Revolutionizing Data Independent Acquisition on q-OT-IT Tribrid Mass Spectrometers

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Overview

Purpose: Demonstrate the application of data independent acquisition (DIA) techniques for the complete quantification of a whole cell lysate using a new state of the art mass spectrometer system with improved sensitivity.

Methods: Several data dependent acquisition experiments were performed and the results used to build a large spectral library on two non-small lung cancer cell lines, one which shows resistance to a tyrosine kinase inhibitor, Erlotinib. Several classicDIA experiments were performed on both current and new MS platforms and the resulting data quantified.

Results: Over 5200 proteins and 44000 unique peptides were able to be quantified from the results of the DIA analysis with run to run coefficients of variation of less than 20%. Of these, nearly 1200 significantly regulated targets were identified with p-values of <0.01. A noticeable increase in quantified proteins was seen for the new q-OT-IT mass spectrometer using test HeLa cell lysate over the previous instrument, especially at lower sample loads.

Introduction

DIA represents a powerful screening technique for the comprehensive and reproducible quantitation of biological samples, particularly their protein component. As the method sequentially collects MS/MS fragmentation spectra on all ions within a given m/z range, it affords the opportunity for retrospective analysis of unknowns and new targets of interest. Obtaining peak performance from a DIA analysis demands an optimum balance of instrument speed, sensitivity, and selectivity to overcome the complexity that results from using larger isolation windows while still maintaining reproducibility of quantitation for low abundance analytes. While increasing instrument speed is certainly an attractive opportunity, without a concomitant improvement in sensitivity, the effect is simply a reduction in data quality as ions have less time to accumulate. The Thermo Scientific™ Orbitrap Fusion™ Lumos™ MS furnishes both: the speed of the current Orbitrap Fusion, but with enhanced sensitivity due to critical upgrades to the quadrupole and front-end optics.

Methods

Sample Preparation

Before committing valuable sample, instrument performance was verified using a standard Pierce HeLa cell digest with Biognosys HRM kit spiked in 1:10 according to the manufacturer's instructions. Next, two non-small cell lung cancer lines, one showing marked resistance to the tyrosine kinase inhibitor Erlotinib were used. The purified protein extract from these cell lysates was reduced, alkylated, and digested overnight. The Biognosys HRM kit was spiked in 1:10 according to the manufacturer's instructions.

Liquid Chromatography

Reversed Phase High Performance Liquid Chromatography was used to separate peptide analytes. A binary mobile phase configuration consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was employed for these experiments. A 120 minute gradient from 5% to 25% B followed by an additional 35 minutes from 25 to 40% B at 300 nl/min flow rate was performed using a Easy nLC 1000 nano-HPLC system coupled to a 75 um x 500 mm PepMap C18 EasySpray column.

Mass Spectrometry

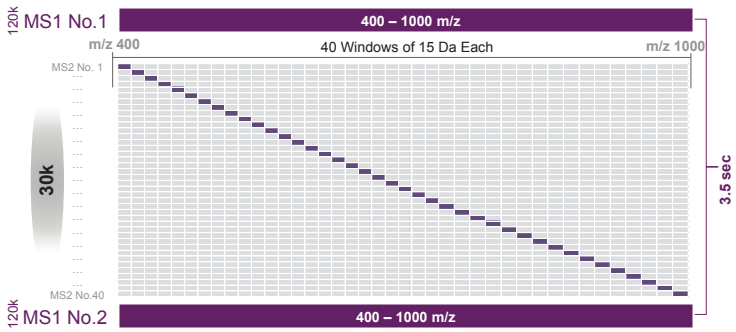
Using an Orbitrap Fusion Lumos mass spectrometer, several discovery experiments were performed using a data dependent acquisition scheme with an MS1 full scan resolution of 120,000 FWHM (at 200 m/z) followed by as many subsequent MS/MS scans on selected precursors as possible within a 3 second maximum cycle time. MS/MS was performed using HCD in the Orbitrap at a resolution of 30,000 FWHM. A data independent acquisition method followed using small (15 Da) sequential MS/MS windows at 30,000 resolution and an 120,000 resolution MS1 full scan. The range surveyed by these experiments was between 400 and 1000 m/z with a maximum injection time of 60 msec.

Data Analysis

Spectral .raw files from data dependent acquisition were analyzed using Thermo Scientific™ Proteome Discoverer™ 1.4 software using the SEQUEST®HT search engine, constrained with a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da. Carbamidomethylation (+57.021 Da) of cysteine was considered a fixed modification, while phosphorylation (+79.966 Da) of serine, threonine, and tyrosine, methionine oxidation (+15.996 Da) and deamidation of asparagine and glutamine (+0.984 Da) were considered as dynamic peptide modifications.

