A Comparison of Label-Free Quantitative Technologies: MaxQuant (MS) vs. Skyline (MS) vs. Spectral Counting (MS/MS) vs. Average TIC (MS/MS) vs. Top3 TIC (MS/MS)

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Abstract

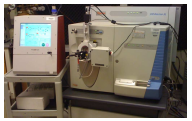
The proteomics community continues to develop strategies for label-free quantification from shotgun LC-MS/MS experiments but no single technique has emerged as universally acceptable. Researchers use a variety of both MS level quantification (Peak Area) and MS/MS level quantification (Total Ion Current and Spectral Counting) and all have shown both advantages and disadvantages depending upon the application. Here, we examined several different methods of label-free quantification from two 48-human protein mixtures, Universal Protein Standards (UPS1 and UPS2) from Sigma. UPS1 contains equal concentrations and UPS2 contains varying concentrations. We compared the quantitative difference in terms of ratio between UPS2 and UPS1 and assesses the methods showing the highest accuracy.

Methods

We digested the using trypsin and divided the final peptide mixtures into 4 separate aliquots. The digested UPS1 standard contained 1.25 pmol per tube and the UPS2 standard contained a range from 12.5 pmol to 125 amol per tube. We ran the UPS standards separately and in duplicate using shotgun/data-dependent LC-MS/MS with a Thermo Orbitrap XL mass spectrometer coupled to a Proxeon EASY-nLC at 300 nL/min using a 15 cm x 75 μm C_{18} microcapillary column with a 120 min gradient. MS was collected in FT profile mode at 30,000 resolution and MS/MS (Top6 IT-CID) in centroid mode. MS/MS spectra were searched using Sequest for Scaffold TIC quantification, Mascot for Skyline MS1 quantification and Andromeda for MaxQuant MS1 LFQ quantification. The reversed and concatenated IPI Human_v3.87 was used. In some cases, the lowest concentration of UPS2 proteins were not detected and a baseline value was used for calculations.

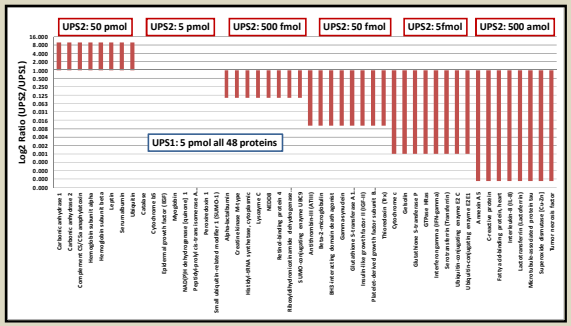
We used the following methods to compare label-free quantification:

1. MaxQuant 1.2.2.5 for MS1 Label-Free Quantification (LFQ)
2. Skyline 1.3 for Total Area MS1 Precursor Filtering
3. Scaffold 3.5 for Average TIC
4. Scaffold 3.5 for Top 3 TIC
5. Scaffold 3.5 for Total TIC
6. Scaffold 3.5 for Spectral Counting

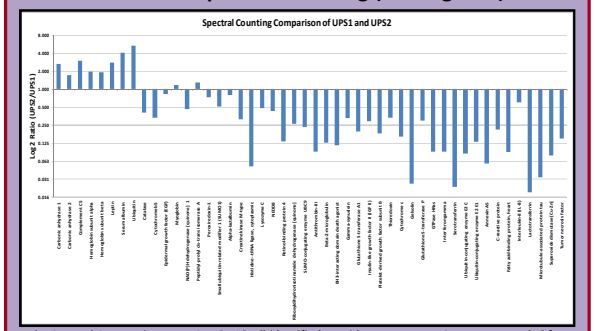


The question: ***"Which label-free method best recapitulates the known concentration difference between UPS1 and UPS2"?***

Actual Concentration Ratios for Universal Protein Standards (UPS) 1 & 2 for 48-Protein Mixture

