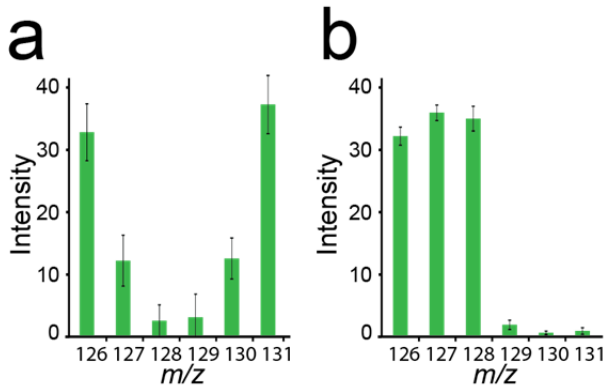
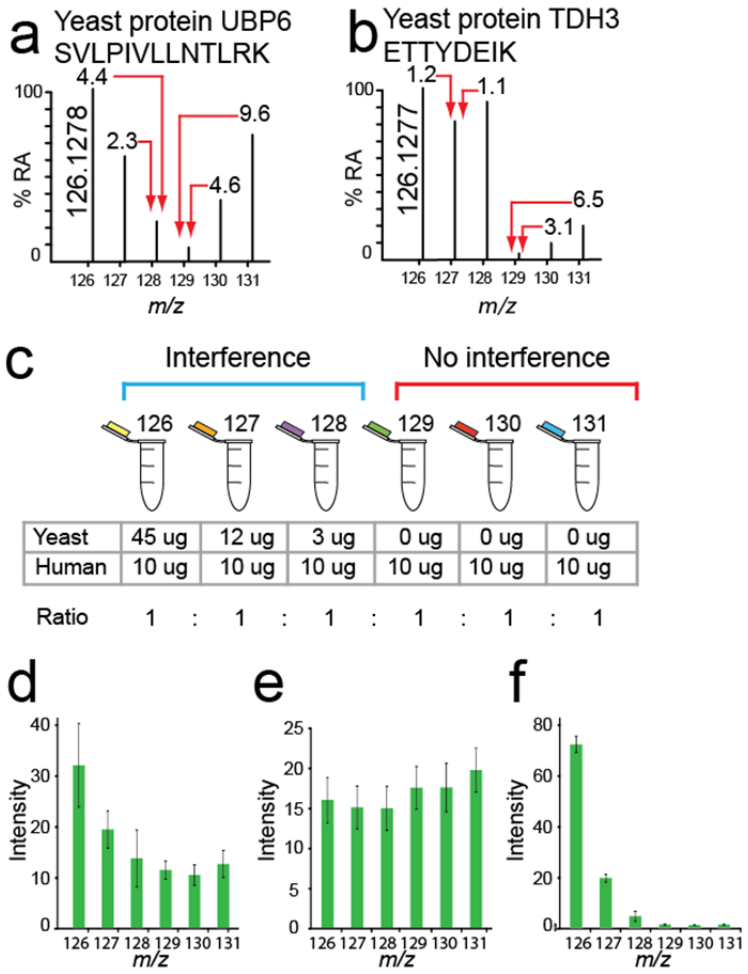
Supplementary Figure 1. Validation of the human-yeast interference model.



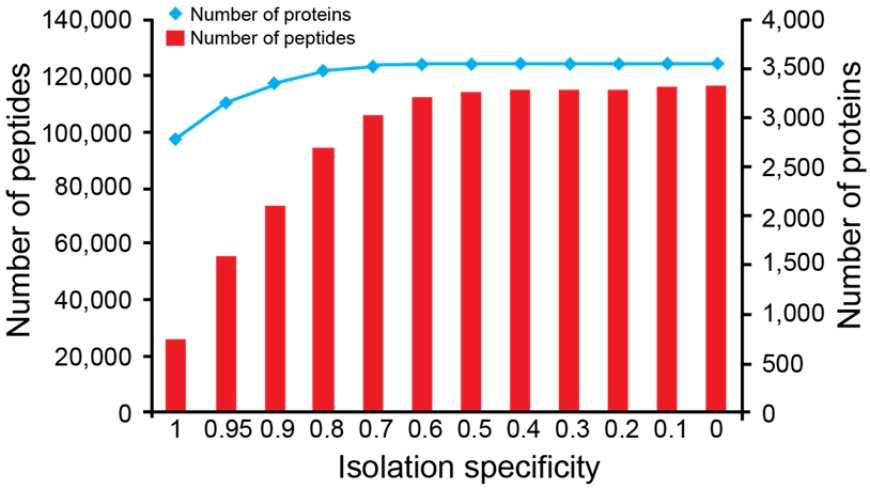
Yeast (**a**) and human (**b**) peptide combinations were made separately and measured prior to the final combination of human to yeast mixtures. The expected 10:4:1:1:4:10 ratio across all channels in the yeast-only mixture validates that yeast peptides were correctly mixed for the interference experiment. The 1:1 ratio across the first three channels in the human only mixture confirms that the human peptides were correctly mixed for the experiment. These data show that a mixing problem did not occur. Error bars represent one standard deviation.

