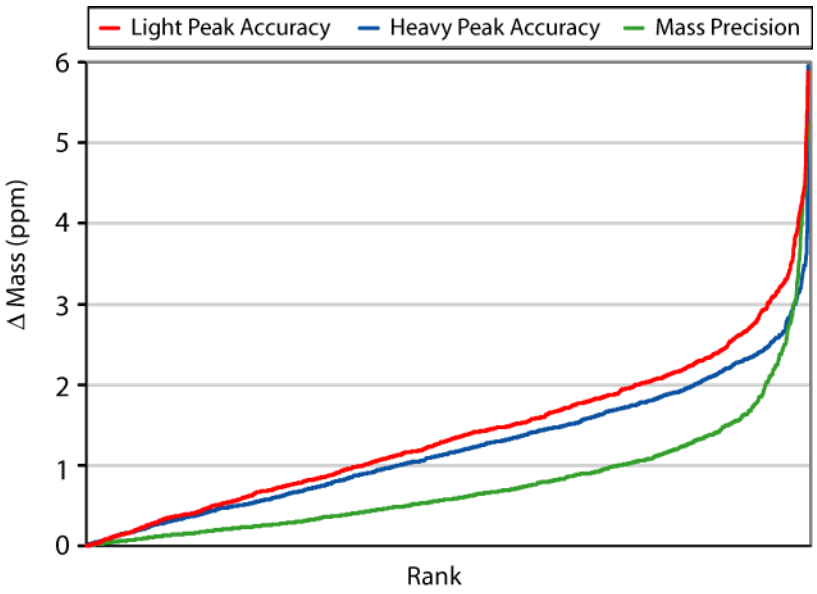
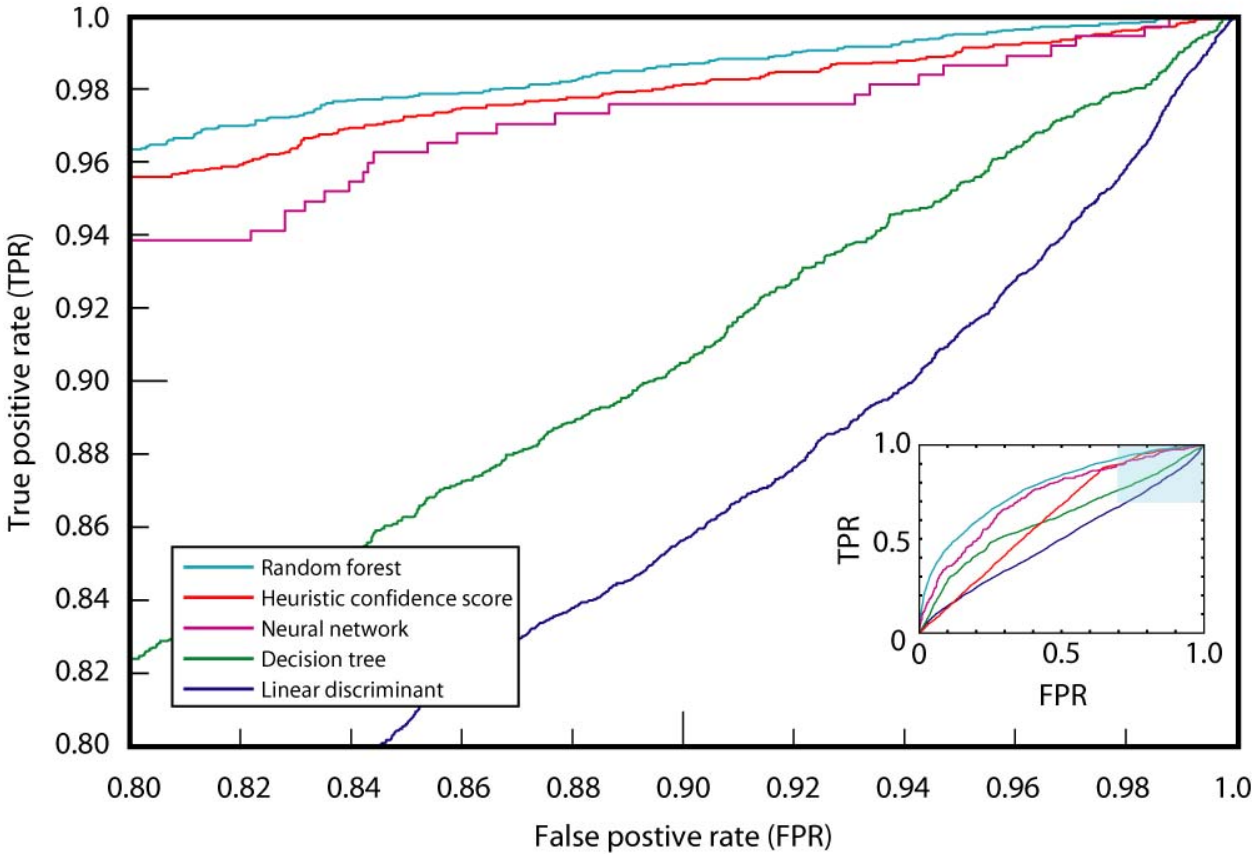