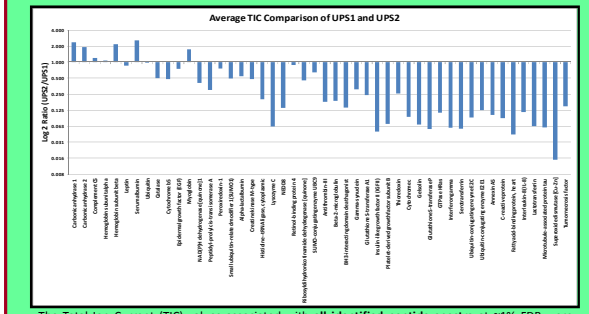


Scaffold: Spectral Counting (unweighted)



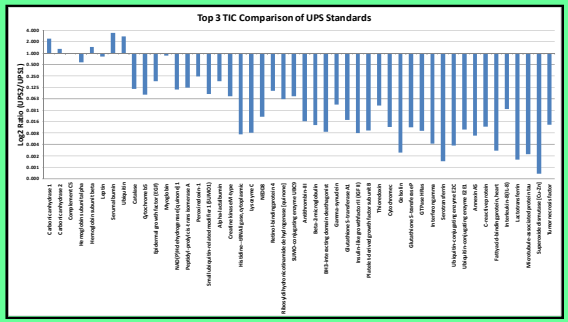
The Spectral Count values associated with **all identified peptide spectra** at ~1% FDR were **totaled** for each identified protein using Scaffold 3.5 software. Replicate LC-MS/MS runs for UPS1 and UPS2 were averaged and the ratios of UPS2/UPS1 were calculated and plotted above.

Scaffold: Average MS² TIC (all peptides)



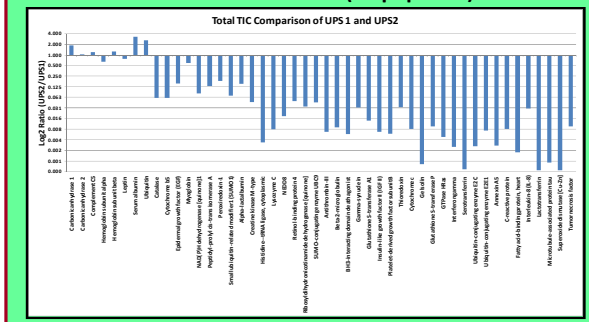
The Total Ion Current (TIC) values associated with **all identified peptide spectra** at ~1% FDR were **averaged** for each identified protein using Scaffold 3.5 software. Replicate LC-MS/MS runs for UPS1 and UPS2 were averaged and the ratios of UPS2/UPS1 were calculated and plotted above.

Scaffold: Average Top 3 MS² TIC (3 most intense peptides)



The Total Ion Current (TIC) values associated with the **top 3 most intense peptide spectra** at ~1% FDR were **averaged** for each identified protein using Scaffold 3.5 software. Replicate LC-MS/MS runs for UPS1 and UPS2 were averaged and the ratios of UPS2/UPS1 were calculated and plotted above.

Scaffold: Total MS² TIC (all peptides)



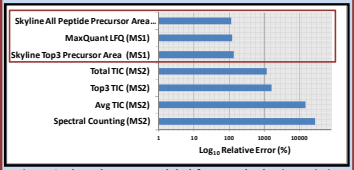
The Total Ion Current (TIC) values associated with **all identified peptide spectra** at ~1% FDR were **totaled** for each identified protein using Scaffold 3.5 software. Replicate LC-MS/MS runs for UPS1 and UPS2 were averaged and the ratios of UPS2/UPS1 were calculated and plotted above.

Reproducibility for Replicate Runs

UPS1	Label-Free Method	UPS2
R^2 = 0.927	Skyline Top 3 Precursor Intensity-MS1	0.985
0.920	Spectral Counting (Scaffold)-MS2	0.993
0.875	Skyline All Peptide Pecursor Intensity-MS1	0.957
0.787	MaxQuant Precursor LFQ-MS1	0.998
0.631	Total TIC (Scaffold)-MS2	0.996
0.338	Top 3 TIC (Scaffold)-MS2	0.960
0.264	Avg TIC (Scaffold)-MS2	0.767

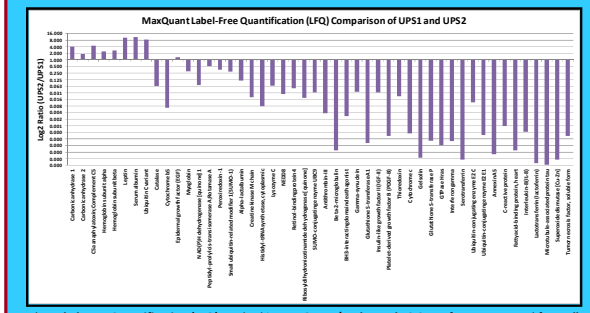
LC-MS/MS shotgun runs were run in replicates from different aliquots for UPS1 and UPS2 48-protein mix standards. The table shows the **coefficient of determination (R^2) from replicate runs** analyzed by each label-fee method.

