Using stable isotope labeling by amino acids in cell culture (SILAC) to improve data independent acquisition (DIA) relative quantification

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Background and Unmet need

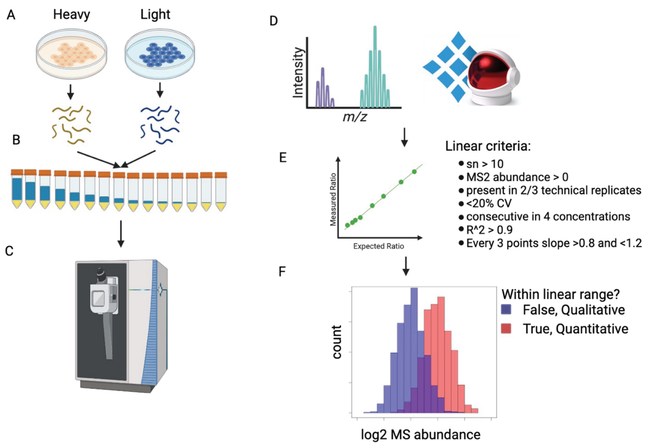
• Deeply profiling clinical samples is important to discover and develop biomarkers that aid in drug development. Mass spectrometry based proteomics approaches are well suited to address this.

• Shotgun data dependent acquisition (DDA) is stochastic making quantitation unreliable, and using labeling approaches is expensive and limits sample numbers.

• Data independant acquisition (DIA) methods have shown promise in delivering complex proteomes and identifying thousands of proteins with less stochasticity. However, relative quantitation and its robustness for DIA runs is poorly understood.

• Using serial dilutions of SILAC labeled cells and a script that was developed and described herein, linear ranges for peptides in any matrix can be defined and quantitative thresholds can be established to help filter datasets for high confidence relative quantitation.

Experiment Design

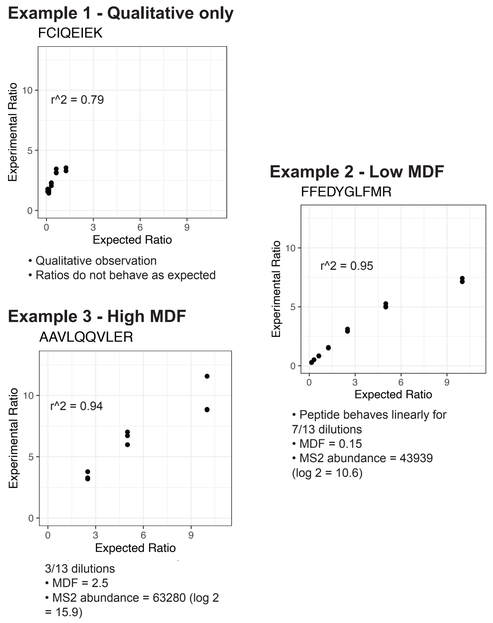


A) Heavy and light SILAC labeled cells are grown separately and frozen into cell pellets. Cells are lysed, proteins are subsequently reduced, alkylated, and digested into peptides. **B)** Serial dilutions are created with light peptides, while heavy peptides are held at a constant rate. **C)** Peptides are injected and spectra are acquired on an Orbitrap Exploris 480*. **D)** Mass spectrometry data is searched in Spectronaut. **E)** Peptides are filtered based on quantitative criteria specified by the user. Sn= signal to noise, CV = coefficient of variation, Every 3 points slope = piece wise slope analysis for every 3 concentrations within a peptide's linear concentration range. **F)** Peptide abundances are plotted and included as within the linear concentration range of a peptide (above MDF, Quantitative) or below the linear concentration range of a peptide (below MDF, Qualitative)

*65 minute data aquisition independent method. In brief, MS1: 120,000 resolution, scan range: 350-1400 m/z, DIA scans: 15,000 resolution, 20 m/z window with 1 m/z overlap, precursor scan range: 400-900m/z.

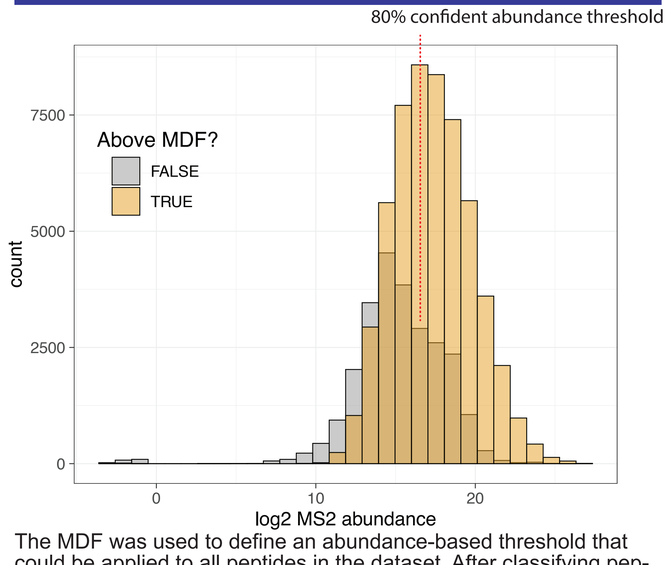
Results

Linear range and "MDF"



