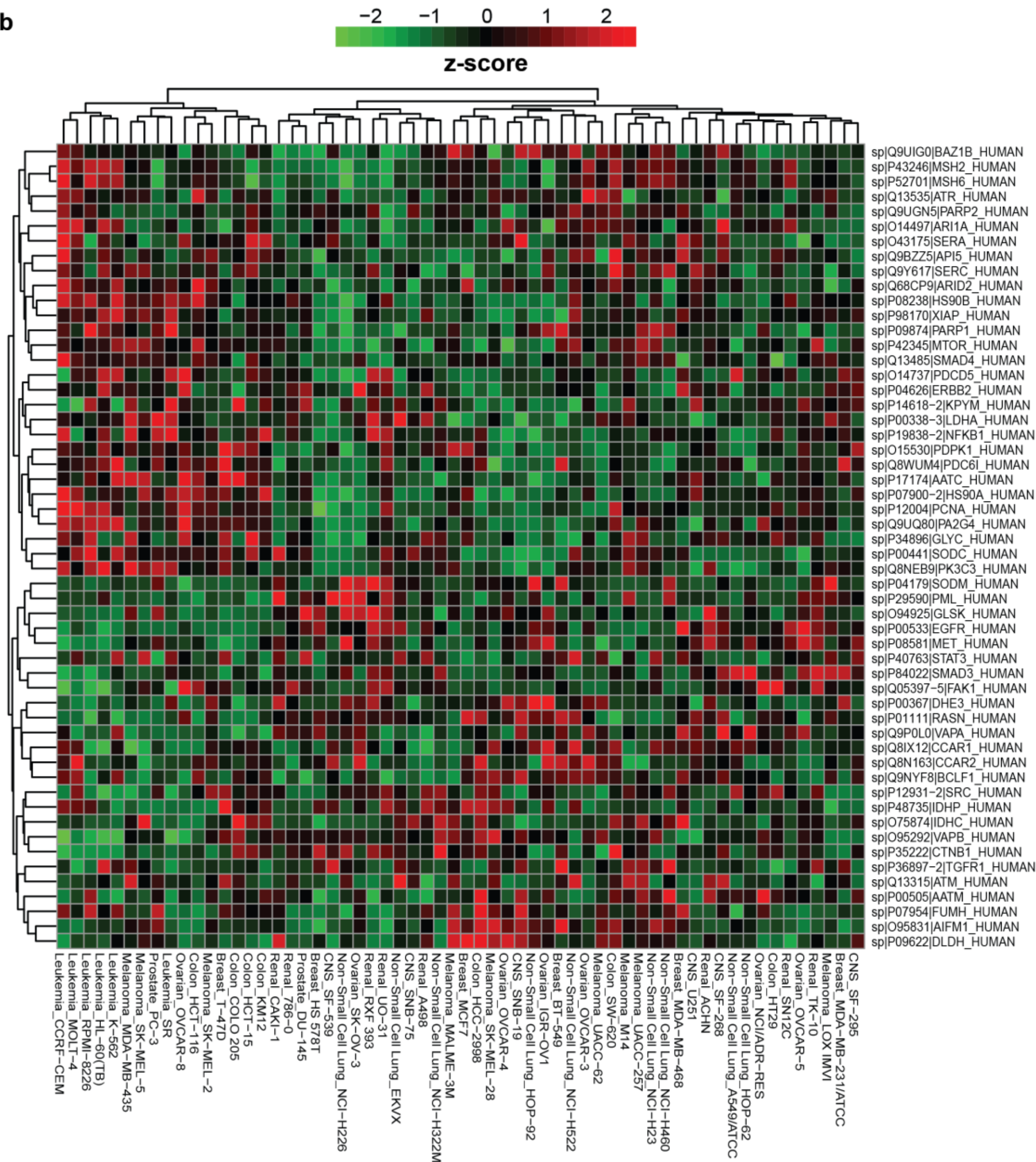
Supplemental Figure 2. Absolute quantification with TOMAHAQ, related to Figure 2. (a) TOMAHAQ can be used to determine absolute quantification by labeling a standard peptide with the isobaric label used to quantify endogenous peptides. Here, the 130 TMT10 tag was used to label an internal standard peptide that was added to the sample at the same level as the higher amount for sample 1 (3.2 fmol) or 2 (800 amol). This quantitative channel was interference free and was used to calculate an absolute amount on column for the 127 and 129 channels. (b) Absolute quantification for single replicates of each sample. The black lines represent the median, which should be 3.2 fmol or 400 amol for sample 1 and 800 amol or 100 amol for sample 2. The box represents the inner quartile range and reflects of the precision of the measurement. (c) Absolute quantification of technical triplicates for the 800 and 100 amol on column channel for Sample 2 (bars represent the mean ± 1 s.d.).



