Combining the Data-driven and Hypothesis-driven Approaches in One Go via a Novel Intelligent Data Acquisition Hybrid-DIA Mass Spectrometry Strategy

Yue Xuan[1], Min Huang[2], Xiangyun Yang[2], Kyle Fort[1], Jesse D. Canterbury[3], Daniel Lopez Ferrer[3], Tao Bo[2], Alexander Makarov[1], Andreas F. Huhmer[3]
1. Thermo Fisher Scientific (Bremen) GmbH, Hanna-Kunath Str. 11, Bremen 28199, Germany; 2. Thermo Fisher Scientific (China) Co. Ltd, Shanghai, China; 3. Thermo Fisher Scientific, San Jose, CA, USA

ABSTRACT

Purpose: To develop a novel intelligent data acquisition "Hybrid-DIA" MS strategy that enables comprehensive proteome profiling via high resolution MS1-based data independent acquisition (HRMS1-DIA) MS and on-the-fly intelligent switching of the acquisition mode to parallel reaction monitoring (PRM) for sensitive and absolute quantification of the markers, substantially increasing throughput and reducing sample consumption.

Methods: Experiments comparing standard DIA methods to the novel Hybrid-DIA methods were performed using a nanoLC system coupled to the Orbitrap Exploris 480 MS and Orbitrap Lumos MS. The Hybrid-DIA method is programmed in C#, utilizing the iAPI.

Results: The novel Hybrid-DIA method was able to improve signal-to-noise ratio, enhance limit of detection and quantitation, and reduce interferences for biomarker quantification, while simultaneously comprehensively profiling proteomes with one single shot LC-Hybrid-DIA analysis.

INTRODUCTION

Translational scientists face the dilemma to choose between comprehensive profiling and sensitive targeted quantitation, especially. with large sample cohorts. Proteomic profiling is commonly used to discover biomarkers, having a great potential for identifying prognostic and predictive biomarkers; however, it lacks the sensitivity to quantify all the markers of interests. Therefore, targeted quantitation experiments of the potential markers are analyzed in the validation phase. This leads to high costs, time losses and more sample consumption. To address these challenges, we have developed a novel intelligent data acquisition "Hybrid-DIA" MS strategy that enables comprehensive proteome profiling via high resolution MS1-based data independent acquisition (HRMS1-DIA)[1] MS and on-the-fly intelligent switching of the acquisition mode to parallel reaction monitoring (PRM) for sensitive and absolute quantification of the markers, substantially increasing throughput and reducing sample consumption.

MATERIALS AND METHODS

Sample Preparation

Pierce™ Hela digest was spiked with 6X5 LC MS/MS Peptide Reference Mix (Promega), which is a mixture of 6 sets of 5 isotopologues of the same peptide sequence (30 peptides in total). The isotopologues of each peptide are present in a series of tenfold differences in concentration. The final solution contains 25fmol/ul of the highest isotopologues peptide and 250ng/ul of Pierce hela digest. 2ul and 4ul of the sample was load onto the LC column for the analysis, respectively.

Plasma sample are depleted using High-Select™ Top14 Abundant Protein Depletion Mini Spin Columns and protocol. PlasmaDeepDive™ reference preptides (Biognosys) were prepared according to the factory protocol, then mixed with plasma digest sample for a final concentration of 250 ng/ul depleted plasma sample. 4ul of the sample was load onto the LC column for the analysis.

LC-MS Method

Samples were separated with a Thermo Scientific™ UltiMate™ 3000 LC system using Acclaim™ PepMap™ columns (trap column PN 164946 and analytical column PN 16494) and analyzed with a Thermo Scientific™ Orbitrap Exploris™ 480 or Orbitrap Fusion Lumos™ Tribrid™ mass spectrometer in data independent acquisition (DIA) and intelligent Hybrid-DIA mode with different LC gradients from 25mins to 60min. Detailed gradient information appears below in Table 1. Details of the standard DIA and Hybrid-DIA methods are presented in Table 2.

Data Analysis

DIA data analyses were performed using Spectronaut™ v.15 software (Biognosys) with 1% FDR using house-prepared spectral libraries. The DIA scans of the Hybrid-DIA raw files were analyzed in Spectronaut v15, and Skyline software was applied for extracting fragments from the intelligently triggered (msx)PRM scans and performed the quantitation evaluation.

