Accelerating proteotyping transformation into clinical routine via an intelligent data acquisition Hybrid-DIA MS based proteomics solution

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ABSTRACT

Purpose: The hybrid-DIA method aims to comprehensively digitize a clinical specimen while simultaneously enhancing measurement sensitivity for a specific set of markers of clinical interest. Diagnostics level-1 markers (of current clinical interest; 1%) & level-2 markers (of potential clinical interest & pathways; 9%) can be accurately monitored in an absolute fashion via highly sensitive and intelligent on-the-fly triggered multiplexing PRM scans, while simultaneously discovery-driven DIA allows to establish the clinical proteotype of level-3 information (markers of currently unknown clinical value; 90%) in single hybrid-DIA MS experiment.

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

Results: The preliminary data demonstrate that the peptides of interests can be detected with a higher signal-to-noise ratio, lower limit of detection and quantitation, fewer interferences, and lower CVs at lower concentration for Hybrid-DIA MS than for standard DIA MS, while simultaneously comprehensively profiling proteomes with one single shot LC-Hybrid-DIA analysis.

INTRODUCTION

MS-based proteotyping has been widely employed for biomarker discovery over the past two decades, yet the clinical/translational proteotyping community requires strategies that not only enable the discovery of novel biomarker candidates but can also boost the probability of establishing protein-based biomarker assays, enhance analytical and clinical validation speed, and resolve the issue of data missingness in a quantitative protein matrix (Fig.1). Here, we present a novel intelligent "Hybrid-DIA" data acquisition MS strategy that enables comprehensive digitization of a clinical specimen on the proteotype level via high resolution MS1-based data-independent-acquisition (HRMS1-DIA) MS (Xuan et al. 2020[1]), while simultaneously enhancing measurement sensitivity for a specific set of markers of clinical interest via "on-the-fly" intelligently inserting the parallel reaction monitoring (PRM) scans. (Fig.2)

MATERIALS AND METHODS

Sample Preparation

SpikeTides™ Set TAA - heavy and light (JPT technologies) were resuspended in LC-LOAD (Preomics) at an approximate concentration of 10 pmol/ml. Dilutions from these stock solutions were prepared accordingly. Homemade Hela lysate was prepared digesting 100 mg of protein input material using the Preomics kit. Homemade Hela lysate was used at a concentration of 1 mg/ml as background matrix in the dilution series.

Methods

The quantitation of the TAA marker panel and the global proteome profiling performance of the hybrid-DIA MS methodology were characterized and benchmarked to the standard PRM and DIA methods. Samples were separated with a Thermo Scientific™ EASY-nLC™ 1200 system using a PepMap™ column (ES803) and analyzed with a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer in standard data independent acquisition (DIA), parallel reaction monitoring (PRM), and intelligent data acquisition Hybrid-DIA mode with 2hour LC gradient with a target list of 120 and 179 , respectively. Three technical replicates are analyzed per condition.

The hybrid-DIA method is programmed in *C#*, utilizing the instrument API on an Orbitrap Exploris 480 MS.

Data Analysis

Targeted quantitation data analysis pipeline for Hybrid-DIA (msxPRM scans) is shown in Fig. 3, where the data were processed and quantified to calculate the Heavy/Light ratio using Python, Skyline, and shinnyR.

The .HTRMS converter (Biognosys) was applied to extract the full scan and DIA scans from the Hybrid-DIA files into the .HTRMS files, which were then analyzed in Spectronaut™ v.15 software (Biognosys) using spectral-library free directDIA search. Standard DIA raw data files were directly analyzed in Spectronaut v.15 (Biognosys) using directDIA search. Standard PRM raw data files were analyzed using Skyline software.

RESULTS

Intelligent Data Acquisition Hybrid-DIA MS Strategy

Hybrid-DIA MS can minimize sample consumption and measurement time by merging different acquisition schemes into one LC-MS run, reducing data missingness, enabling researchers to combine DIA-driven discovery phase and targeted-driven validation phase in one step and in one single experiment.

Figure 1. Intelligent data acquisition Hybrid-DIA strategy enables the comprehensive digitization of a clinical specimen on the proteotype level while at the same time enhancing measurement sensitivity for a specific set of markers of clinical interest .