Supplementary Figure 2. Yeast peptide interference examples and the inverse human-yeast interference model.



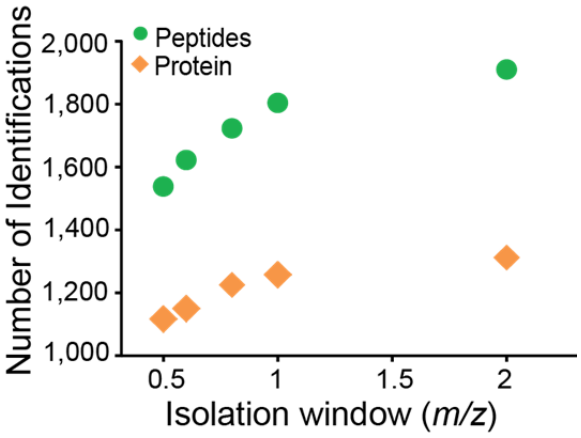
(**a**) Example of a yeast peptide with a low level of human peptide interference. The channels with human interference, where 10:1 and 4:1 ratios were expected, were compressed to 4.4:1 and 2.3:1, respectively. (**b**) Example of a yeast peptide with a high level of human peptide interference. The channels with human interference, where 10:1 and 4:1 ratios were expected, were compressed to 1.2:1 and 1.2:1, respectively. (**c**) Human protein was combined 1:1 in all six TMT channels, while yeast was combined 15:4:1 in the first three channels. Yeast protein was combined at a 50% interference level. (**d-f**) Normalized TMT channel intensities for the inverse interference human-yeast experiment where error bars represent one standard deviation. The ratios from the last three channels of the human-yeast mixed sample (**d**) were as expected (1:1); however, ratios from the first three channels were inflated to 2.5:1 (126/128) and 1.5:1 (127/128) due to the interference effect of interfering yeast peptides. Human (**e**) and yeast (**f**) peptide combinations were made separately and measured prior to the final combination of human to yeast mixtures. The 1:1 ratio across all channels in the human-only mixture validates that human peptides were correctly mixed for the inverse interference experiment. The 15:4:1 ratio across the first three channels in the yeast only mixture confirms that the yeast peptides were correctly mixed for the experiment.

Supplementary Figure 3. The effect of isolation specificity thresholds on post-experimental data filtering.



