Improvements to ProSightPD nodes in the Thermo Scientific Proteome Discoverer Software Framework

David M. Horn,[1] Tara L. Schroeder,[1] Ioanna Ntai,[1] Richard D. Leduc,[2] Ryan T. Fellers,[2] Joseph B. Greer,[2] and Neil L. Kelleher[2], [1]Thermo Fisher Scientific, San Jose, CA, [2]Northwestern University, Evanston, IL

ABSTRACT

Purpose: Here we demonstrate the use of the ProSightPD™ nodes for the Thermo Scientific™ Proteome Discoverer™ software framework to analyze complex top down proteomics data. We also demonstrate how sliding window deconvolution results from Thermo Scientific™ Biopharma Finder™ can be used to determine differentially expressed proteoforms.

Methods: For this work, Study 1 data from Ntai *et al*[1] were used for the analysis. Both the "High/High" and "Low/High" GELFrEE fractions were analyzed using ProSightPD Base and ProSightPD High Mass, respectively. The High/High data were analyzed using a five-step search to maximize the number of identifications, while the Low/High data were analyzed using a three-step search. PrSMs were filtered by a minimum C score of 3. Sliding window deconvolution in the Biopharma Finder software was used to process the High/High data using Xtract deconvolution. The quantitative results were integrated with the filtered human PrSMs from ProSightPD via a Perl script. Normalization and p-value calculations were produced using InfernoRDN and Microsoft® Excel.

Results: The High/High runs identified 2,374 proteoforms total with 735 producing a C-score of 3 or better. The Low/High runs identified 254 proteoforms with 38 proteoforms having a C-score of 3 or better. Many of the proteoforms that were identified had masses greater than 40 kDa. Integration of the sliding window deconvolution results with the High/High data produced over 400 proteoforms that differ significantly between the WHIM2 and WHIM16 samples.

INTRODUCTION

ProSightPC™ is the leading search engine for top-down proteomics because of its sophisticated tools for proteoform identification. ProSightPC has several search modes for identification of intact and truncated proteins and uses the information annotated in UniProt to identify heavily modified and processed proteins. The user interface of ProSightPC is well suited for comprehensive characterization of single datasets, but it can be challenging to perform multi-stage searches against large datasets with many data files.

The ProSightPD nodes for the Proteome Discoverer software framework were created to analyze such complex datasets. The workflow layout makes it straightforward to create multi-step searches and the results interpretation tools are well suited for complex data. In the ProSightPD 1.1 release, additional nodes for sequence tag prefiltering prior to absolute mass or biomarker searches were added as well as the C-score[2] for confidence in proteoform characterization. Here we present further improvements to the ProSightPD nodes, including new result tables better suited for top down proteomics as well as integration of a fragment map similar to ProSight Lite.[3]

MATERIALS AND METHODS

High/High data analysis

The study 1 top down dataset from Ntai *et al* was downloaded from the CPTAC website. The multidimensional fractions for the High/High and Low/High data for the WHIM2 and WHIM16 samples were loaded into the Proteome Discoverer using the Add Fractions option. Four of the databases were downloaded in XML or flat text format from UniProt and indexed with various levels of complexity. The WHIM2 and WHIM16 sequence variant databases that were the same as used for the paper.

The High/High data were run using the five-step search shown in Figure 1, closely following the strategy reported in the supplementary information in Ntai *et al*. First, the High/High cRAWler performs Xtract deconvolution on the precursor and product ion spectra. The first search was performed against the mouse proteome with high proteoform complexity with narrow mass tolerances, identifying intact mouse proteoforms from the mouse host of the xenograft tissue. Any PrSMs with P-values lower than 1e-10 are passed to the next search through the Spectrum Confidence Filter node. The second search identified intact proteoforms of the sequence variants integrated with the rest of the information from the UniProt entries. While there are fewer protein entries in this database, the proteins in the database were indexed with high complexity. The third search identified intact proteoforms from a complex human proteome database. The fourth search detected truncated products of human proteins and the fifth search was an error tolerant delta-m search to find protein products that may significantly deviate in sequence or PTMs from the UniProt database.

Because the second step of the analysis requires a different database for the WHIM2 and WHIM16 samples, the processing workflow was cloned for the two different datasets. This is shown in Figure 2. The search results were subsequently combined into a single result using the consensus workflow.

Figure 1. Five-step workflow used for the analysis of the High/High WHIM2 and WHIM16 data.