Data was searched against a Swiss-Prot® complete human database with a 1% FDR criteria using Percolator. Analysis and interpretation of DIA data were performed using Biognosys Spectronaut 7. A library was built from the combined search outputs of Proteome Discoverer 1.4 using a high confidence peptide filter and fragment filter settings of 300 to 1800 m/z with fragments greater than y3 or b3. Minimum and maximum number of fragments was set to 3 and 6 respectively. Peak detection employed a dynamic iRT retention time prediction model and a non-linear iRT calibration strategy. Peptide identification was performed taking into account MS1 scoring and a dynamic score refinement. Interference correction was utilized during quantitation and cross-run normalization was done. Important regulated candidates were selected using a student's t-test based on peak area and with a q-value threshold of 0.01.

FIGURE 1. Isolation scheme and ion density map used for the Data Independent Acquisition Method for 3 minute representative window



Results

Improvements seen in test data set using the Orbitrap Fusion Lumos MS

An increase in the number of quantifiable protein groups resulted using the Orbitrap Fusion Lumos MS over the previous generation Orbitrap Fusion MS. This effect was especially pronounced for lower sample loads on column, with an observable 30% difference in quantifiable (CV ≤ 10%) protein groups at 100 ng load and approximately 25% more quantifiable unique peptides at this threshold. On the merits of these performance enhancements, a decision was made to commence analysis using the limited amount of H358 sample.

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Library development and searching

Replicate data dependent acquisition analyses on the Orbitrap Fusion Lumos MS were able to identify approximately 32000 unique peptides and 4900 protein groups per run while compilation of all experimental runs resulted in a total of approximately 60000 unique peptides and 6700 protein groups as a complete spectral library. Total processing time to perform detection, alignment, extraction, scoring, and normalization was approximately 14 minutes for three replicate files of each condition, searched against the complete library from above. Nearly 96% of all peptides from the library were able to be identified at less than 0.01 q-value. The use of an exclusion list in replicate DDA experiments was used to further enhance the comprehensiveness of the library and increase the search depth for subsequent DIA identification/quantitation. (Figures 2 and 3)

Quantitation and Candidate Selection

Amongst the 6498 protein groups in the spectral library detected in the DIA analyses a median coefficient of variation of 9.2% was observed. Closer inspection of the data revealed about 5300 protein groups with coefficients of variation below 20%. With a p value threshold of 0.01, more than 1200 significantly regulated protein groups were detected showing expression changes of greater than 2-fold, many belonging to important signaling pathways previously implicated in earlier TMT analysis. (Figure 4).

Correlation with Orthogonal Methods

For the vast majority of proteins assayed in this analysis, results obtained processing the data independent acquisition spectra with Spectronaut agreed quite well with orthogonal data collected on the same sample and instrument type using TMT10-plex labelling. In almost all observed cases, the pattern of regulation was highly conserved between the two methods, although in some cases the magnitude of the change was found to be greater using the DIA results. More than likely this results from some ratio compression seen in the TMT experiment which was run without the benefit of the SPS-MS3 method's improvement in reporter ion accuracy (Figure 5).

Where available, for a few key proteins, a high degree of relation was also seen between the DIA results and SILAC quantitation performed previously. Finally, the configuration of regulation obtained herein align extremely well with that seen previously from data collected on the Q Exactive HF using the multiplexed DIA strategy and integrated using Skyline.

FIGURE 2. Nearly 6500 proteins were identified with a q-value of less than 0.01. Of these 5200 could be quantified with coefficients of variation less than 20%.

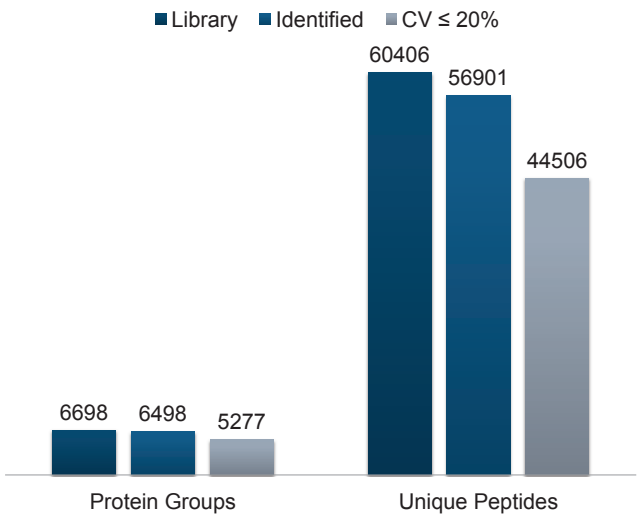


FIGURE 3: Determination of Cscore cutoff criteria for <0.01 q-value. Scrambled library peptide sequences were used as a decoy model for truly absent peptides.