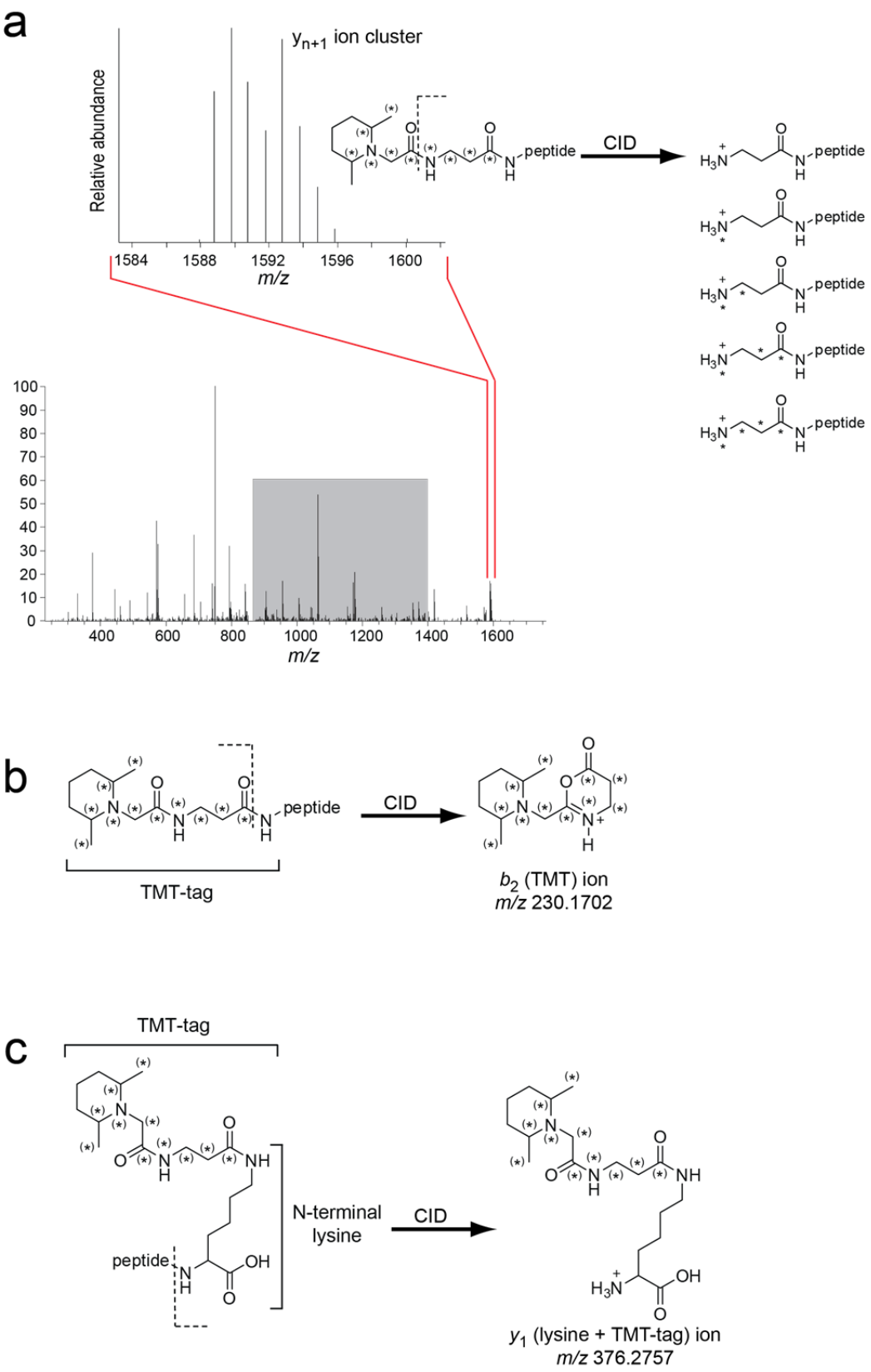
Isolation specificity is defined as the proportion of signal from the total ion current that belonged to the precursor ion. Isolation specificity was graded on a scale from 0 to 1, where 0 represented 100% interference (none of the signal belonged to the precursor); and 1 represented 0% interference (the entire signal belonged to the precursor). In a simulated yeast experiment, where a yeast peptide sample was split equally across the 6 TMT channels, 80% of all identified proteins had an isolation specificity of 1.0, and 94% of proteins had an isolation specificity of ≥ 0.9.

Supplementary Figure 4. The effect of decreasing precursor isolation specificity on peptide and protein identifications using an HCD-MS2 method.



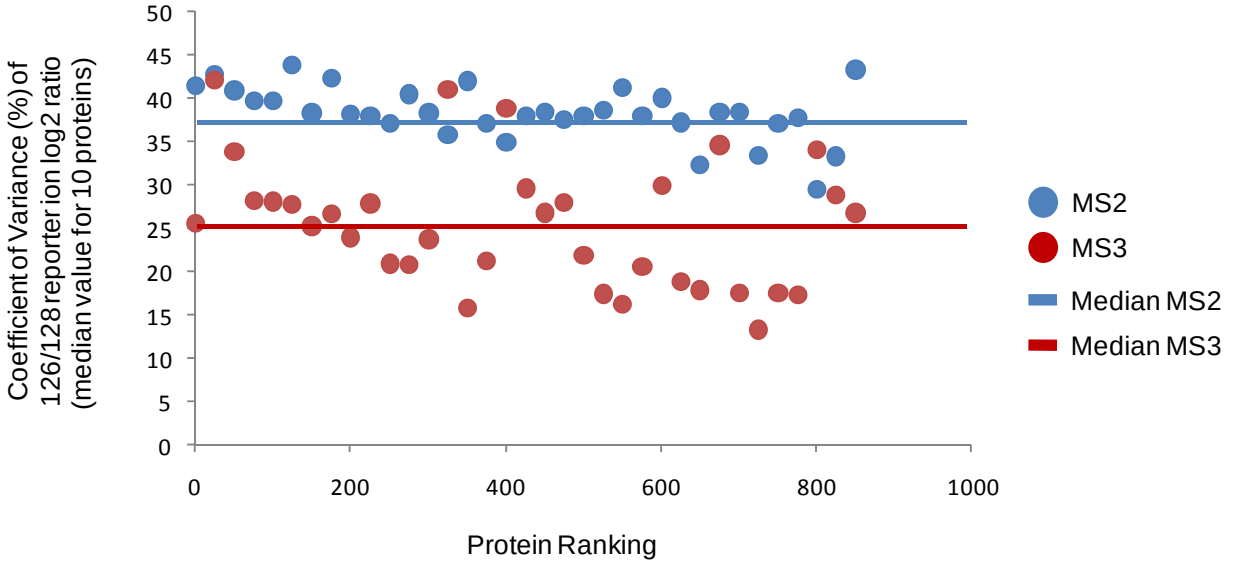
One out of 20 SCX fractions collected from the described multi-proteome sample was used in this analysis. As isolation width was decreased, there was a reduction in the identification of peptides and proteins.

