Reproducible and Quantitative Precipitation of Low Starting Protein Quantities in the ProTrap: Comparison to Conventional Precipitation

Jessica L. Nickerson[*1], Victoria A. Miller[1], Sara Lahsaee Little[1], Jean-François Noël[2], Angela Giraldo[2], Hugo Gagnon[2]*

[1]Allumiqs Inc., Halifax, Canada; [2]PhenoSwitch Bioscience, Sherbrooke, Canada

jnickerson@allumiqs.com

Summary

Maximized recovery for bottom-up proteome characterization is particularly important for precise quantitation and analysis of limited sample quantities and dilute biofluids. The ProTrap HT semi-automated filter plate exploits an optimized organic solvent-based precipitation workflow to enable efficient and high throughput sample preparation while maximizing total proteome recovery.

The present work aims to evaluate the performance of the ProTrap HT while processing minimal sample quantities (ranging from 1 to 10 µg total protein).

Total protein recovery of a BSA test sample suggested quantitative precipitation efficiency was achieved down to 0.5 µg starting material. A human mononuclear cell (hMNC) line was used to evaluate total sample coverage and digestion efficiency, showing effective label-free quantitation of up to 23,000 precursors and 2900 proteins.

The ProTrap HT was subsequently compared against a precipitation workflow in a conventional microcentrifuge tube (microtube) for the preparation of 1-10 µg human cerebrospinal fluid (CSF) protein. At the lowest loading of 1 µg, the high throughput filter plate enabled the identification of 25% more precursors and 49% more proteins than the manual approach. Quantitative precision at the protein level was equivalent between the two workflows at the 2 µg load with a 3% difference in mean CV at 5 µg.

Methods

Bottom-up Sample Preparation

Cells were extracted with an SDS-based lysis buffer (1% SDS, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl) and total protein content was quantified by BCA assay. Protein lysates were reduced and alkylated with DTT and IAA and brought to final concentrations of 1, 2, 5, and 10 µg/100 µL. 100 µL aliquots were transferred in triplicate to either Eppendorf microcentrifuge tubes (vials) or the ProTrap HT 96-well filter plate. Samples were combined with 400 µL acetone/methanol (3.5/0.5 parts respectively) and homogenized by gentle mixing. Samples were precipitated for 30 min at room temperature followed by isolation of the protein pellet by positive pressure in the ProTrap HT and centrifugation in the microtubes. Resulting pellets were twice washed with cold methanol prior to trypsin digestion.

Pellets were re-solubilized and digested overnight at 37°C at a 30:1 substrate/enzyme ratio with a trypsin/LysC mixture (Promega V5072) in the presence of 0.2% SDC, 10% methanol and 50 mM Tris-HCl (pH 8.0).

Digested samples were quenched with 1% formic acid and collected by positive pressure in the ProTrap HT and recovery of the supernatant following centrifugation of microtube digests. A Strata X SPE clean-up was performed and eluted samples dried by SpeedVac.

LC-MS/MS

Dried SPE-eluted samples were re-suspended in 3% DMSO/0.2% formic acid. LC-MS/MS analysis of proteome digests proceeded by online reversed phase separation on a Kinetex XB C18 column at 60 °C across a 30-min ethanol gradient. MS acquisition was performed on an ABSciex TripleTOF 6600 via electrospray. Information Dependant Acquisition (IDA) mode was used to establish the ion library. The samples were analyzed in SWATH acquisition mode.

Data analysis

Bottom-up SWATH MS/MS spectra were searched in DIA-NN using an IDA library and quantified by label-free MS1 intensities. Peptide and protein identifications were compared across 1-10 µg loadings to determine overlap in sample coverage based on starting protein quantity. Peptides were also sorted based on miscleavage frequency to estimate relative digestion completion. Subcellular location was mapped using the UniProt database. Protein abundance distributions were compared across loading quantities based on the PAXdb protein abundance database.

Results

Bottom-up Proteome Coverage of hMNC with Low Protein Quantities in the ProTrap

Total Precipitation Recovery

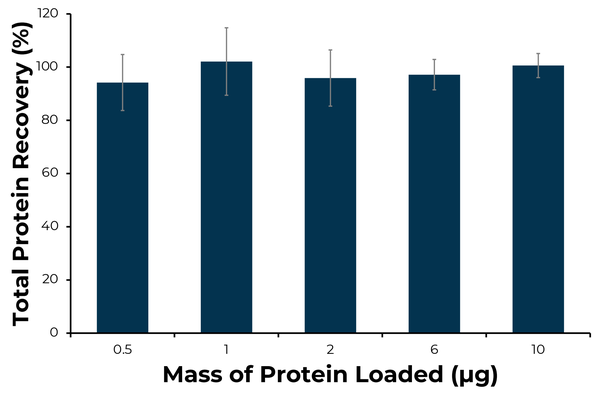

Figure 1. Total protein recovery following rapid precipitation of low starting quantities of BSA in the ProTrap XG. Total protein recovered in the pellet was quantified by a BCA colorimetric assay.

