Using Pathway Analysis to Develop and Verify Targeted Proteomics Studies

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233

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Introduction

The discovery of new biomarkers may be driven by proteomics, metabolomics, or genomics experiments and can result in a list of proteins of interest for subsequent targeted analysis. Preclinical validation of these putative biomarkers requires assays that are both specific and quantitative for target proteins and such assays are typically multiplexed, multiple reaction monitoring (MRM) analyses to achieve the required high-throughput. New pathway analysis and data visualization software facilitates the development of such targeted proteomics experiments by allowing simultaneous, integrated, multi-omics analysis and pathway visualization. A list of proteins of interest can then be exported and analyzed. The quantitative results from this targeted analysis can then be imported into the same software for statistical analysis and pathway visualization thus facilitating both discovery and verification. This workflow will be illustrated using a model sample set.

Experimental

Samples: In order to model this workflow, treated and unstimulated HeLa cell lysates were purchased (Millipore). The unstimulated (US) cell lysate was used as the control. The treated lysates had been exposed to 1) heat shock and arsenite (HS-Ars), 2) interferon α (IFN), 3) tumor necrosis factor (TNF) and 4) epidermal growth factor (EGF).

Sample preparation: For each sample, 100 or 200 µg of total protein was reduced, alkylated (carbamidomethyl) and digested with trypsin using a modified FASP protocol using 2,2,2-trifluoroethanol for solubilization and denaturation. After overnight digestion, the trypsinized lysate was acidified with formic acid and stored at -80°C until use.

LC/MS/MS Analysis: For each injection, 500 ng tryptic digest was loaded. Each cell lysate was analyzed multiple times (minimum of n=4) on the HPLC-Chip/MS system interfaced to a 6490 QQQ with iFunnel technology using a 90 minute gradient method. The HPLC-Chip used was the new Polaris chip (G4240-6230) which is comprised of a 360 nL enrichment column and a 150 mm x 75 µm analytical column which were both packed with Polaris C18-A, 3 µm material (Agilent). Data was in acquired with retention-time scheduling using dynamic MRM mode.

Data processing: The QQQ data was first processed by MassHunter Quantitative Analysis software to automatically extract the MRM chromatograms and calculate areas. The Quantitative software also have very convenient data review tools for assessing a large data set.

Area results for all targeted peptides were imported for into Mass Profiling Pro 12 (MPP), a data analysis and visualization package that includes an optional Pathways Analysis package.

Workflow

MassProfiler Pro
- Pathway analysis of differentially expressed proteins
- Export of protein accession numbers from pathways of interest

Creation of MRMs
- MRM Selector in Spectrum Mill for export of DMRM method based on discovery data
- Peptide Selector or Skyline for *in silico* prediction of transitions

MRM analysis on target peptides
- 6490 iFunnel QQQ
- Retention-time scheduled dynamic MRM mode based on discovery results

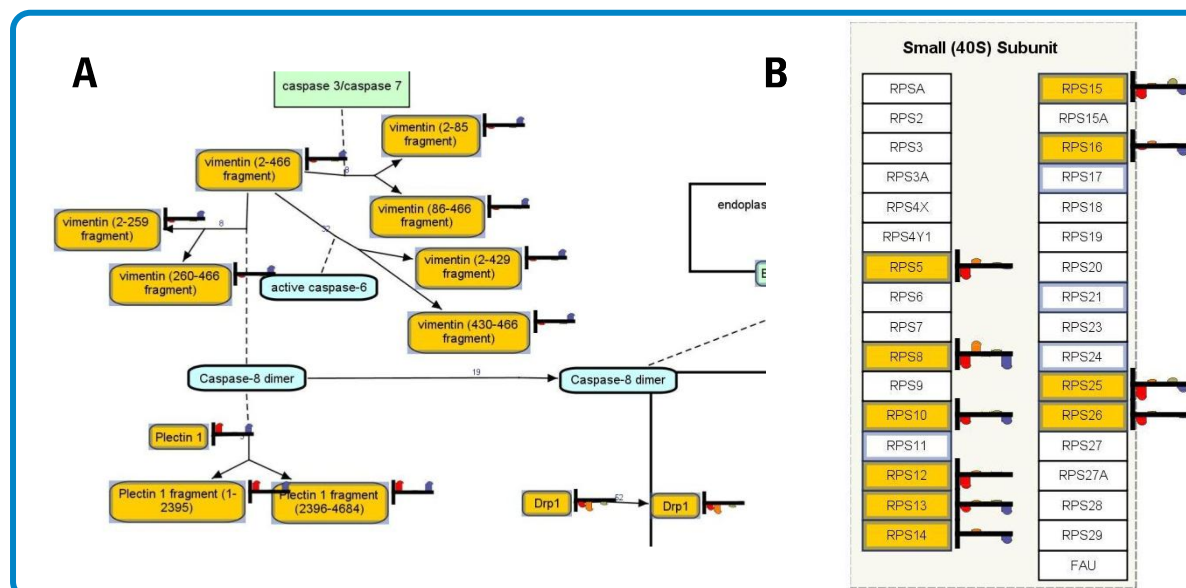
MassHunter Quantitative Analysis
- Data browser to review quantitative results
- Export of peak areas for all target compounds to MPP