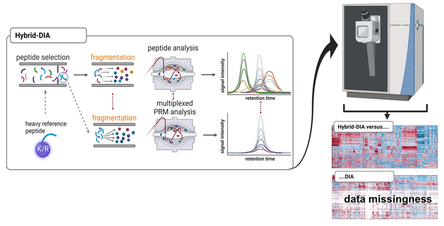


Figure 2A. Standard high-resolution MS1-based quantitative data independent acquisition (HRMS1-DIA) : all ions, including the markers, are fragmented and detected in the wide isolation window DIA MS/MS scans and ion injection time is calculated for all the ions in the DIA window.

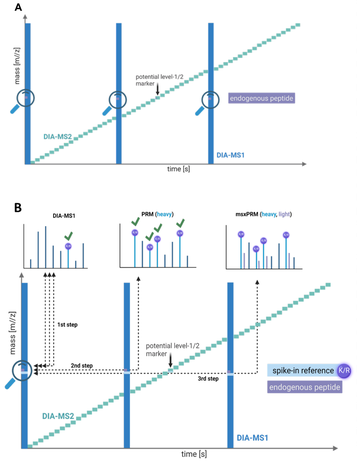


Figure 2B. Intelligent Data Acquisition Hybrid-DIA MS :

1st step: The Hybrid-DIA strategy consists of a standard DIA scan cycle, where MS1 scans are followed by several DIA MS/MS scans. High resolution accurate mass MS1 scans can detect the isotope-labeled reference peptides at low S/N ratio.

2nd Step: Fast (multiplexed) PRM MS/MS scans are triggered based on the detection of isotope-labeled reference peptides and serve as a second layer of confirmation.

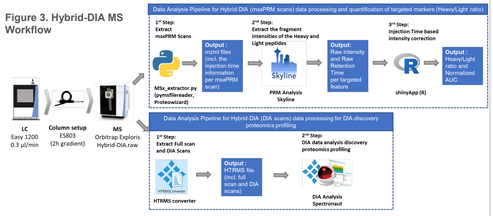
3rd Step: Successful isotope-labeled peptide detection triggers the high-quality measurement of the corresponding endogenous counter-peptide, multiplexed (msx) with the isotope-labeled peptide through msxPRM MS/MS scans acquired with narrower isolation window width and maximizing ion injection time for each species.

Hybrid-DIA MS Workflow

Comprehensive proteome profiling with Hybrid-DIA

In the context of digitization of larger clinical cohorts, Hybrid-DIA MS could provide clinically actionable information and new research insights into disease development and treatment trajectories as the basis for new AI-driven biomarker/diagnostic protein pattern detection. The Hybrid-DIA workflow is shown in Fig. 3.

Figure 3. Hybrid-DIA MS Workflow

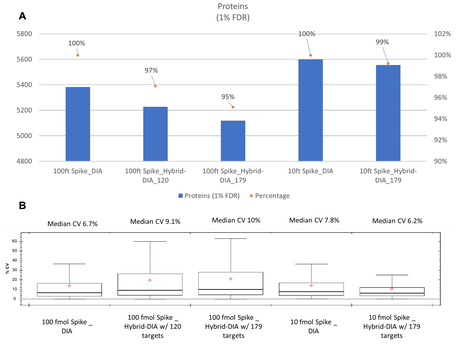


Global Proteome Profiling

Comprehensive proteome profiling with Hybrid-DIA

The global profiling performance of Hybrid-DIA MS was investigated and benchmarked against the standard DIA MS method by analyzing a HELA cell lysate digest spiked with a mixture of crude stable isotope labelled peptides with a concentration of 10 fmol and 100 fmol, respectively. 120 and 179 targets were monitored per LC-Hybrid-DIA experiment, respectively. In given the crude stable isotope labelled (IS) peptides mixture has an extremely high background, > 5000 proteins are identified with 1% FDR by all DIA and Hybrid-DIA experiments (Figure 4A). While increasing the number of targeted peptides from 120 to 179 per 2-hour LC-Hybrid-DIA experiment, Hybrid-DIA methods demonstrate consistent and comparable proteome profiling capabilities as the standard DIA methods with both samples. Not only were a similar number of proteins identified, but Hybrid-DIA methods alsoshow good quantitation precision of the proteins and peptides with the median CVs below 10%. (Figure 4B).