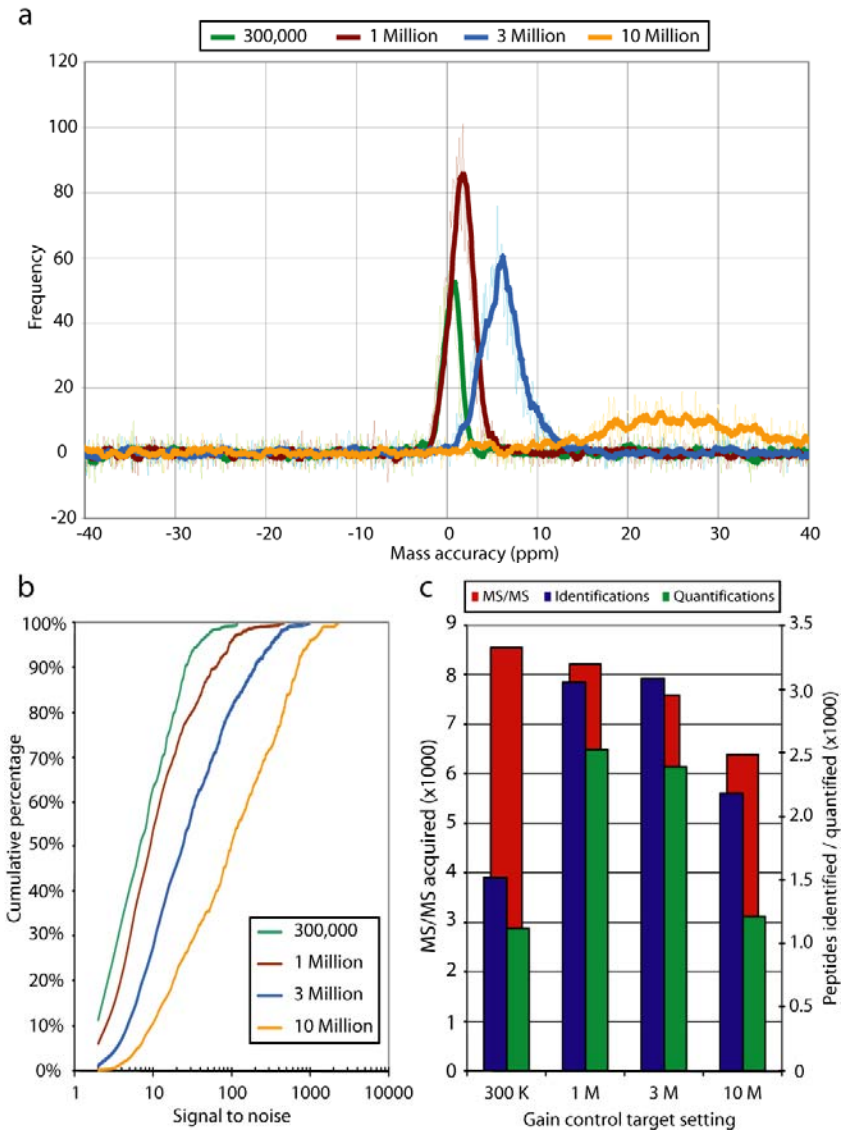


