Improved Metabolome Coverage and Increased Confidence in Unknown Identification Through Novel Automated Acquisition Strategy Combining Sequential Injections and MS^n

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ABSTRACT

Purpose: Develop a workflow that maximizes the number of unique metabolites interrogated by MS/MS and MS^n, while minimizing the acquisition of uninformative data, resulting in comprehensive metabolome coverage.

Methods: Human plasma (NIST SRM1950) was analyzed with a Thermo Scientific™ Orbitrap ID-X™ Tribrid™ mass spectrometer. The data were analyzed using Thermo Scientific™ Compound Discoverer™ 3.0 software for metabolite identification.

Results: The Orbitrap ID-X Tribrid MS with AcquireX resulted in more unique precursor ions fragmented and consequently more metabolites identified in human plasma.

INTRODUCTION

Metabolite identification is a current bottleneck in the broad implementation of metabolomics, hindering biological interpretation of results. The development of fast-scanning, high-resolution, accurate-mass spectrometers has increased the number of metabolites detected in biological samples. However, many metabolites remain unidentified because of sample complexity and limitations in data-dependent acquisition (DDA). Data-independent acquisition (DIA) approaches can provide fragmentation for all precursor ions simultaneously, but result in complicated fragmentation spectra, further convoluting the identification process. Here, we describe a data-informed workflow that maximizes the number of metabolites interrogated by MS/MS and MS^n, while minimizing the acquisition of uninformative spectra. This innovative workflow was used to analyze human plasma resulting in high confidence identifications, deeper metabolome coverage, and enhanced biological knowledge generation.

MATERIALS AND METHODS

Sample Preparation

Human plasma (NIST SRM1950) was purchased from NIST. Metabolites were extracted by addition of methanol at a ratio of 3:1 (methanol:sample). After centrifugation, the supernatant containing the metabolites was evaporated. Dried metabolites were resuspended in water containing 0.1% formic acid.

Instrumentation

Two microliters of the resuspended metabolites were injected on a Thermo Scientific™ Hypersil GOLD™ column (15 cm × 2.1 mm ID). The mobile phase consisted of solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). Instrumentation included a Thermo Scientific™ Vanquish™ UHPLC system, in-line with an Orbitrap ID-X Tribrid mass spectrometer.

Data Analysis

Data were analyzed using Compound Discoverer software. Traditional DDA was compared to the intelligent acquisition approach, called AcquireX, offered by the new instrument control software of the Orbitrap ID-X MS.

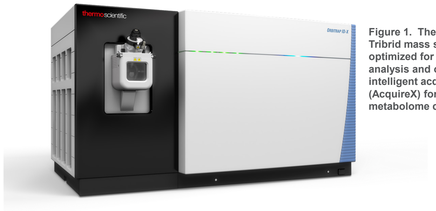


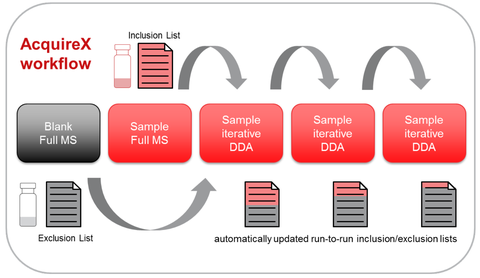
Figure 1. The Orbitrap ID-X Tribrid mass spectrometer is optimized for small molecule analysis and offers an intelligent acquisition method (AcquireX) for comprehensive metabolome coverage.

RESULTS

AcquireX – A New Acquisition Paradigm

To overcome the current bottleneck of metabolite identification in DDA-based metabolomics, we developed an improved workflow that allows for the direct interrogation of all detected metabolites and applied it to the analysis of human plasma.