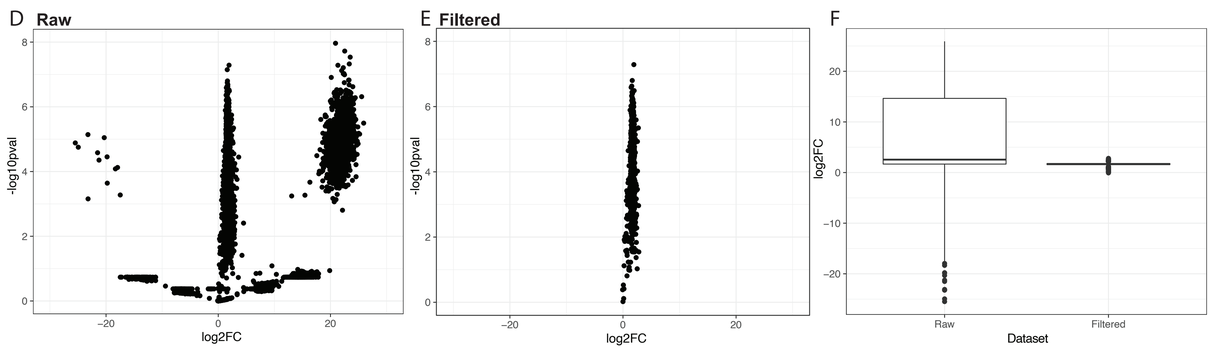
A list of peptides that behave linearly when serially diluted are generated through a series of checks that the user can adjust to their own discretion. Peptides were classified as either quanlitative or quantitative, and for the quantitative peptides an MDF (minimal dilution factor) was determined.

Quantitative Threshold



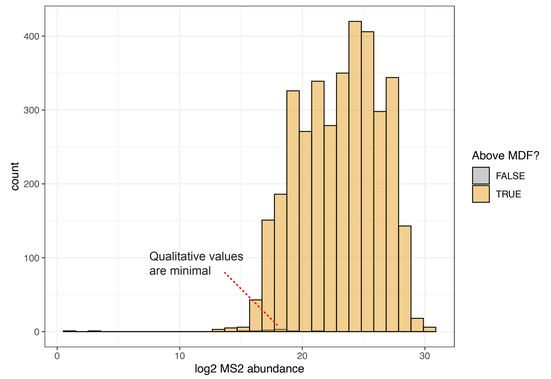
The MDF was used to define an abundance-based threshold that could be applied to all peptides in the dataset. After classifying peptide MS2 abundances as relatively quantitative or qualitative, protein MS2 abundances were plotted. The above histogram displays an inflection point of mostly quantitative peptides versus qualitative peptides. Using a thresholding algorithm from the workflow, the user can define the desired percentage of quantitative versus qualitative peptides (e.g. 80% = 0.8) and will receive an abundance intensity at the respective cutoff. Here, the threshold is a log2 abundance value of 16. As evidenced, there are still quantitative peptides below the threshold of 16, so this filter will help to select the highest confidence relatively quantitative peptides, but not all.

Application of quantitative threshold



Two known dilution ratios were compared - 0.31 and 1.25 - with an expected log2 fold change of 2. Fold changes were assessed before and after the applied 80% quantitative threshold (D, E). Using the 80% threshold, 1218 peptides (15%) passed the filter criteria and fit the expected 2 fold change with a mean fold change of 1.66 and a standard deviation of 0.27 (F). From the application of this workflow, the user can define an abundance threshold filter to apply to all peptides and measurements and can remove low confidence results to retain only peptides with the highest confidence relative quantitation in the dataset.

Application to PQ500 serial dilutions



PQ500 is a set of ~500 peptides optimized for digestion and mass spectrometry acquisition. We serially diluted PQ500 peptides alone at 6 different concentrations and asked how many peptide abundances were above or below the MDF based on the following criteria: sn >10, MS2 abundance >0, consecutive abundances in 4 concentrations, and an R^2 of 0.9. Here, we see that most peptides are above the MDF, with a small set of qualitative abundances averaging ~18. From this result, we observe that with a set of well characterized peptides in a relatively simple system, there is essentially no qualitiative range and DIA performs similarly to a targeted MRM assay: if peptides are detected, they are quantitative.

Conclusion

• Application of this technique demonstrates that data independent acquisition mass spectrometry can produce high accuracy quantitation for a subset of peptides.

• This approach can allow a user to QC their DIA datasets by defining a range in which most peptides have dilutional linearity.

• This approach can be applied to different matrices to establish quantitative thresholds for different clinical sample types

Workflow on github:
https://github.com/casavae2/SILAC_DIA_Quant

Youtube tutorial:
https://youtu.be/BhsQhfxIa70

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