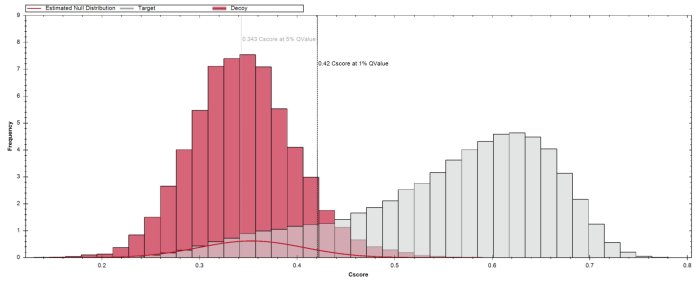


FIGURE 4. A student's t-test of the data independent acquisition data revealed nearly 1200 significantly regulated proteins with more than 2 fold up or down regulation at a p-value threshold of 0.01

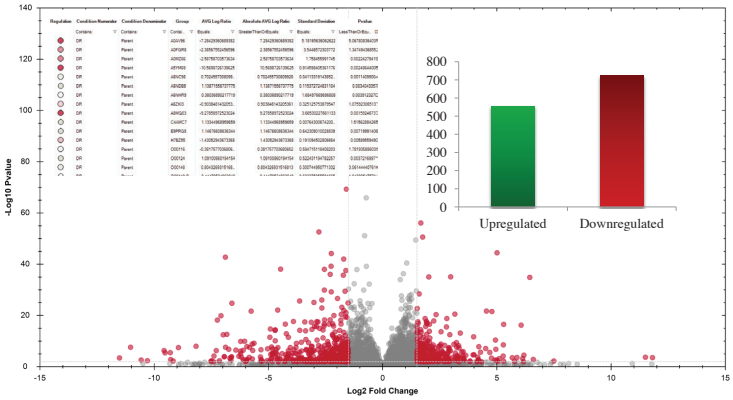
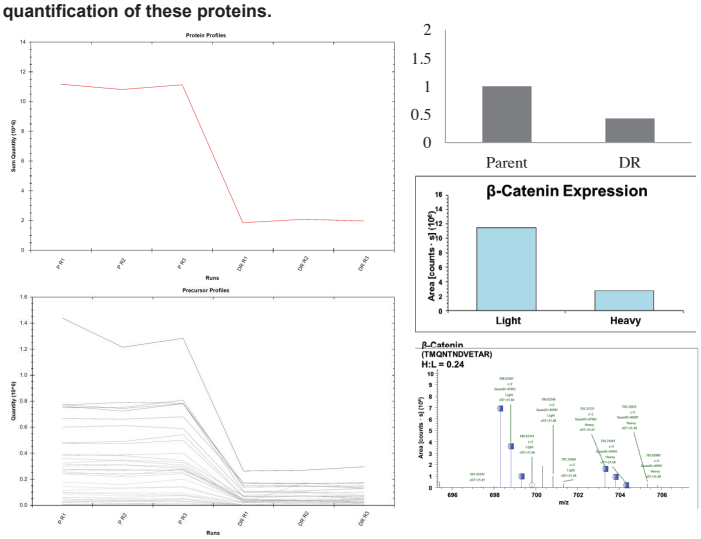
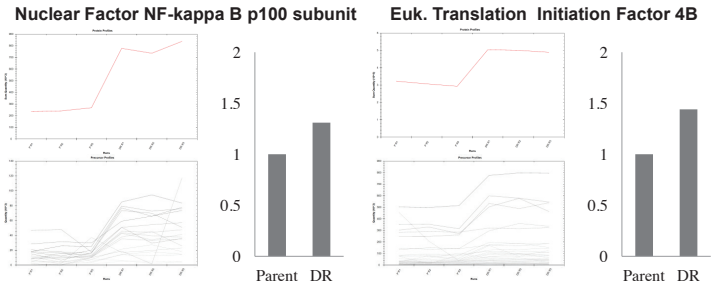
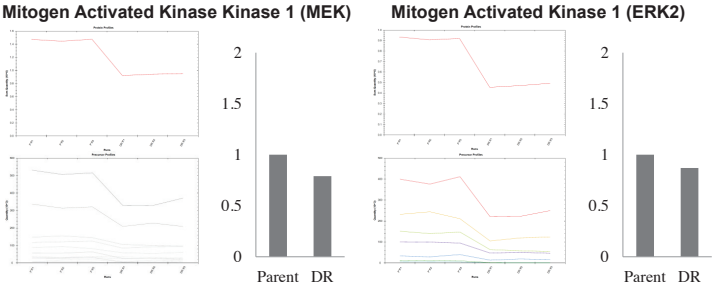