Supplementary Figure 5. Fragment ion selection for MS3.



A typical CID-MS2 spectrum of a doubly charged peptide ion that was acquired in an analysis using the described MS3 method. The grey area in panel A shows the 110-160% *m/z* range relative to the precursor ion *m/z*, from which the most intense fragment ion was selected for HCD-MS3. The *m/z* range for fragment ion selection was limited to avoid MS3 on ions common to the target precursor peptide ion and contaminating ions such as: (a) y_{n+1} ion clusters (insert) carrying a part of the TMT balancing group (asterisks in the chemical structures are used to indicate the position of ^{13}C or ^{15}N stable isotopes; asterisks are in parentheses indicate positions with either light or heavy isotopes), (**b**) b_2-type ions resulting from fragmentation at the amide bond between the TMT tag and the peptide ion (*m/z* 230.1702), (**c**) y_1-type ions composed of the TMT tagged N terminal lysine (*m/z* 376.2757), and neutral loss products with *m/z* values slightly lower than the precursor *m/z*.

Supplementary Figure 6. The precision of quantitative data from the MS3 method is superior to that from the MS2 method over a broad range of protein abundances.



The plot shows the coefficient of variance (CV, %) of the log2 126/128 reporter ion ratio (10:1) for yeast proteins quantified using the MS2 and the MS3 method. The proteins are ranked based on their decreasing number of quantified peptides per protein; each data point gives the median CV calculated for 10 proteins. Only proteins quantified based on at least three peptide spectra with a de-normalized (see Method Section) 128-reporter ion intensity of at least 100 counts were considered. For each protein peptide log2 126/128 ratios were subjected to the Grubb's outlier test (p-value, 0.1) and outliers were removed from the dataset. Furthermore, proteins with an average log2 126/128 ratio determined as an outlier value (Grubb's outlier test; p-value, 0.1) considering all protein log2 126/128 values were not considered in this analysis.

Supplementary Table 1. A comparison of the number of peptide and protein identifications and quantifications from 20 SCX fractions of the human yeast multi-proteome model using the MS2 and the MS3 methods (extended from Table 1).

			Total peptides	Unique peptides	Total proteins	% MS2 method[h]
Identifications[a]		MS2	117,849	43,656	8,216	
		MS3	96,202	38,122	7,578	92%
Quantifications[b]	MS2	Human all 3; Yeast all 6[c]	109,967	41,804	8,803	
	MS3	Human all 3; Yeast all 6	75,330	33,849	7,089	88%
	MS2	Human all 3; Yeast outer 4[d]	112,304	42,171	8,134	
	MS3	Human all 3; Yeast outer 4	81,901	35,576	7,314	90%
Quantifications (human only)	MS2	All 3 channels[e]	86,026	33,626	6,074	
	MS3	All 3 channels	65,565	29,214	5,617	92%
Quantifications (yeast only)	MS2	All 6 channels[f]	23,941	8,178	2,009	
	MS3	All 6 channels	9,765	4,635	1,472	73%
	MS2	Outer 4 channels[g]	26,278	8,545	2,060	
	MS3	Outer 4 channels	16,336	6,362	1,697	82%

[a] All human and yeast identifications.
[b] All human and yeast quantifications.
[c] Human all 3 represents human peptides with signal > 0 in TMT channels 126, 127 and 128. Yeast all 6 represents yeast peptides with signal > 0 in all TMT channels.
[d] Human all 3 represents human peptides with signal > 0 in TMT channels 126, 127 and 128. Yeast outer 4 represents yeast peptides with signal > 0 in TMT channels 126, 127, 130 and, 131.
[e] All 3 channels represents peptides with signal in TMT channels 126, 127, and 128.
[f] All 6 channels represents peptides with signal in all TMT channels.
[g] Outer 4 channels represents peptides with signal in channels 126, 127, 130 and, 131. Peptides were filtered to give a protein FDR of 1.5%.
[h] The proportion of identified or quantified proteins using the MS3 method compared to the MS2 method

We have chosen to represent yeast identifications and quantifications using two different filters: “all 6” and “outer 4” to provide numbers based on two different levels of stringency. “Yeast all 6” or “all 6 channels” is the most conservative, where all six TMT reporter ions are required to be present (that is, signal > 0). “Yeast outer 4” or “outer 4 channels” is less stringent, where TMT channels 128 and 129 are allowed to have an absence of signal. These two channels are allowed to be empty due to the experimental design of the human yeast multi-proteome model, where channels 128 and 129 have 10-fold less protein compared to channels 126 and 131, and 4-fold less protein compared to channels 127 and 130.