Table 1. LC gradients with buffer A (0.1% FA in water) and buffer B (0.1% FA in 80% ACN)

Time (min)	Flow (nl/min)	Mixture (%B)
0	800	1
3	300	8
18 / 33 / 53	300	30
22 / 37 / 57	300	95
25 / 40 / 60	300	95

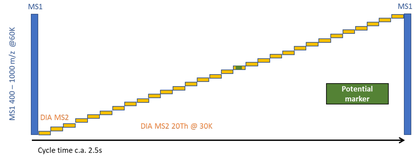
RESULTS

Intelligent Data Acquisition: Hybrid-DIA MS Strategy

The hybrid-DIA strategy consists of a standard DIA scan cycle, where MS full scan is followed by DIA MS/MS scans. Fast (multiplexed) PRM MS/MS scans are triggered from MS data based on isotope labelled peptide signals and are used as a second layer of confirmation for isotope labelled peptides. Successful isotope labelled peptide detection triggers the high-quality measurement of corresponding endogenous peptides multiplexed (msx) with the labeled peptides through (msx)PRM MS/MS scans acquired with narrower isolation window width and maximizing ion injection time for each species. This data acquisition scheme maximizes instrument productivity while resulting in only minor increases in DIA duty cycle time (Figure 1).

Figure 1. Standard DIA and the novel Hybrid-DIA MS Strategy

Standard Data independent acquisition (DIA): all ions, including the markers, are fragmented and detected in the wide isolation window DIA MS/MS scans and ion injection time is calculated for all the ions in the DIA window.



Novel Hybrid-DIA is an intelligent data acquisition strategy using isotope labelled standards (IS) to on-the-fly trigger (msx)PRM scans with a narrow isolation window and maximizing the ion injection time of the endogenous peptide, while simultaneously acquiring the DIA data of the entire proteome.

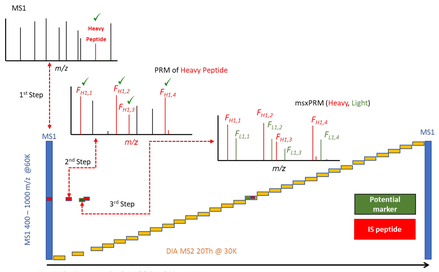


Table 2. Standard DIA & Hybrid-DIA methods

	Mass Range (m/z)	AGC target	Maximal ion injection time (ms)	Resolution (@ 200 m/z)	LC gradients (min)
MS1	400 – 1000	3e6	Auto	60,000	25 / 40 / 60
DIA MS2	20 / 15 / 10	5e5	Auto	30,000	25 / 40 / 60
(msx)PRM MS2	2	5e5	200 (light peptide) 10 (heavy peptide)	30,000	25 / 40 / 60

Global Proteome Profiling

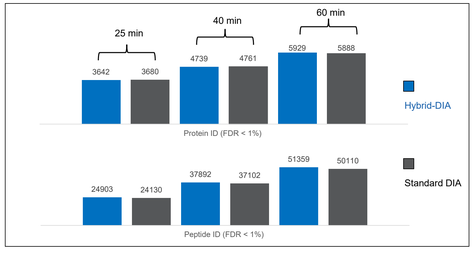
Comprehensive proteome profiling with Hybrid-DIA

The global profiling and quantitation performance of Hybrid-DIA MS was investigated and benchmarked against the standard DIA MS methods by analyzing a mixture of stable isotope labelled peptides with a concentration range of 4 orders of magnitude, which were spiked in a HELA digest on the same LC system coupled to the Orbitrap Exploris 480 MS. Similar number of proteins and peptides were identified with 1% FDR and quantified with medium CV<10% by both the Hybrid-DIA and DIA experiments (Table 3 and Figure 2).

Table 3. Hela Proteome Profiling via Hybrid-DIA vs. Standard DIA @ different LC gradients

LC gradient	Methods	Protein ID (FDR < 1%)	Protein medium CV(%)	Peptide ID (FDR < 1%)	Peptide medium CV(%)
25 min	Hybrid-DIA	3642	4.8	24903	4.5
25 min	Standard DIA	3680	5.1	24130	4.3
40 min	Hybrid-DIA	4739	5.7	37892	5.8
40 min	Standard DIA	4761	3.6	37102	3.5
60 min	Hybrid-DIA	5929	4.8	51359	5.1
60 min	Standard DIA	5888	6.3	50110	6.4

Figure 2. Hela Proteome Profiling via Hybrid-DIA vs. Standard DIA @ different LC gradients



Sensitive and Accurate Targeted Quantitation

Improved LOQ/LOD of the targeted peptides with Hybrid-DIA

The narrow isolation window and maximized ion injection time of the msxPRM scans improve the selectivity and sensitivity of quantitation, as well as the confidence of detection and quantitation esp. with high background matrix (Figure 3). The XICs of the fragments from the targeted peptides with Hybrid-DIA provide much cleaner chromatographic peaks and higher signal-to-noise ratio comparing to DIA experiment (Figure 3A). LOQ and LOD of the peptides are improved with Hybrid-DIA vs standard DIA method. (Figure 3B).