Supplementary Figure 1. Mass precision is a more consistent indicator of signal peaks than mass accuracy. Comparing the mass accuracy of the light peptide (red), heavy peptide (blue), and the difference between the two values (green) for over 3,000 confidently-assigned peptides from a 1:1 test mixture in ascending rank order illustrated that the difference between the light and heavy peptide species' deviation (mass precision) from the theoretical mass was lower than either species' deviation from its theoretical value (mass accuracy). This principle was exploited in the creation of the mass precision (MP) score.

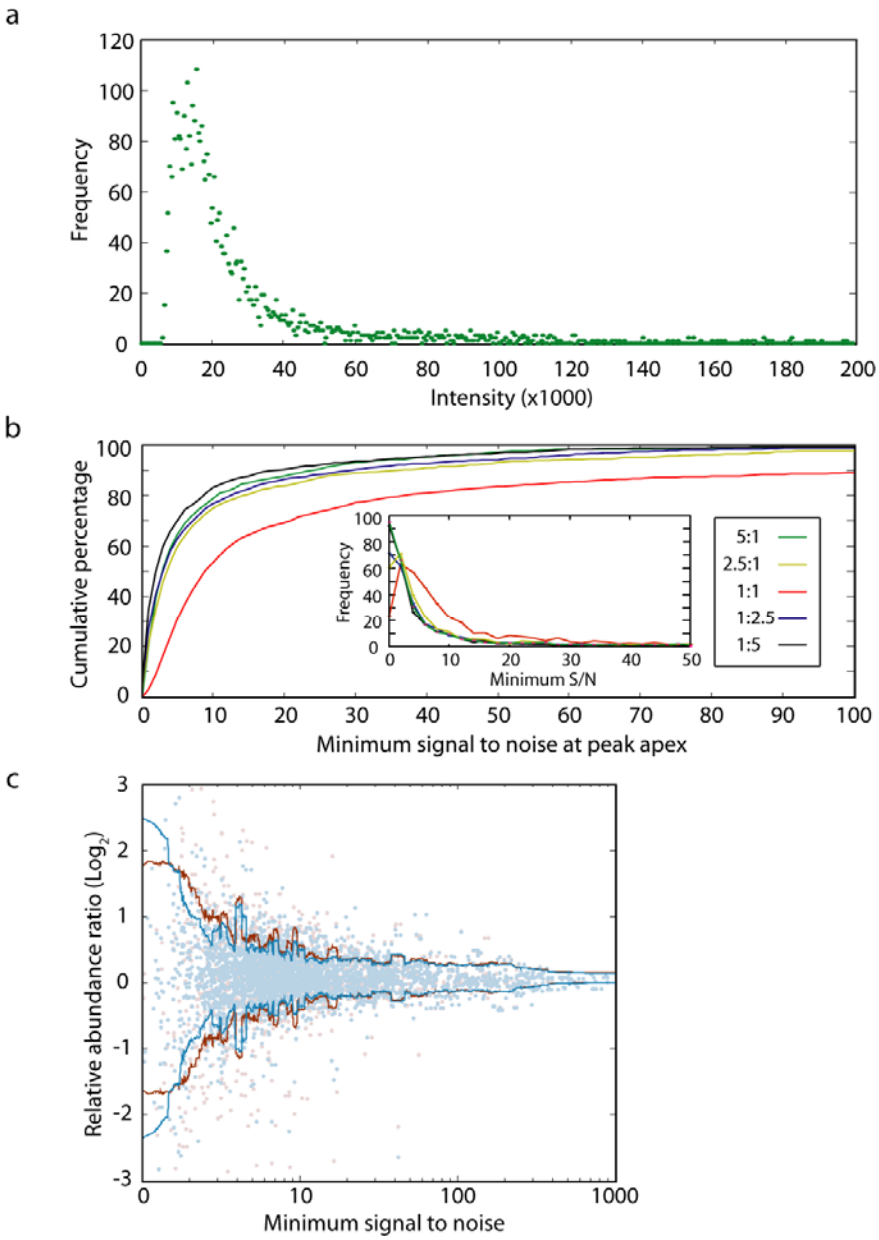


Supplementary Figure 2. Receiver operating characteristic (ROC) curves for common machine learning algorithms applied to assess ratio reproducibility. Feature information was gathered from 15,044 separate quantitation events taken from duplicate instrument analyses of five SILAC samples mixed at different proportions (5:1, 2.5:1, 1:1, 1:2.5, and 1:5). Each quantitation event was classified as correct if the relative abundance ratios generated from repeated analyses of the same peptide in duplicate runs differed by no more than 5%. This data was then used to test and train five different machine learning algorithms using a five-fold cross validation approach. Linear discriminant analyses and binary decision trees (pruned with a maximum of 10 observations per leaf node) were generated from the data. A heuristic score, developed in-house, weighed the outcome of a number of Boolean predictors of conditions which correlate with reliable quantitation (high S/N, large number of observations across the chromatographic peak, etc.). For the neural network analysis, a single-layer feed-forward network was used with 20 hidden nodes and 2 output nodes. The random forest algorithm was run in classification mode with replacement to grow 500 trees with a terminal node size of 1 or greater. The inset depicts the ROC curve across the entire range of true and false positive rates.



