Optimized DDA+ and HR-DIA workflows for standardized, reproducible, precise and robust label-free quantitation of proteomes

Aaron S. Gajadhar[1], Oleksandr Boychenko[2], Xin Zhang[3], Yue Xuan[4], and Andreas Huhmer[1], Thermo Fisher Scientific, San Jose[1], CA, USA; Germering, Germany[2]; Sunnyvale, CA[3], USA; Bremen, Germany[4]

ABSTRACT

Purpose: To overcome the limitations of current label-free proteome quantitation (LFQ) protocols, we sought to develop an end-to-end, complete LFQ workflow solution for improved robustness, reproducibility, and throughput while still retaining high-performance.

Methods: A configuration of high-performing, yet robust and easy-to-use components, were optimized to satisfy the analytical requirements necessary for: standardized operation; reproducibly comprehensive results; a readily scalable workflow; and quantitatively precise measurements of proteomes.

Results: Complementary label-free precursor-ion based MS1 quantitation workflows — data-dependent acquisition plus (DDA+) and high-resolution DIA (HR-DIA) — allow highly sensitive and robust measurements to be collected in a cap-flow high-throughput based configuration.

INTRODUCTION

Quantitative proteomics studies require implementation of highly robust, analytical platforms to deliver accurate and reproducible peptide/protein quantitation, along with productive throughput. There is a need for optimized, analytical approaches to overcome the typical challenges of bottom-up LFQ of proteomes, such as inherent unreliability of nano-flow LC, and irreproducibility of conventional MS acquisition strategies. Here we describe a complementary set of label-free precursor-ion based MS1 quantitation workflows, DDA+ and HR-DIA, to achieve highly precise and reliable protein measurements. Each component of the analytical platform and workflow – HPLC, separation column, mass spectrometer and data analysis – has been optimized in an integrated fashion to achieve high sensitivity, robustness, throughput and reproducibility while eliminating missing values across samples.

MATERIALS AND METHODS

A standardized, label-free precursor-ion based MS1 quantitation workflow was configured with the following components. A capillary-flow Thermo Scientific™ UltiMate™ 3000 RSLCnano system was set up for pre-concentration mode using a 300 µm x 5 mm trap column (P/N 160454) in back-flush configuration. Rapid sample loading (100 µL/min flow rate) onto the trap cartridge was performed with the micro-flow loading pump integrated into the UltiMate 3000 RSLCnano system. Concurrently, a second low-flow high-pressure gradient pump delivers flow to equilibrate the separation column and mix the gradient. Peptides were separated using a mobile phase composition of consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) using a 45 minute gradient from 5% to 40% B at 1.2 µL/min flow rate. A Thermo Scientific™ EASY-Spray™ LC column, 15 cm × 150 µm (P/N ES801), was connected to the system and coupled to a Thermo Scientific™ Q Exactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer, with a Thermo Scientific™ EASY-Spray™ source (P/N ES981). The temperature of the EASY-Spray source and UltiMate 3000 RSLCnano system heating compartment was set at 40ºC. Mass spectroscopy (MS) analysis for the DDA+ workflow was performed using the following data dependent settings. MS1 full scans were acquired for 350-1400 m/z at 120,000 resolution, 60 ms maximum IT, 3e6 AGC target. MS2 HCD scans were acquired for Top 40 precursors at 7,500 resolution, 11 ms maximum IT, 1e5 AGC target, NCE=28, at an isolation window of 1.2 m/z. The dynamic exclusion time was set to 20 seconds and charge state screening was enabled to reject unassigned and singly charged ions. HR-DIA settings included MS1 full scans acquired for 350-1400 m/z range at 120,000 resolution, 60 ms maximum IT, 3e6 AGC target. Eighty, 10 Da wide DIA isolation windows were performed for each cycle. Various amounts of HeLa cell line protein digest (Pierce) spiked with peptide retention time calibration mixture (PRTC, Pierce) or iRT peptides (Biognosys) were analyzed to characterize the workflows. Label-free quantitation was performed using Thermo Scientific™ Proteome Discoverer™ 2.2 software for DDA+ and Spectronaut (Biognosys) for HR-DIA. Spectral raw files were analyzed using Proteome Discoverer 2.2 software using 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 oxidation (+15.995 Da) of methionine was considered a dynamic peptide modification. Data were searched against a SwissProt complete human database (Taxonomy ID: 9606, June-2017) with a 1% FDR criteria using Percolator. Label free quantitation was conducted using Feature Mapper and Precursor Ions Quantifier nodes in the Consensus Step and Minora Feature Detector node in the Processing Step with default settings. All HR-DIA data were directly analyzed in Spectronaut v10 software. Total peak areas of the isotopic envelope were chosen for quantification. All results were filtered by a Q value of <0.01 (equals a FDR of 1% on peptide level). Each experiment was performed in technical triplicates. The results are shown from technical triplicates.