Supplemental Figure 3. Comparing peptide and protein level quantitation from a full proteome to TOMAHAQ analysis, related to Figure 3. Peptide and protein quantitation from a full proteome and TOMAHAQ analyses were compared for eight cancer cell lines (a). For some peptides, the peptide level Pearson correlation was less than the protein level correlation. (b) The full proteome method utilized a data-dependent SPS-MS3 method. The ten most intense peaks (excluding a range (-50, +5 *m/z*) near the precursor) were selected for SPS isolation. In some instances, interfering product ions can result in perturbed ratios. (c) TOMAHAQ utilizes targeted SPS ions as well as online filters to remove impure product ions. As displayed, ions exceeding the fragment ratio tolerance are excluded (red boxes). (d) The quantitation for peptide, 'VEITYTPSDGTQK', is displayed for the full proteome measurement (red circles) and the TOMAHAQ method (blue circles). The boxplots display the quantitation distribution for 51 peptides of IDH1. The median abundance of the peptides for each cell line is well aligned with the TOMAHAQ derived profile.

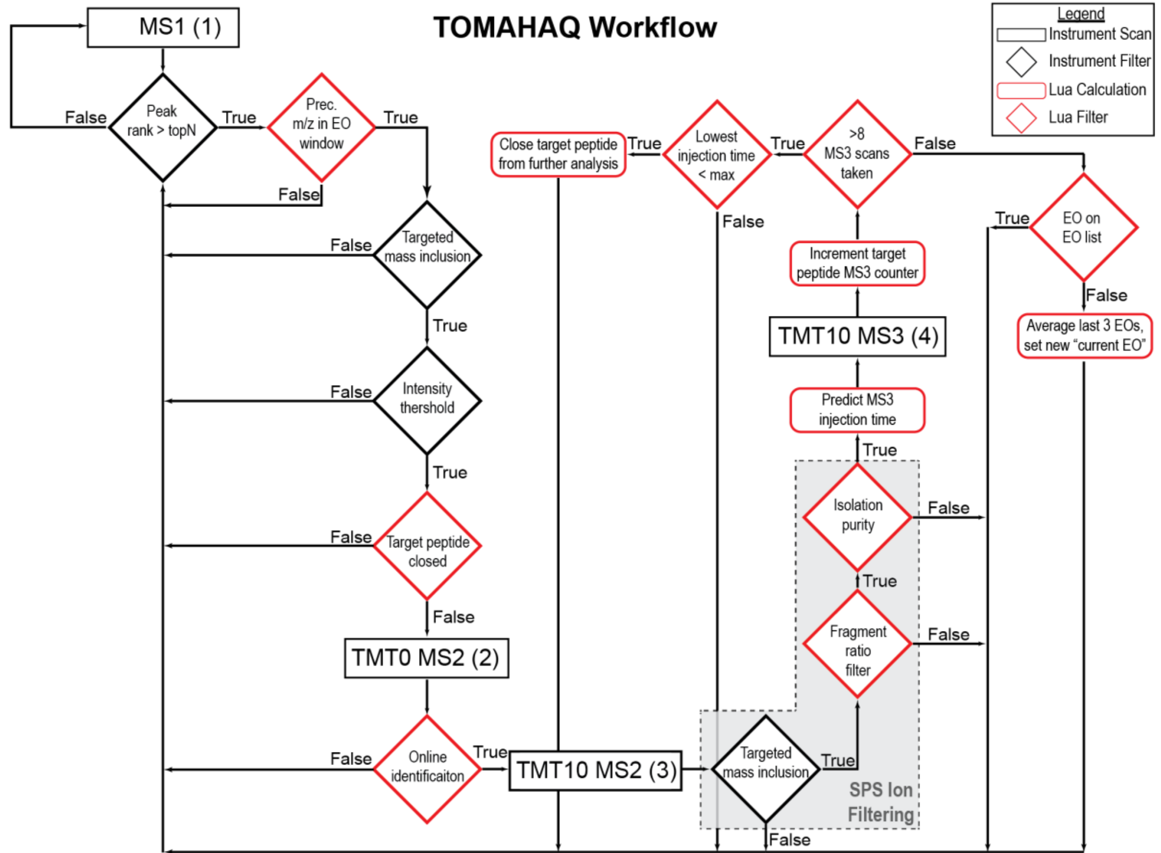
a

	Targeted	Trigger identified	Quantified (≥1 cell line)	60 cell lines (≥1 reps)	60 cell lines (≥ 2 reps)	60 cell lines (all 3 reps)
Peptides	131	128 (98%)	126 (98%)	83 (66%)	83 (66%)	65 (52%)
Proteins	69	69 (100%)	68 (99%)	57 (84%)	54 (80%)	44 (65%)