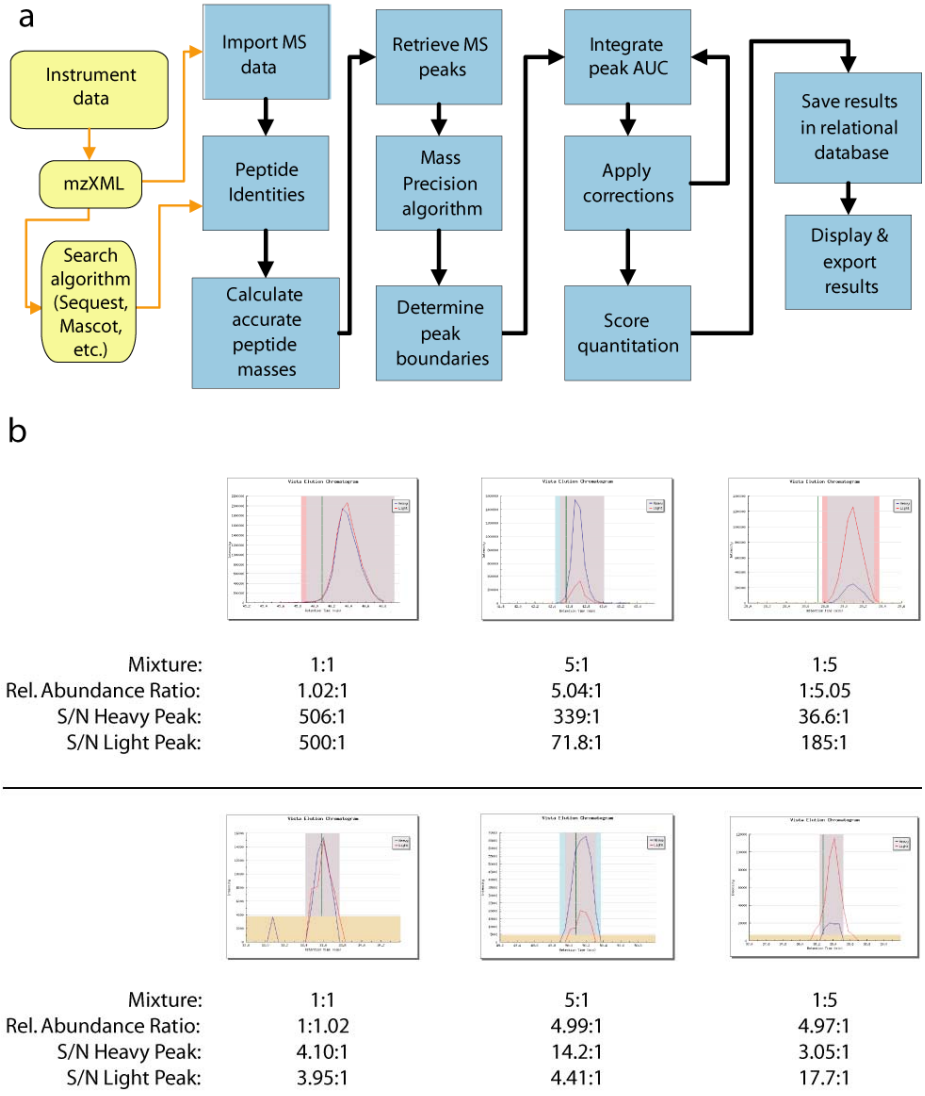
Supplementary Figure 3. Relationship between mass accuracy, signal-to-noise ratios, and instrument gain control settings in FT-ICR-based experiments. Increasing the number of ions permitted into the ICR cell of the mass spectrometer produces tradeoffs between mass accuracy and signal-to-noise. For quantitative analyses, it should be desirable to optimize both. **(a) Mass accuracy inversely correlated with increasing ion count.** Four separate 80-min LC-MS/MS analyses were collected from a 1:1 SILAC-labeled mixture, where the maximum number of ions admitted into the ion trap was varied via the automatic gain control setting from 300K to 10M ion counts. Data are shown as a 5-point moving average of peptide observations binned in 0.1 mass unit increments. Increasing the number of ions within the ICR cell reduced the mass measurement accuracy due to ion space-charging effects. **(b) Signal-to-noise ratios improved with increasing instrument gain control.** S/N ratios for peptide ions generated from confident peptide assignments were determined for the four analyses described above. Any point along the graph describes the percentage of peptides identified at or below a particular S/N threshold. Increasing the number of ions within the ICR cell produced an overall increase in the relative S/N levels for peptides identified in that particular instrument run. **(c) Relationship between instrument gain control settings, peptide identifications, and successful quantitation events.** As the number of ions analyzed increased, the duty cycle lengthened (not shown) and fewer MS/MS spectra were collected per analysis (red bars). The number of correct peptide identifications (blue bars) and corresponding quantification events (green