Average Accuracy of Label-Free Methods



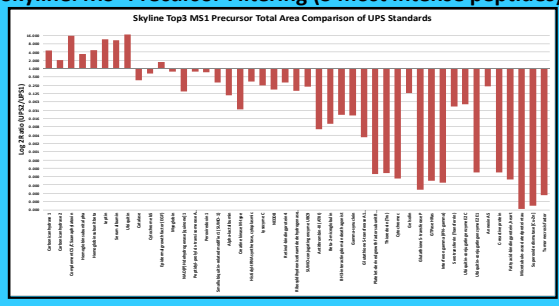
The **MS1 based precursor label-free methods** showed the **highest quantitative accuracy**. Some MS2 TIC methods fared well, though spectral counting showed the lowest accuracy.

MaxQuant: MS¹ LFQ Precursor Filtering (all peptides)



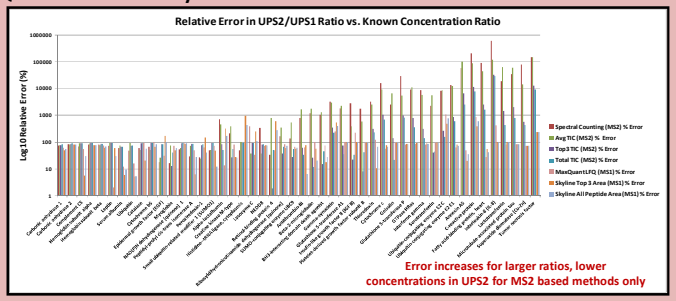
The **Label-Free Quantification (LFQ)** method in **MaxQuant/Andromeda 2.2.5** software was used from **all identified MS1 precursor peptide intensities** at 1% FDR for each identified protein. A minimum LFQ ratio count of 1 was chosen and retention time matching between runs was not used. Replicate LC-MS/MS runs for UPS1 and UPS2 were averaged and the ratios of UPS2/UPS1 were calculated and plotted above.

Skyline: MS¹ Precursor Filtering (3 most intense peptides)



Precursor ion filtering in **Skyline 1.3** software was used for the **Top3 total area MS1 precursor peptides** at 95% Probability for each identified protein. All high quality peptide **total area** values were **averaged** for each identified protein. Peptides were matched between runs and validated manually. Replicate LC-MS/MS runs for UPS1 and UPS2 were averaged and the ratios of UPS2/UPS1 were calculated and plotted above.

Quantitative Accuracy for Label-Free Method vs. Known Concentration Ratios



Error increases for larger ratios, lower concentrations in UPS2 for MS2 based methods only

The **accuracy** of each **label-free** method was assessed versus the known **ratio of UPS2/UPS1** by calculating a **percent error (%)** for each of the 48 proteins using the MS1 and MS2 based methods. The accuracy was poor for the low level proteins in the UPS2 mixture by the MS2 methods and we detected a systematic error of ~70%. **MS1 label-free methods were consistent across concentration ratios.**

Conclusions

- The highest scoring methods in terms of *accuracy* to known UPS2/UPS1 concentration ratios were based on MS1 peak intensity filtering (MaxQuant LFQ and Skyline Precursor Total Area)
- The most reproducible methods between replicate runs calculated by R^2 were MS¹ based methods (MaxQuant LFQ and Skyline Total Area Top 3 Peptides) and Spectral Counting (Scaffold)
- The MS1 based methods were consistent in quantitative accuracy across all concentrations and ratios while the MS2 methods failed to produce accurate results at low concentration/large ratios
- MS1 and MS2 based methods have advantages and disadvantages: Reproducibility may be more important for biomarker discovery while accuracy is more critical for stoichiometric calculations
- We detected a systematic error in the detected UPS2/UPS1 ratio