Repeatability of Peptide Identifications

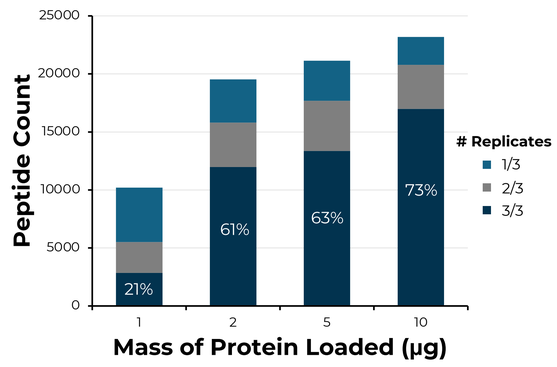

Figure 2. Total bottom-up peptide identifications following SWATH DIA LC-MS/MS, identified in 1, 2, and 3 of 3 preparative replicates. Loading 10 µg protein enables greater overlap across replicates than observed for 2-5 µg and 1 µg.

Miscleavage Analysis

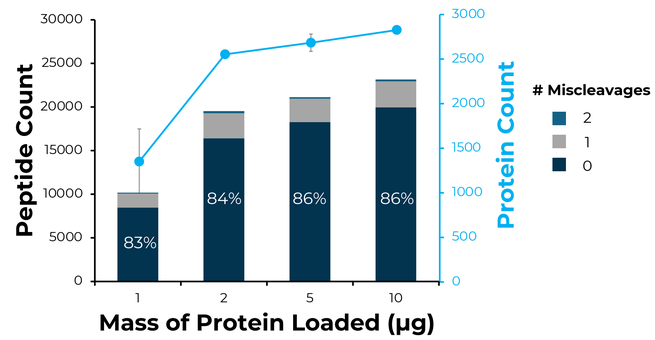

Figure 3. Total bottom-up protein identifications alongside peptide precursor identifications sorted based on miscleavage frequency.

Cellular Coverage

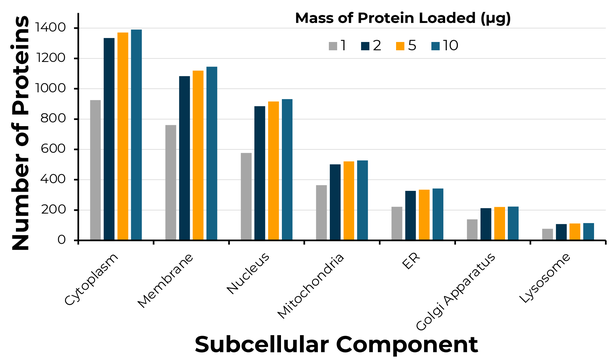

Figure 4. Comparison of cellular coverage based on total hMNC protein processed. All loadings enable similar coverage of subcellular components, with just 29-30% of proteins being cytosolic in all samples.

Venn Diagram of Protein Identifications

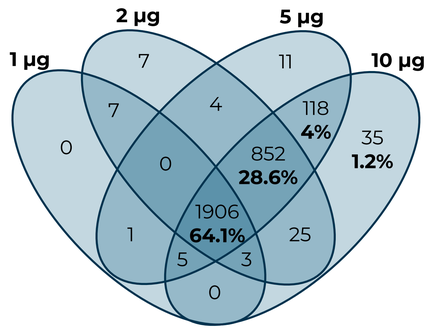

Figure 5. Venn diagram of bottom-up protein identifications following preparation of 1, 2, 5, and 10 µg initial hMNC protein in the ProTrap HT filter plate. 64% of proteins are common between all samples, while 29% are excluded from the lowest load of 1 µg.