RESULTS

DDA+ and HR-DIA Label-free Precursor Level Quantitation (LFQ) workflows

Two complementary LFQ workflows allow flexibility for both data-dependent acquisition and data independent acquisition methods in an optimized complete workflow for the rigors of standardized LFQ proteomics. The workflows leverage the user-friendly capillary-flow UltiMate 3000 RSLCnano system, the new 150 µm i.d EASY-Spray columns to balance sensitivity and robustness, the performance attributes of the next-generation Q Exactive HF-X mass spectrometer and data informatics empowered by Proteome Discoverer 2.2 software or Spectronaut DIA software to virtually eliminate the 'missing value' problem of traditional LFQ approaches.

Thermo Scientific™ UltiMate™ 3000 RSLCnano HPLC system

- Nano, cap, micro-flow rate range
- Direct inject or pre-concentration mode
- Easy connections with nanoViper fittings

Thermo Scientific™ EASY-Spray™ 150 µm LC column

- Thermo Scientific™ Acclaim™ PepMap™ C18 LC column 2 µm
- Sensitivity and robustness
- RT stability <1% CV observed for 350 injections

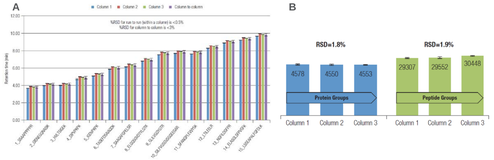
Thermo Scientific™ Q Exactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer

- Improved ion transmission
- Increased acquisition speed
- Advanced peak determination (APD)
- Same # of protein IDs in half the time

Reliable Performance - RT and Column Robustness

The DDA+ workflow was evaluated using HeLa cancer cell line digest with the new 150 µm EASY-Spray coupled to the UltiMate 3000 capillary LC system and an optimized acquisition method on the Q Exactive HF-X mass spectrometer consisting of 60 minutes total analysis time (Figure 1A). Intra-column (20 runs for each column) and inter-column retention time consistency was high with low variation between runs and columns. Excellent column-to-column consistency was also observed, including coverage of >4500 proteins, and ~30000 peptides all with RSD<2% between columns (Figure 1B).

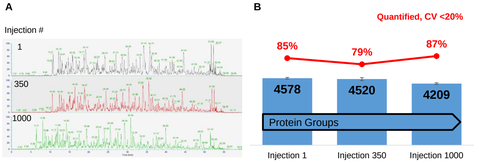
Figure 1. A) Intra and inter-column reproducibility of representative peptides from a PRTC standard (n = 20 injections). B) Inter-column consistency for proteomics applications (n = 3).



Workflow Durability for 1000+ Injection Study

A large cohort analysis was simulated by evaluating the performance of the DDA+ workflow following 1000 injections of HeLa digest (Figure 2). We observed a durable response factor upon inspection of MS1 base peak traces. Long-term quantitative performance was not compromised either as comparable proteome coverage and quantitative precision was observed throughout the 1000 injection experiment. This dependable, robust performance is essential for large scale proteomics initiatives.

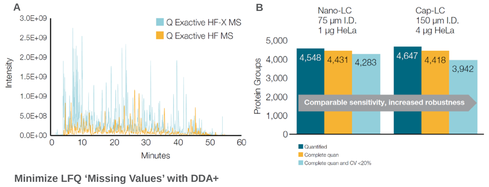
Figure 2. A) Durable long-term quantitative performance over 1000 injections shown by representative MS1 base peak traces. B) Dependable performance with <5% RSD in proteome coverage across 1000 injections of HeLa digest, and comparable number of precisely quantified proteins (n=3 from sequential injections).



Maintaining Sensitivity with Increased Robustness

The combination of the high-throughput capillary flow LC system and brighter ion-optics of the Q Exactive HF-X mass spectrometer allows a balance between sensitivity and robustness to be maintained. Evaluation of 1 µg HeLa digest injected on the Q Exactive HF-X mass spectrometer shows that the MS1 base peak trace is clearly higher in intensity than the same injection done on a Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap mass spectrometer, demonstrating improved sensitivity of this new system (Figure 3A). This enhanced sensitivity essentially allows the higher ID 150 µm EASY-Spray in capillary flow mode to achieve results similar to a traditional nano-LC setup with respect to quantified proteins, completely quantified proteins across triplicate analysis, as well as completely quantified highly precise measurements (Figure 3B).

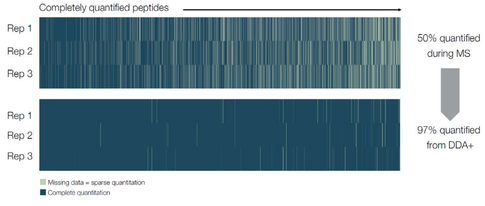
Figure 3. A) 1 µg HeLa digest was analyzed using the 150 µm EASY-Spray column and 60 min LC-MS analysis. The MS1 base peak chromatograms on each system are overlaid and shown. B) The improved sensitivity of the Q Exactive HF-X MS allows quantitative results comparable to nano-LC (300 nL/min flow to be obtained using the 150 µm EASY-Spray column and capillary-LC (1.2 µL/min flow).