Figure 4. Hybrid-DIA methods w/ a predefined targeted list of 120 and 179 peptides, respectively vs. standard DIA method on Hela digest spiked w/ a mixture of crude peptides with a concentration of 10fmol and 100fmol. The number of identified proteins with the standard DIA method is set as 100% (Fig. 4A). The median CV values of proteins for each run is shown in Fig. 4B.



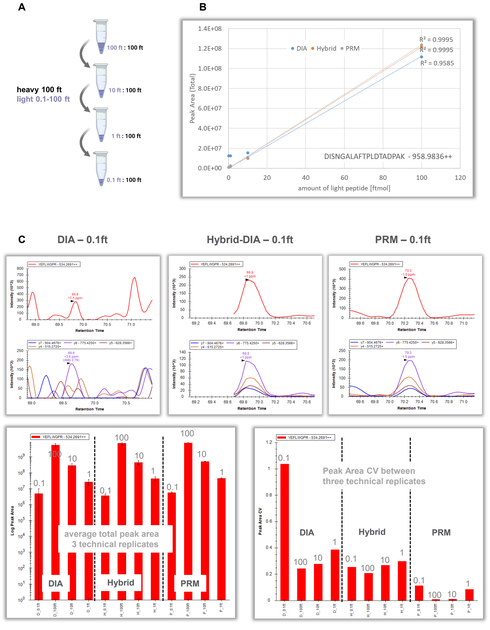
Quantitation of diagnostic markers

Improved quantitation of diagnostic markers with Hybrid-DIA

We tested the Hybrid-DIA method on a pool of 252 representative proteotypic peptides for tumor associated antigens (TAA) derived from 61 annotated human proteins. We generated mixtures containing both the heavy reference peptide as well as its synthetic light isotope. Whereas the heavy reference peptide was kept constant in all samples at approximately 100 femtomole (injected onto column), its light counterpart was measured in a dilution series ranging from 100 femtomole to 100 atomole. We monitored 179 TAA peptides at a time in a scheduled fashion using high-resolution msxPRM while at the same time recording the DIA traces in Hybrid-DIA.(Fig. 5A)

The narrow isolation window and maximized ion injection time of the msxPRM scans of Hybrid-DIA method improve the selectivity and sensitivity of quantitation, as well as the confidence of detection and quantitation, especially with high background matrix (Fig. 5). The linearity of quantitation of detected peptides were improved to 0.9995 for Hybrid-DIA method, better than the standard DIA method (0.95), and as good as standard PRM methods (0.9995) (Fig. 5B). Compared to the standard DIA method, hybrid-DIA is able to improve the LOD/LOQ and achieve better quantitation precision with lower CVs of the targeted peptides, especially for the low abundant endogenous peptides at low concentration ranges (Fig. 5C).

Figure 5. Hybrid-DIA method vs. standard DIA method in quantifying the peptides of interest



CONCLUSIONS

This novel Hybrid-DIA MS methodology presents a new capability to combine the data-driven and hypothesis-driven approaches in one experiment, enabling broad proteotype digitization via DIA scans and simultaneous sensitive quantitation of the markers of interests by on-the-fly triggered targeted scans to support clinical decision-making, substantially increasing throughput and reducing sample consumption. .

- Here, we present the novel intelligent data acquisition Hybrid-DIA MS strategy on sensitively and accurately quantifying diagnostics level-1 markers (of current clinical interest; 1%) & level-2 markers (of potential clinical interest & pathways; 9%) in the complex human cell lysate sample, while simultaneously proteotyping level-3 information (markers of currently unknown clinical value; 90%) in a single hybrid-DIA MS experiment.
- Compared to the standard DIA methods, the Hybrid-DIA MS strategy can improve signal-to-noise ratio, enhance limit of detection and quantitation, reduce interferences, improve the coefficient of determination of the calibration curve, achieve better quantitation precision at lower concentration ranges, by on-the-fly intelligently inserting (msx)PRM scans of the targeted peptides (markers).
- In terms of DIA-driven proteomics discovery capabilities, Hybrid-DIA methods demonstrate consistent and comparable proteome profiling performance as the standard DIA methods. Not only are similar number of proteins identified with 1% FDR, but Hybrid-DIA methods also show good quantitation precision of the proteins and peptides with median CVs below 10% while simultaneously targeted quantifying 179 peptides per 2-hours of LC-Hybrid-DIA MS experiment.

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

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PO66165-EN0422S