Protein Abundance

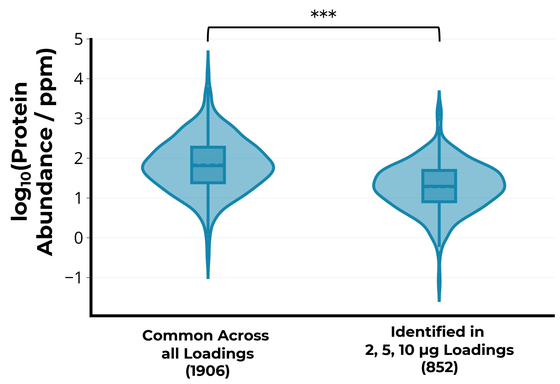

Figure 6. Violin plot of estimated protein abundances of proteins identified in all samples vs. those that were identified in the 2-10 µg samples but excluded from the 1 µg preparation. The difference in abundance distributions ($p<0.0001$) shows a loss of low-abundance proteins when <2 µg is processed.

Comparing CSF Characterization following Preparation in the ProTrap HT vs. a Conventional Vial

Precursor and Protein Identifications

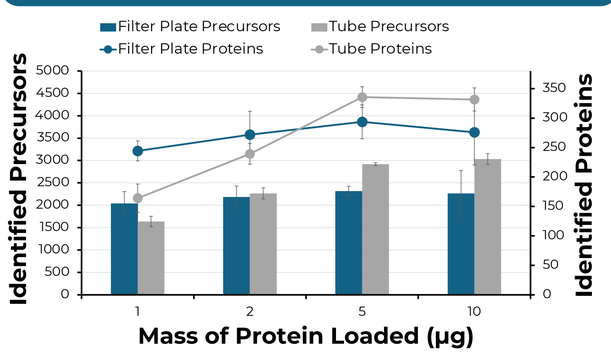

Figure 7. Comparison of total bottom-up precursor and protein identifications following preparation of 1, 2, 5, and 10 µg total CSF protein in a conventional vial vs. the ProTrap HT filter plate. At the lowest loading of 1 µg, the ProTrap HT enabled the identification of 25% more precursors and 49% more proteins than the conventional vial.

Comparing Intensity of Common Peptides

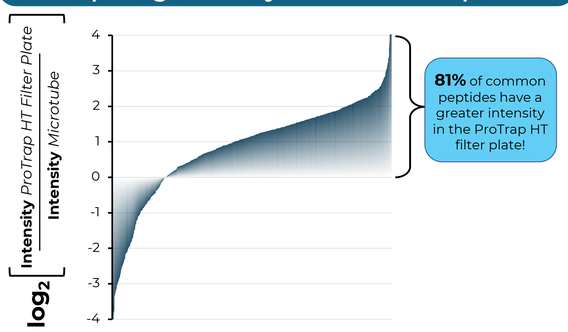

Figure 8. Waterfall plot of the ratio of SWATH precursor intensities following preparation of 2 µg CSF protein in the ProTrap HT filter plate vs. a conventional microcentrifuge tube.

Protein Quantitation

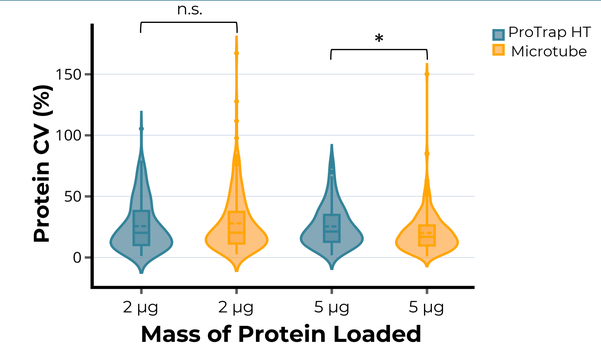

Figure 9. Violin plot of protein coefficients of variation (CV) following preparation of 2 and 5 µg total CSF protein in the ProTrap HT vs. a conventional microcentrifuge tube. Similar quantitative precision is observed at 2 µg, while precision is slightly enhanced by the conventional approach at 5 µg. ** $p<0.01$*

Conclusions

- A ProTrap-based precipitation workflow facilitates maximized recovery for samples containing as little as 0.5 µg total protein.
- The ProTrap HT uses a high throughput automated workflow to enable deep sample coverage of low protein quantities, with optimal results for ≥2 µg while reducing hands-on processing and total preparation time.
- At a low loading of 1 µg, the ProTrap HT outperformed a conventional/manual precipitation workflow, identifying more peptides and proteins with greater total intensity.
- Ongoing studies are working to further enhance the recovery and digestion efficiency of sub-microgram sample quantities in the ProTrap HT to improve the identification and quantitation of low-signal proteins

Acknowledgements

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