

Abstract

iTRAQ labeling has opened the doors for the easy quantitative assessment of proteins using tandem mass spectrometry. iTRAQ labeled experiments are often limited to 4- or 8-plex but daisy chaining can be employed to link several samples together. There are, however, inherent difficulties in normalizing within and between iTRAQ experiments due to variation in reagent and protein concentrations. We have developed Scaffold Q+ to address the challenges of normalization in iTRAQ labeled quantitative experiments. In a single experiment the intra-channel reporter ion ratios are median normalized. An experimental design in which multiple MS/MS analyses are daisy chained together is median normalized between references first. Our method also allows users to normalize to housekeeping or spiked proteins. Finally, Scaffold Q+ gives users the power to combine multiple iTRAQ datasets consisting of technical replicates and multiple MS/MS runs labeled with common reporter ion tags.

Methodology

The iTRAQ labeling method uses isobaric reporter ion tags for relative quantitation of biological samples. Each sample is labeled independently and then pooled for MS/MS analysis. Spectral reporter ion intensities are used to determine relative quantitation for peptides and proteins in the experiment.

iTRAQ data is commonly normalized using the average reporter ion intensity. This method is susceptible to outlier ratios. iTRAQ data can be particularly tricky because it can be difficult to estimate extreme ratios due to variability in the noise level as well as isobaric impurities inherent to iTRAQ experiments.

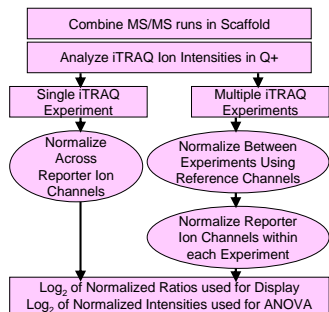


Figure 1: Multiple MS/MS runs can be examined using Scaffold Q+.

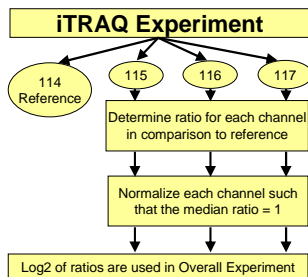


Figure 2: The individual reporter ion channels within an iTRAQ experiment are normalized by adjusting the median ratio between reporter ion and reference ion to 1. The reference ion channel is selected based on experimental design.

Reporter ion values below a certain threshold should be treated as missing values for which the specific intensity is impossible to determine. As the intensity of the reference or reporter ion decreases the error in their ratio increases dramatically. This error can be transferred to all values in a sample when using average normalization.

In situations when multiple iTRAQ experiments are combined, common approaches directly link reporter ion intensities between proteins across the references. This approach masks the technical variance between experiments. This variance is often larger than intra-sample variance and is important to consider.

In this work MS/MS samples are loaded into Scaffold after database searching is completed. In Scaffold proteins and peptides can be filtered to specific statistical confidence levels. Once protein assignments are confirmed, reporter ion intensity information is extracted from the spectra associated with proteins that meet the defined filter settings.

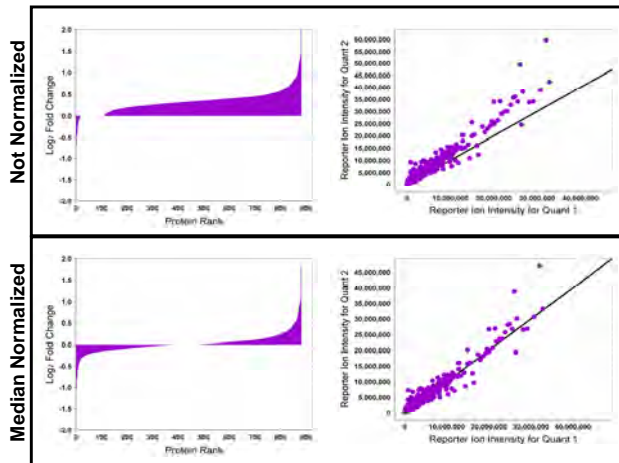


Figure 3: The two samples in this data set were technical replicates. Before normalization this data set shows an uneven distribution of protein fold change and a skewed reporter ion intensity scatter plot. The false discovery rate is 9.3% at 1.5 fold and 1.6% at 2.0 fold. After normalization, the data set shows symmetric distribution of fold change across the sample and the reporter ion intensity scatter plot is grouped around a 45° line. The false discovery rate is reduced by a factor of three to 2.5% at 1.5 fold and 0.5% at 2.0 fold.

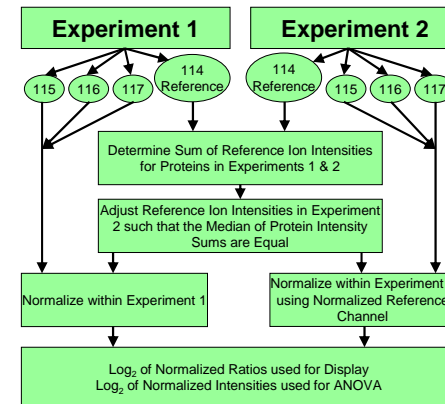


Figure 4: When combining multiple iTRAQ experiments Q+ first normalizes between all experimental reference channels. The normalized references are then used to further normalize ratios within each individual experiment.

Reporter ion channels are typically median normalized with respect to a user-defined reference sample. Employing median ratio normalization ensures that the calculated ratios are no longer dependent on missing or suspect ion intensities because those ratios are not used in the calculation.

An experimental design that daisy chains multiple iTRAQ experiments together using a single reference (but possibly different quantitative samples) can be normalized with an additional step. Before intra-experiment normalization is done, the separate experiments are linked together. The normalization factor is calculated as the median of the summed reporter ion intensities for each protein in the linked reference channels. Normalizing across reference samples in multiple iTRAQ experiments reveals the technical variance between those samples, which can be considered in the final analysis.

Log₂ values of the reporter ion to reference ratios are used to force symmetry between up and down regulated proteins. Statistical test such as the t-Test and ANOVA in Scaffold Q+ are based on Log₂ of the underlying median normalized reporter ion intensities. Testing using the ratios alone ignores variance in the reference channel. Testing using the reporter ion intensities ensures that reference channel variation is considered, while log normalization forces the tests to be magnitude independent.

Conclusion

Scaffold Q+ allows users to robustly analyze multiple iTRAQ experiments simultaneously. Scaffold Q+ has also been extended to analyze iTRAQ 8-plex and TMT data. A free trial is available at <http://www.ProteomeSoftware.com>