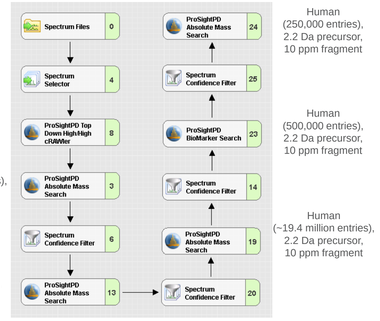
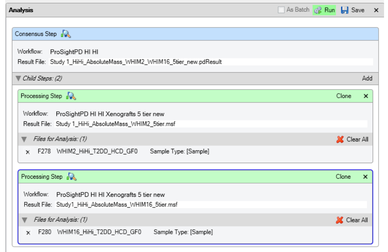


Figure 2. Two processing workflows were used to search the WHIM2 and WHIM16 data with different databases in the second search as shown in Node 13 in Figure 1. The results of the two searches are integrated by the consensus step.



Low/High data analysis

The Low/High data were analyzed using a three-step search in a similar manner described for the High/High data. For these data, the workflow employed the Low/High cRAWler, which uses the ReSpect™ deconvolution algorithm to determine average mass values for precursor proteins while still using Xtract deconvolution for the MS/MS spectra. In this search, there were three absolute mass nodes in sequence searching intact proteoforms for mouse, human, and human sequence variants. Since the MS1 data were acquired using ion trap mass spectrometry, a wider precursor tolerance had to be used (1000 ppm), but 10 ppm tolerance was still used for the high resolution MS/MS spectra.

Sliding window deconvolution and p-value calculations

All High/High and Low/High data were analyzed using sliding window deconvolution in a prerelease build of Biopharma Finder™ 3.1 to produce mass, apex retention time, and abundance for the MS1 data. For the High/High data, the Xtract deconvolution algorithm was used to produce monoisotopic masses. The PrSMs with C scores >= 3 from the human searches were integrated with the sliding window deconvolution results using a Perl script. The integrated results were loaded into InfernoRDN (Pacific Northwest National Laboratories) and Microsoft Excel for LOESS normalization and calculation of p-values.

RESULTS

High/High WHIM2 versus WHIM16

The combined search results returned 30,104 PrSMs corresponding to 2,374 proteoforms and 602 protein isoforms. Table 1 below shows the list of PrSMs with a P-value for 1e-10 or better for each search shown in Figure 1.

Table 1. PrSM counts the WHIM2 and WHIM16 samples at each step in the ProSightPD workflow.

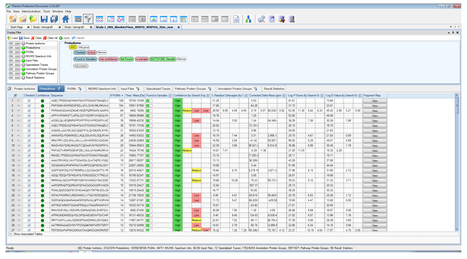
Search Order	Search type	# WHIM2 PrSMs	# WHIM16 PrSMs
1	Mouse intact proteoforms	4063	3067
2	Human sequence variants	89	53
3	Human intact proteoforms	2611	2204
4	Human truncations	48	236
5	Human error tolerant	10709	7206

There were significantly more PrSMs detected in the WHIM2 sample, indicating that there was more starting material for this sample than for WHIM16. Further, there were significantly more mouse proteoforms than human proteoforms, but this is likely the case because many of these mouse proteoforms have significant homology to those from human. Thus, many of these PrSMs may actually be human proteins, but it is not possible to distinguish whether or not they are mouse or human proteins. Finally, there are a lot of PrSMs identified in the final step, indicating that there are many proteoforms that differ from what is predicted from the UniProt entry.

The PrSM table was further filtered to those that are likely to be fully characterized by using a threshold C score of 3. Using the Proteome Discoverer software feature "Check All In This Table and All Associated Tables," all of the proteoforms and protein isoforms associated with these characterized PrSMs can be checked and filtered. There were 735 proteoforms with C scores 3 or more after filtering.

The "Found in Samples" columns were subsequently used to find proteoforms that were identified in only one of the two samples. Figure 3 shows the list of proteoforms to those that were only identified in the WHIM2 sample by filtering by "Not Found" in the WHIM16 sample. The list is sorted by decreasing numbers of PrSMs, which means the proteins at the top of the list with over 100 PrSMs are highly up-regulated in WHIM2 relative to WHIM16.

Figure 3. Proteome Discoverer view of proteoforms detected only in the WHIM2 sample.

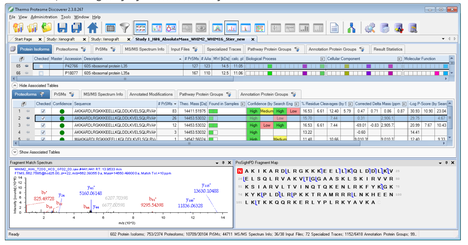


In total, there were 78 proteoforms with five or more PrSMs that were identified only in the WHIM2 sample, while there were 75 proteoforms with five or more PrSMs that were identified only in the WHIM16 sample. Table 2 shows the top five proteoforms by PrSM count unique to either the WHIM2 or WHIM16 sample. While many of these proteoforms are for protein isoforms only identified in one of the two samples, Figure 4 shows example of a protein that was identified in both samples. In this case, there were two proteoforms only identified in both samples and two proteoforms identified only in the WHIM2 sample. The most abundant proteoform was unmodified while the others were acetylated at three different sites.