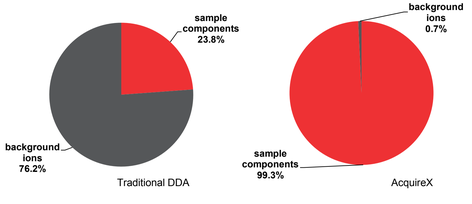
Figure 2. AcquireX represents a new acquisition paradigm. First, an exclusion list is generated from a blank run. Then, an injection of the sample followed by feature detection and component assembly populates the inclusion list with compounds detected in the samples. A series of iterative DDA injections follow. Each injection is informed from the previous one, minimizing redundant fragmentation spectra and maximizing relevant spectra and metabolite annotations.



Background Exclusion – Minimizing the Acquisition of Irrelevant Data and Enabling Fragmentation of More True Sample Components

During data-dependent MS/MS, ions are selected based on abundance, without any knowledge of biological relevance or type of ion. Often, irrelevant spectra, resulting from fragmentation of solvent, plasticizer, and other background ions dominate the duty cycle, limiting the capacity of the instrument to acquire informative spectra. In a typical DDA experiment, we determined that >70% of MS/MS spectra could be attributed to background ions. By enabling the automatic generation and implementation of a background exclusion list based on real-time feature detection in LC-MS data, background ion MS^2 spectra were practically eliminated, allowing for the analysis of more true sample components.

Figure 3. During a traditional DDA injection, background ions dominate the duty cycle and 76% of MS^2 scans could be attributed to background ions. With AcquireX, acquisition is focused on true sample components and fragmentation of background ions is minimized.

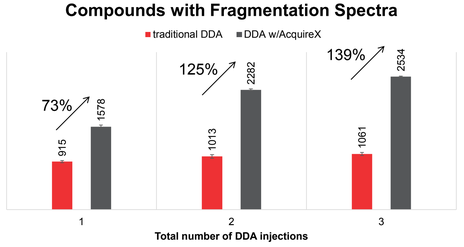


IMPROVED METABOLOME COVERAGE

AcquireX Provides Fragmentation for More Compounds than Traditional DDA

Small molecules form different types of adducts and cluster ions during electrospray ionization. Highly abundant compounds, in the form of a parent ion or any of its accompanying features, such as isotopes and adducts, may prevent the fragmentation of metabolites of lower abundance. By populating the inclusion list with the preferred ion for each metabolite, more compounds can be sampled by MS/MS in a single run. Additionally, by automatically updating inclusion and exclusion lists after each injection during analysis, we can ensure that compounds not selected for MS/MS will be prioritized during a subsequent injection.

Figure 4. The AcquireX workflow delivers improved MS/MS sampling by automatically excluding background ions and focusing acquisition on true sample components. AcquireX increased the number of compounds with fragmentation data in human plasma (NIST SRM1950) over traditional DDA by 139% after three injections.



AcquireX Minimizes Redundant Fragmentation and Enables Fragmentation of Low Abundance Compounds

In the traditional DDA workflows, each injection is independent of the previous one, resulting in redundant fragmentation spectra. With AcquireX, inclusion and exclusion lists are automatically updated after each injection, minimizing redundant fragmentation and allowing for more analytes of lower abundance to be sampled with subsequent injections. In the analysis of human plasma (NIST SRM1950), the most abundant compounds were only fragmented in injection one resulting in a 5-fold decrease in average intensity of compounds fragmented from injection 1 to injection 3.

Figure 5. Unlike traditional DDA workflows, AcquireX avoids redundant fragmentation across a series of subsequent injections, in favor of interrogating new compounds.

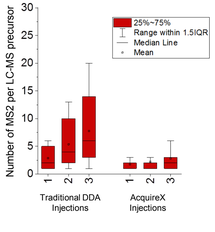
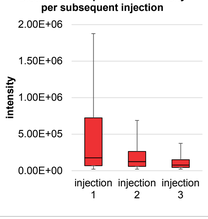


Figure 6. AcquireX triggers fragmentation for more unique precursors with every reinjection allowing for more analytes of lower abundance to be sampled.