FIGURE 5. Focused examination of one of the candidate proteins, β-catenin (CTNB1). Approximately a 5 fold change is observable at the protein level between the parent and drug resistant cell lines. This expression pattern is consistent with orthogonal methods on the same sample, including SILAC and TMT10plex. High peptide-to-peptide reproducibility result in a low p-score for quantification of these proteins.







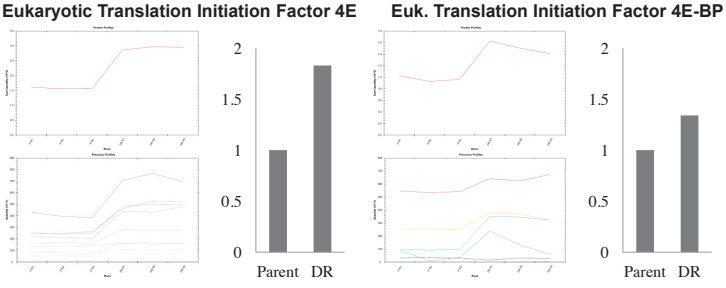
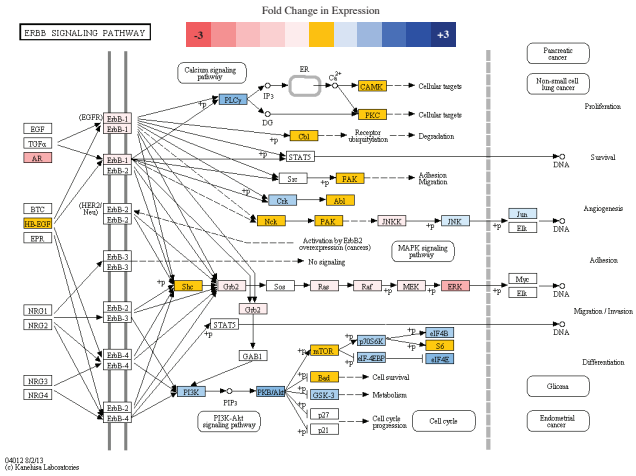


FIGURE 6. The ErbB Signaling pathway showing fold regulation changes between the parent cell line and drug resistant cell line. Degree of down regulation is shown in shades of red, while upregulation is seen in blue. Unidentified proteins are shown in white.



Protein Expression in the Drug Resistant Cell Line

Many proteins of the canonical MAPK pathway, especially MEK and ERK, are subtly but consistently underexpressed in the drug resistant cell line as seen in previous analyses. This is consistent with what might be expected given Erlotinib' s inhibition of EGFR phosphorylation. In contrast, several of the downstream targets of the mTOR pathway, including EIF4E and EIF4B and P70S6K are noticeably upregulated in this cell line suggesting a possible mechanism for drug resistance. Interestingly, AKT3 is significantly upregualted in the drug resistant cell line while both AKT1 and AKT2 are unchanged or slightly downregulated.

Conclusion

- The results obtained showed a general reduction in expression of proteins belonging to the canonical MAPK pathway and an increase in abundance of downstream proteins of the mTOR pathway for the drug resistant cell line, consistent with previous results.
- However, a remarkable reduction in total analysis time was noted and a substantially higher number of peptide targets were identified at 1% FDR and quantified than in previous analysis thanks to the high throughput capabilities of Spectronaut.
- The Orbitrap Fusion Lumos outperformed the Orbitrap Fusion by 25% with regard to quantified proteins below 10% coefficient of variation at lower loads on column thanks to improvements in sensitivity.
- The large number of identifications obtained from Spectronaut provided an inclusive list from which to select significantly regulated proteins of interest for future targeted analysis.

References

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2. Senko, et al. (2013) Analytical Chemistry, 85, 11710-11714
3. Bruderer, et al. (2015) *Mollecular and Cellular Proteomics*, 27 February, Epub ahead of printing.

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