Figure 3A. XICs of the fragments with 10ppm mass tolerance from four Promega peptides at 50amol

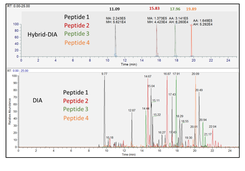
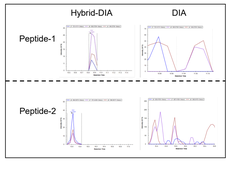


Figure 3B. Hybrid-DIA improves the LOQ and LOD of the endogenous peptides



Comprehensive plasma profiling and sensitive biomarkers quantitation in single-shot LC-Hybrid-DIA MS/MS

Plasma Profiling Performance

Plasma sample spiked with a mix of isotope labelled plasma reference peptides representing relevant targets for biomarker screening were analyzed by the standard DIA MS and Hybrid-DIA MS methods. 20, 40, and 60 peptides are targeted with 60min LC-Hybrid-DIA analysis, respectively. The novel Hybrid-DIA method was able to improve single-to-noise ratio, enhance limit of detection and quantitation, and reduce interferences for biomarker quantification, while simultaneously profiling more than 500 protein groups in human plasma with one single-shot LC-Hybrid-DIA analysis (Figure 4 and Figure 5).

Figure 4. Single-shot plasma profiling by Hybrid-DIA vs Standard DIA method. The number of identified protein groups and peptides with standard DIA method is set as 100%.

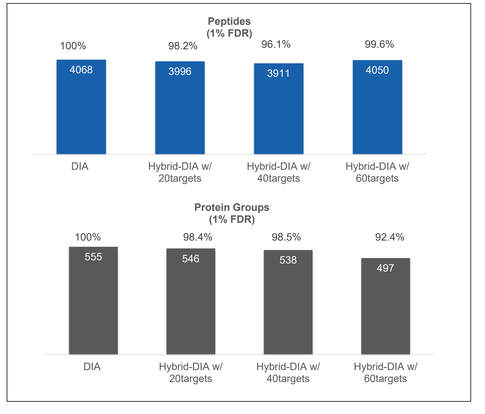
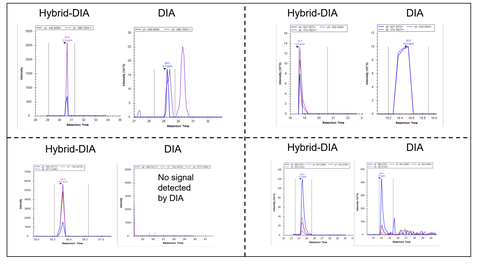


Figure 5. Hybrid-DIA method improves single-to-noise ratio, enhances limit of detection and quantitation, reduces interferences, more accurately quantifying plasma biomarker peptides



CONCLUSIONS

This novel Hybrid-DIA MS methodology presents a new capability to combine the data-driven and hypothesis-driven approaches in one go, enabling broad proteotype digitization via DIA scans and simultaneously sensitive quantitation of the markers of interests to support clinical decision-making.

- Similar number of proteins and peptides are identified with 1% FDR and quantified with median CV < 10% by both the Hybrid-DIA and DIA experiments at different LC gradients.
- By on-the-fly intelligently inserting (msx)PRM scans of the targeted peptides, much narrower isolation window and maximized ion injection time for the targeted peptides are applied, resulting in highly specific and sensitive quantitation.
- Hybrid-DIA MS strategy has demonstrated its capability to improve siignal-to-noise ratio, enhance limit of detection and quantitation, and reduce interferences for biomarker quantification, while simultaneously comprehensively profiling ~ 6000 protein groups from hela digest and > 500 plasma protein groups with a single-shot analysis in 60min.

REFERENCES

[1] Xuan, Y., Bateman, N.W., Gallien, S. et al. Standardization and harmonization of distributed multi-center proteotype analysis supporting precision medicine studies. Nat Commun 11, 5248 (2020). https://doi.org/10.1038/s41467-020-18904-9

TRADEMARKS/LICENSING

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