Minimize LFQ 'Missing Values' with DDA+

DDA+ data analysis and LFQ reproducibility was improved with the Minora Feature Detector and Feature Mapper algorithms of the Proteome Discoverer software, version 2.2. MS1 mass accuracy, isotope pattern, charge state and retention time were used to link and quantify peptides across sample datasets. With this new algorithm, you only need to identify a peptide once across samples to quantify that peptide in all samples. This resulted in increased quantitation and less missing values across samples as 97% of peptides could be reproducibly quantified in triplicate HeLa DDA+ analysis, compared to only 50% of peptides in traditional DDA (Figure 4).

Figure 4. A triplicate analysis of 4 µg HeLa protein digest was analyzed using the Q Exactive HF-X mass spectrometer and DDA+. The heat maps indicate peptides that were commonly quantified across replicates (dark green) and those that were not quantified in one or more replicates (light green). The improved label free quantitation algorithm of DDA+ boosts quantitative information resulting in a more comprehensive analysis.



DDA+ Enables Quantitative Confidence Through High Precision and Reproducibility

The quantitation variance from the 4 µg triplicate HeLa analysis was evaluated. The overall precision of the measurements was assessed by plotting the distribution of the coefficient of variation for all the proteins and peptides that were quantified in the experiment (Figure 5). Virtually all of the measurements have a CV of <20%, and in fact ~90% of the protein measurements have a CV<10%. This precision becomes important when monitoring very subtle biological changes that require very high fidelity quantitation. Additionally, the reproducibility of the measurements was evaluated across 15 HeLa digest analysis. As you would expect with traditional DDA the probability of detecting the same target decreases with more replicates, and after 15 samples you have fully quantified 62% of proteins and only 20% of peptides. The DDA+ algorithm really boosts these numbers quite substantially to now well over 90% of proteins and >80% of peptides which helps drive the reproducibility needed in quantitative studies (Figure 6).

Figure 5. DDA+ LFQ delivers highly precise protein and peptide quantitation

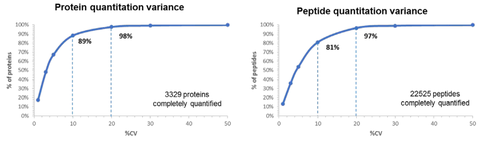
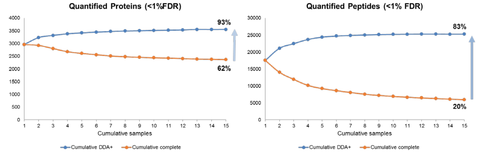


Figure 6. DDA+ LFQ workflow allows high reproducibility across samples and injections



HR-DIA: Unparalleled Coverage and Reproducibility

The HR-DIA workflow is uniquely suited for large scale studies due to its ability to rapidly co-isolate and fragment all precursors in the target *m/z* range for identification and precursor-based LFQ. The high resolving power and mass accuracy of the Thermo Scientific™ Orbitrap™ analyzer, and improved speed and sensitivity of the Q Exactive HF-X mass spectrometer empowers the HR-DIA LFQ workflow. A HR-DIA method using an optimized resolving power of 120,000 for MS1 scans and multiple 30,000 wide-isolation window DIA MS2 scans yields comprehensive identification and quantitation results (Figure 7). One of the challenges with optimizing DIA methods is determining the proper number of windows to ensure adequate sampling across the peptide elution profile. Under-sampling results in unreliable quantitation since the error may exceed acceptable limits. To facilitate method optimization, we recommend use of a scouting method that was developed by Biognosys (Figure 8). Using a selected chromatography setup and gradient, the DIA scouting method can capture information about cycle time and peak widths. The information is used to calculate desired peak sampling parameters and create an optimized method. This method and tutorial is available upon request.

Figure 7. A triplicate analysis of HeLa protein digest was analyzed using an optimized HR-DIA method (120K MS1, 30K DIA, 10 m/z x 80 windows). A) Peptide groups quantified from 4 µg HeLa protein digest. B) Protein groups quantified from a 60 minute MS analysis.

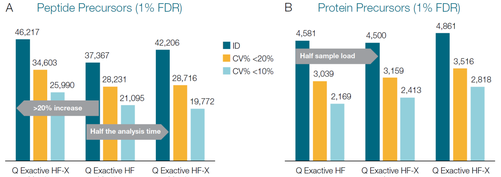


Figure 8. HR-DIA scouting method facilitates optimal DIA method generation.

CONCLUSIONS

We describe a standardized proteomics platform for DDA and DIA label-free quantitation providing sensitivity, throughput and robustness while retaining quantitative reproducibility across experiments. These new DDA+ and HR-DIA workflows for label-free quantitation eliminate missing values across samples, delivering high sensitivity and precision in a user-friendly, complete workflow solution.

REFERENCES

1. A. Gajadhar. Pushing the leading edge in protein quantitation: Integrated, precise, and reproducible proteomic workflows". Slide Presentation. PlanetOrbitrap.com
2. Top reasons to upgrade to a Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer. Technical Guide/Grant Application Resource. PlanetOrbitrap.com
3. X. Zhang, A. Gajadhar, X.Sun, M.Baynham. A highly sensitive and robust 150 µm column to enable high-throughput proteomics. APPLICATION NOTE 21744. PlanetOrbitrap.com

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