CONFIDENT COMPOUND ANNOTATIONS

Improved MS/MS Sampling Translates to More Spectral Matches

AcquireX results in the interrogation of more unique precursors in a sample, which in turn translates to more compounds annotated. One way to confidently annotate compounds is through a spectral match against a library, such as mzCloud™ (www.mzCloud.org)

Figure 7. AcquireX results in more spectral matches against mzCloud over DDA (exact and similarity matches).

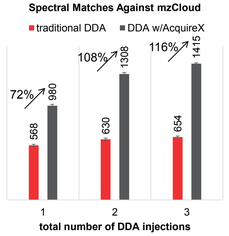


Figure 8. AcquireX results in more mzCloud identifications over DDA (exact match).

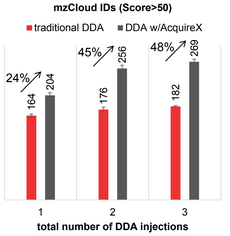


Figure 9. Spectral match against mzCloud increases confidence in compound identification. Below is the MS^2 spectrum of an unknown with an excellent match to the MS^2 spectrum of tyrosine in mzCloud.

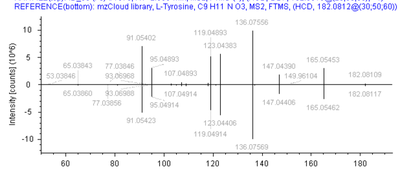
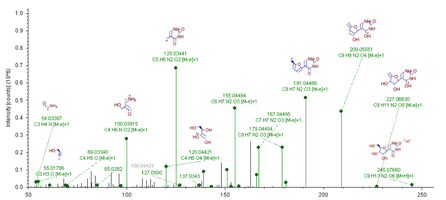


Figure 10. For compounds not in mzCloud, fragmentation data can be used to provide more confidence in ChemSpider™ hits. Below is the structural annotation of the MS^2 spectrum of an unknown at *m/z* 245.0770 supporting the putative ChemSpider annotation of pseudouridine.



INFORMATION-RICH DATA FOR CONFIDENT IDs

AcquireX Offers Flexibility for MS^n Acquisition

AcquireX efficiently collects more data allowing for multiple dissociation techniques and multi-stage fragmentation in the same injection without compromising metabolome coverage. Duty cycle is no longer a limitation, as any compounds not selected during the first injection can be prioritized during subsequent injections.

Figure 11. MS^n data can now be collected for all precursors using AcquireX, resulting in more confident annotations. Below, MS^3 fragmentation of kynurenine provides increase confidence in its identification.

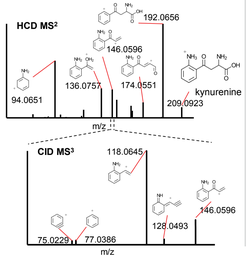
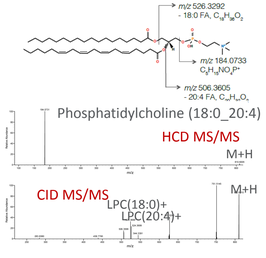


Figure 12. Complementarity of HCD and CID increase the probability of generating information-rich spectra across more compound classes and is illustrated in the spectra of phosphatidylcholine (18:0_20:4) below.



CONCLUSIONS

- The Orbitrap ID-X system delivers improved MS/MS sampling by automatically excluding background ions and focusing acquisition on true sample components. This novel, data-informed approach, AcquireX, utilizes sequential injections to interrogate more unique compounds.
- AcquireX prevents redundant fragmentation of highly abundant compounds allowing for more analytes of lower abundance to be sampled with subsequent injections.
- The Orbitrap ID-X system offers multistage fragmentation (MS^n) and the flexibility of complementary dissociation techniques (HCD, CID) to increase the probability of generating information-rich product-ion spectra across more compound classes. Annotation of more high-quality spectra leads to improved metabolome coverage and enables comprehensive pathway annotation and functional interpretation of results.

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TRADEMARKS/LICENSING

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