Table 2. Top five by PrSM unique proteoforms for each sample.

Sample	Proteoform Mass	Best C score	Protein entry	# PrSMs	Best log E-value
WHIM2	15793.74	1514	Calmodulin-like protein 5	109	73.69
WHIM2	15551.98	814	Cellular retinoic acid-binding protein 2	104	23.81
WHIM2	9402.193	697	High mobility group nucleosome-binding domain-containing protein 4	69	45.43
WHIM2	18654.57	1701	NP_000705-variant:Translocator protein	57	46.80
WHIM2	19506.40	583	Adenine phosphoribosyltransferase	39	30.38
WHIM16	10304.19	1063	Protein S-100P	60	22.3
WHIM16	12635.34	821	SH3 domain-binding glutamic acid-rich-like protein	59	51.27
WHIM16	9546.343	1537	Triple QxxK/R motif-containing protein	48	32.79
WHIM16	10393.22	571	Protein S-100P	46	28.46
WHIM16	7760.944	740	Adipogenesis regulatory factor	43	82.17

Figure 4. 60S ribosomal protein L35 shows two proteoforms identified in both samples and two proteoforms identified only in the WHIM2 sample. Three of these proteoforms have the same mass, differing only by the site of acetylation.



Low/High WHIM2 versus WHIM16

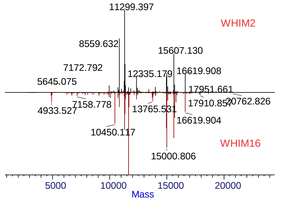
The search of the Low/High data produced more than 17000 PrSMs for 48 protein isoforms and 254 proteoforms. 654 PrSMs had C scores greater than 3, corresponding to 38 proteoforms and 24 protein isoforms. Of the 38 confidently-characterized proteoforms, 15 were larger than 40 kDa demonstrating the power of the low/high method for identification of larger proteins. Many of those 24 confidently characterized protein isoforms were for mouse proteins. However, there were some human proteins uniquely identified in each sample. Transmembrane protein 43 (44 kDa, 20 PrSMs), high mobility group nucleosome-binding domain-containing protein (31 kDa, 5 PrSMs), and reticulon-4 (20.4 kDa, 442 PrSMs) were unique to the WHIM2 sample and parathymosin (11.5 kDa, 2 PrSMs) was unique to the WHIM16 sample.

It is likely that the requirement for wider precursor tolerances reduced the sensitivity of the approach. The most abundant protein in both samples, glyceraldehyde 3-phosphate dehydrogenase, had over 5000 PrSMs between the two samples. However, none of the PrSMs were identified with a non-zero C score. The 1000 ppm precursor mass tolerance is equivalent to +/- 36 Da, which is too wide to distinguish between many of the possible combinations of PTMs. These data would benefit from a "medium/high" acquisition strategy where the precursor spectrum was acquired at 15K resolution. In this case, we could use a much narrower precursor mass tolerance (~30-50 ppm), decreasing the number of theoretical proteoforms to be compared in the database. Further, the majority of the fragment coverage from HCD of this protein was near the N- and C-termini. Another MS/MS mode such as CID will likely produce more fragmentation in the middle of the protein, which would lead to improved C scores.

Sliding window deconvolution of High/High data

Sliding window deconvolution of the WHIM2 and WHIM16 data shows that we should expect significant differences between even common proteoforms between the samples. Figure 5 shows a mirror plot of the sliding window deconvolution results for the first GELFrEE fractions of the WHIM2 (upper plot) and WHIM16 (lower plot) samples.

Figure 5. Mirror plot comparison of GELFrEE fraction 1 for the WHIM2 and WHIM16 samples.



All 18 High/High datasets were analyzed using sliding window deconvolution and integrated with the PrSMs as previously explained. The WHIM2 and WHIM16 data had three replicates each and thus p-values could be calculated. Of the 735 confidently characterized proteoforms, more than 400 proteoforms exhibited a significant difference in expression with p-values <0.01. This is over half of the confidently identified proteoforms, which is higher than one would normally see by quantitative bottom up proteomics.

CONCLUSIONS

- ProSightPD is a powerful tool for analysis of complex top down proteomics data.
- The C-score is an especially important measurement for proteoform identification.
- Integration of sliding window deconvolution results showed the promise for quantitative label-free top down protein ID.

REFERENCES

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TRADEMARKS/LICENSING

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