MassProfiler Pro
- Statistical analysis of quantitative results
- Visualization of differential peptides
- Pathway analysis at the protein level based on peptide targets

Results and Discussion

Pathway Directed Target Analysis

Based on protein identification results from Spectrum Mill, differentially expressed proteins were mapped onto pathways in MPP (see companion poster 232). Two of the pathways (shown below) were selected for further targeted analysis by triple quadrupole mass spectrometry.



Q-TOF based label-free differential analysis displayed on portions of the A) Apoptotic execution phase WP1784_44958 pathway and B) Cytoplasmic Ribosomal Proteins WP477_41118 pathway. The heat strips to the right of each detected protein indicate the relative abundance in each sample treatment group.

Development of MRM-based Methods for Detected Targets

The SwissProt accession numbers for the detected differentially expressed proteins (yellow nodes in figures A and B above) were copied and then pasted into MRM Selector in Spectrum Mill software (below). MRM Selector automatically creates MRM transition lists based discovery data with a number of user-specified criteria. For this study, up to 5 transitions per peptide and 5 peptides per protein were exported from the data-dependent Q-TOF analyses. Because the same LC analysis was applied to the targeted study, retention time information could be exported for direct use in dynamic MRM methods.

Development of MRM-based Methods for Non-Detected Targets

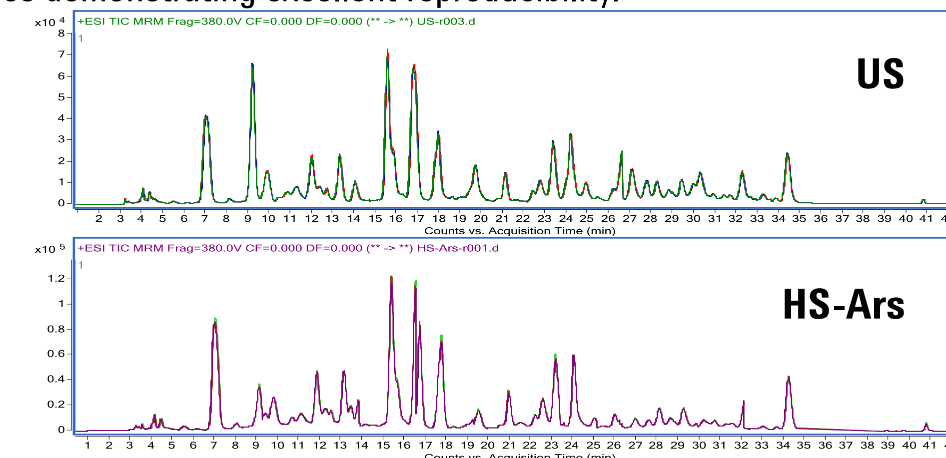
It is also possible to develop MRM assays for any or all proteins in an pathway regardless of detection in the protein discovery phase. In this case, the GAS2 protein from the apoptotic pathway was selected to illustrate that *in silico* MRM transitions can be predicted using either Skyline (A) or Peptide Selector in Spectrum Mill (B). This same approach is used when the pathway-directed experiment is based on genomics or metabolomic information

MRM transitions can be exported and applied to a pooled sample to determine which peptides can be detected.