bars) indicate the 1M and 3M settings produced the highest identification and quantification rates. These settings had a balance of higher S/N levels and high mass accuracy.



Supplementary Figure 4. Signal-to-noise ratios and quantitative accuracy in Orbitrap data. (a) Spectral peak intensity distribution for Orbitrap data. A histogram of spectral peak intensity information, similar to Figure 2a, recorded across a defined retention time (± 20 MS scans) and mass window (± 25 *m/z*) illustrates the distribution of peaks arising from both signal and noise. When compared to FT-ICR data, the range of intensities in Orbitrap data was broader (fewer noise peaks were present). **(b) The majority of peptide identifications from Orbitrap data are from low signal-to-noise events**. Similar to data collected on an FT-ICR instrument, the majority of peptides in the 1:1 mixture were identified with S/N <10, and the median S/N value was 9.2 (n = 4,816). Other mixing ratios produced S/N distributions with more peptides identified at low S/N levels; in the 5:1 mixture, the median value was only 3.03. Inset shows a histogram of the S/N distribution for all mixtures. **(c) Ratio measurement accuracy correlates with signal-to-noise.** A 100-point moving window of the mean standard deviation of 4,184 observed abundance ratios from a 1:1 test mixture, similar to Figure 3a, compares the variance in ratio measurements obtained using only a high mass accuracy filter (red line, red points), or with the mass

precision algorithm (blue line, blue points), against S/N level. Ratio variability was negatively correlated with S/N. As with the FT-ICR, the mass precision algorithm significantly improved the accuracy of ratio measurement at low signal-to-noise (Ansari-Bradley test: $p = 6.1 \times 10^{-4}$; n = 2,107).



Supplementary Figure 5. Overview of the Vista quantitation algorithm and representative output. (a) Overview of the Vista workflow. Spectral data collected from high mass accuracy instruments were loaded into a relational database along with peptide identification data from compatible search algorithms. Accurate peptide masses were then calculated and putative spectral peaks from each labeled peptide were extracted from MS data. Spectral peaks were filtered and chromatographic peak boundaries determined via a mass precision algorithm (see text). The extracted ion chromatogram was integrated by examining intensity over time, appropriate corrections were applied, and the results evaluated for accuracy using a Random Forest classifier and a heuristic score. All results were saved in a relational database for further analysis and export. **(b) Examples of software output at varying relative abundance ratios**

and varying S/N levels. The extracted ion chromatograms for light (red) and heavy (blue) peptide species from samples mixed at three different proportions (5:1, 1:1, 1:5) at both high and low signal levels are displayed. The tan bars at the bottom of each graph indicate the relative noise level, as described in the text.