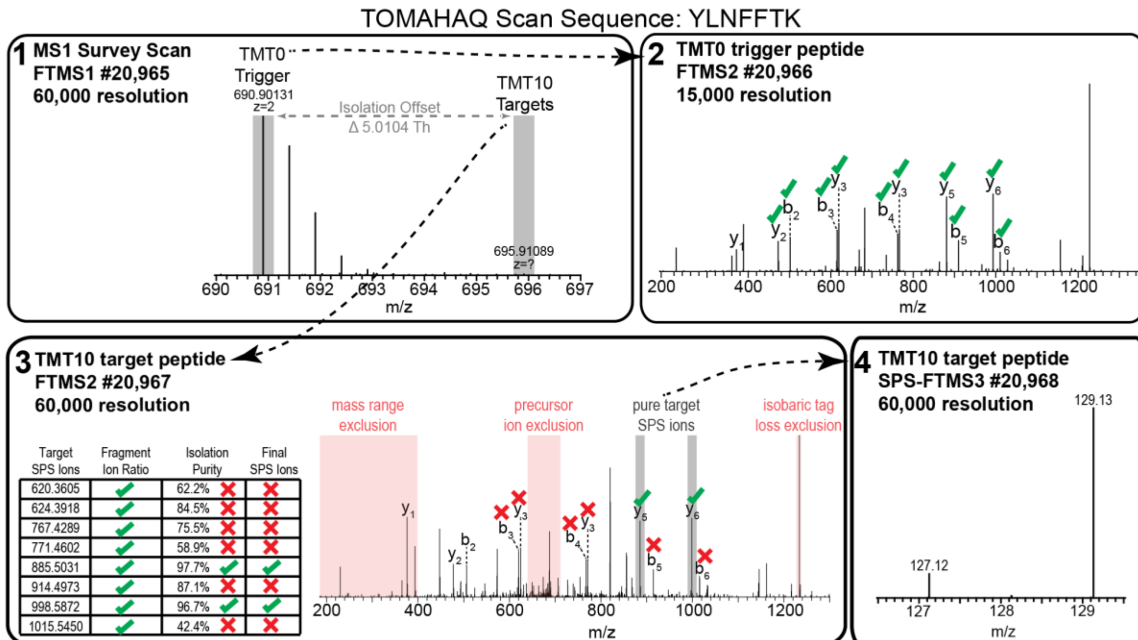


Supplemental Figure 4. TOMAHAQ analysis of the NCI-60 metrics, related to Figure 4. (a) Summary metrics for the TOMAHAQ analysis of the NCI-60. (b) Z-transformed expression profiles for 54 proteins characterized in all 60 cell lines and at least two of the three biological replicates. In each case where the Trigger peptide was identified, a measurement of the Target peptide abundance was made. Target peptide measurements that had a sum S:N of <200 were ultimately discarded.

a



b



Supplemental Figure 5. TOMAHAQ instrument process flow chart and scan sequence, related to Experimental Procedures. (a) A summary of the TOMAHAQ logic workflow. Mass spectrometry scans are represented by black rectangles. Decisions are represented by diamonds; decisions available through the standard method editor are black while custom decisions written in the instrument language (Lua) are red. Custom calculations are represented by red rounded rectangles; these calculations increase the depth and robustness of quantification. (b) A summary of the TOMAHAQ scan sequence: 1) An MS1 survey scan is acquired to determine the presence of the trigger peptide, shown here labeled with TMT0. The trigger peptide is spiked in at an abundance that results in reproducible detection. 2) The trigger peptide is isolated, fragmented by CID, and the resulting FTMS2 spectrum is assessed for the presence of fragment ions belonging to the trigger peptide. If ≥6 fragment ions are present (±10 ppm) the peptide is considered identified and prompts the analysis of the multiplexed target peptides (panel 3), if <6 fragment ions are present subsequent scans are not performed. 3) Identification of the trigger peptide prompts isolation of the target peptides labeled with TMT10, even in cases where the precursor ions is not assigned a charge state. Target peptides are fragmented by CID and the resulting FTMS2 spectrum is assessed to determine the presence of fragment ions belonging to b and y fragments of the target peptide. For all fragment ions that are found the intensity relative to all other fragment ions is calculated and compared to a library spectrum created from an initial analysis of the neat peptides. The isolation purity is then calculated for any ions that pass all previous filters and only ions that have sufficient signal relative to contaminating peaks are isolated and fragmented for quantification. 4) An SPS-FTMS3 scan at 60,000 resolution yields quantification of 10-plex isobaric reagents. For each scan type (1-4) in panel, the corresponding scan within the workflow is noted in panel a (parentheses, square boxes).

Table S1, related to Figure 3 - 5. Target peptides, proteins and associated PubMed links

Table S2, related to Figure 3. Full proteome and TOMAHAQ analysis of eight cancer cell lines

Table S3, related to Figure 4, 5. TOMAHAQ characterization of 180 cancer cell lines

Data File S1. Detailed protocol, related to Experimental Procedures. A comprehensive outline of the sample preparation prior to TOMAHAQ analysis is described above. Recipes for buffers utilized within the preparation are also described.