Results and Discussion

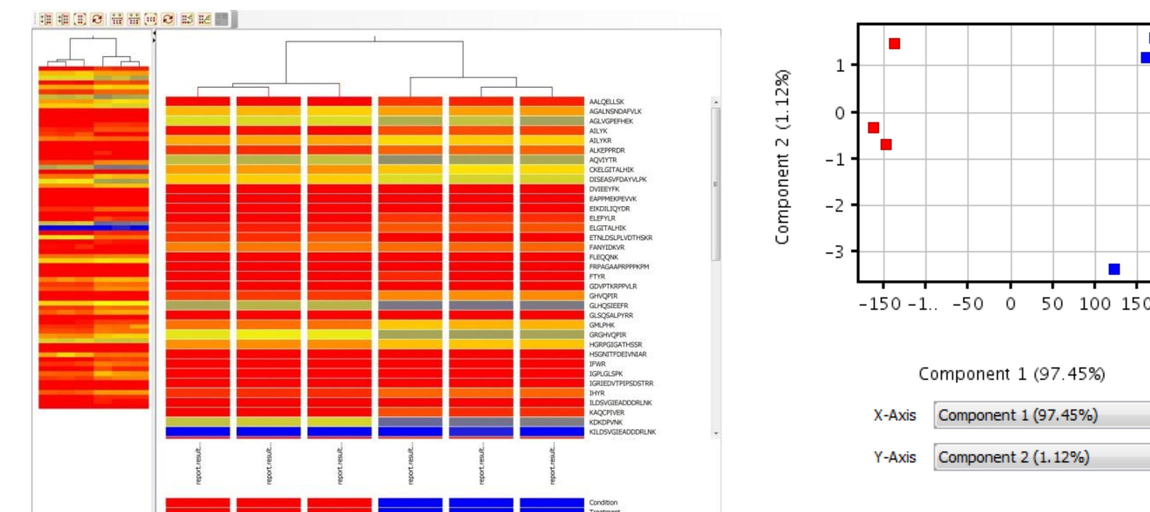
QQQ Reproducibility

Dynamic MRM analysis was performed on the unstimulated and HS-Ars samples. The figure below shows overlaid TIC MRM chromatograms for the replicate analyses demonstrating excellent reproducibility.

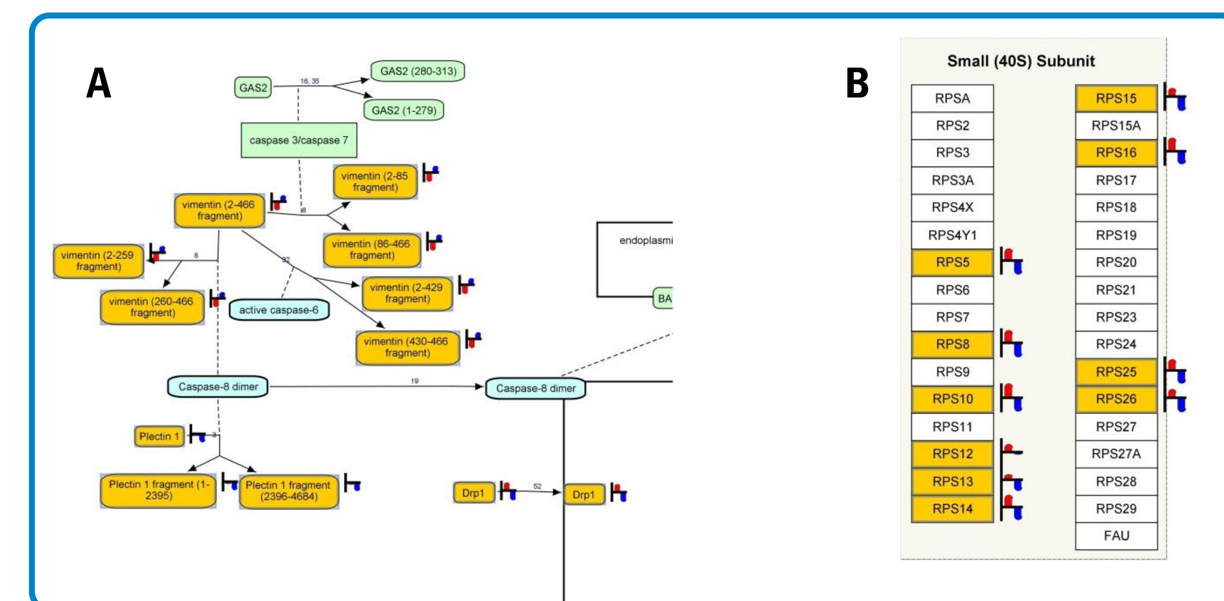


MassHunter quantitative results (below) were exported to MPP.

These results were then subjected to statistical analysis (t-test) to determine the differential features. Again, PCA and hierarchical clustering can be used to visualize the quality of the data.



Because the accession number was included in the MRM table as the "Compound Group", pathway analysis could be performed on the exported results as well (below). Note that the QQQ results quite clearly show expression level changes in these proteins with HS-Ars treatment.



Conclusions

- Pathway analysis based on differential 'omics experiments can facilitate the development of targeted analyses for proteins of interested
- Targeted MRM-based assays can be easily developed based on proteomics discovery results or in silica prediction in combination with "scouting" analyses to confirm the best targets.
- Quantitative results from target analyses can be subjected to the same statistical evaluation, visualization and pathway analysis using MPP software
- This approach in combination with 'omics-based discovery facilitates biologically-driven workflows