Scaffold Analysis of Isobaric Tag Datasets with a Pooled Reference

Open Scaffold, choose Edit/Preferences/Memory, then set Memory to 50-80% of RAM, with a recommended setting of ≥4000 MB. Close, re-open Scaffold and then open the Scaffold file. Choose Protein Threshold: 99%, Min. # Peptides: 2, Peptide Threshold: 95%

Click on Q+, enter these settings after clicking Quant/Update Experimental Design: Analysis Type: Intensity Based Experiment: Between Subjects with (Common/Pooled Reference)

"Edit Sample Names and Categories"

Select the number of categories or conditions in the experiment *including* the pooled reference. Double-click on each cell to rename each quant sample under the "Name" column, which corresponds to its TMT label

"Organize Quant Samples"

Move Reference, Control, & Experimental Samples into proper categories

For experiments with multiple mastermixes be sure to click "Add reference alignment group" for each new mastermix and corresponding samples

"Approve Settings"

Click "Edit Settings", on the Normalization Tab choose Calculation Type: Median, Blocking Level: Unique Peptides, Reference Type: Average Protein Reference, Spectrum Quality Filter: Reference value required, Normalization Between Samples: On. On the Minimum Dynamic Range tab chose 5%; on the Other Settings tab deselect "Use Non-Exclusive Peptides" to *exclude* peptides that below to multiple proteins. Click "Apply" and "Finish"

"Quant/Quantitative Testing"

Select two groups of samples to compare (e.g., treated vs control), use the "Remove" button to remove other samples. Choose Mann-Whitney Test with Benjamini-Hochberg multiple test correction with a significance level of $p<0.05$, then choose the appropriate reference Category (e.g., control) and click "Apply".

Interpreting Results

Set Display Options to Fold Change Ratio, Min FC: 1.5 Fold; Click on Mann-Whitney Test column to rank order proteins by p-value. In addition to the Mann-Whitney test, Scaffold also carries out a Brown-Forsythe population variance test. Orange highlighting indicates proteins whose p-values have significantly *unequal* variances based on this test. The Scaffold tutorial cautions that these p-values "may be based on a small number of measurements or unreliable data" and that they should be examined in more depth.