API Controlled In-Depth Proteome Analysis by Utilizing an Inter-LC Run Logic on Benchtop Orbitrap MS

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Overview

Purpose: The .NET based Application Programming Interface (API) for Thermo Scientific™ Orbitrap™ benchtop mass spectrometer should be used to apply Inter and Intra Run logic decisions during and between LC-MS/MS runs of complex proteomics samples to access optimization strategies of iterative analysis steps.

Methods: The Exactive Series API is used to monitor precursor ion streams at runtime to track the ion flux behavior of potential targets of interest over separation run time level at different LC gradient programs and sample loads. Additionally the API is used to apply intra-run as well as inter-run strategies, for a stepwise performing of iterative LC-MS/MS runs.

Results: Information on the availability of precursors over LC-MS/MS runs can be used at least to adapt the sample load and maximum allowed ion inject times for iterative LC-MS/MS runs to stay at a high sequenceing speed with a maximized number of selectable precursors.

FIGURE 1. API integration into common development environments.

Introduction

Data dependent Top N methods are wideley established for large scale bottom-up tandem MS protein sequencing. But Protein identification is increased only to a certain point, since this method prefer highly abundant precursors, belonging to highly abundant proteins. Thus, the sequence coverage for highly abundant proteins is increased, while lower abundant peptides were not triggered and their belonging proteins were not improved to identified. Iterative runs with an exclusion of already triggered precursors showed a deeper sequencing of low copy number proteins. [1]

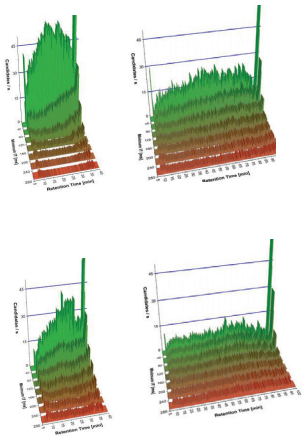

FIGURE 2. Extracted Precursors for a variation of load and gradient

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With a prototype .NET application was generated to communication with the Exactive Series Mass Spectrometer to analyze the scan data stream near real time to track the timely distribution of precursor candidates. The precursor densites can be used to determine the number of accessible candidates per time segments, clustered by their required ion inject time to reach sufficient fragmentation spectral quality (IT bands).

Depending on the sample complexity and applied separation program and sample, the IT bands (see Figure 2.) will provide enough precusors to run at a high sequencing frequency. By continuously updating the data of the retention time IT band matrix by already selected precursors and related species, the scenario for a next iterative analysis step can be determined. A lack of available precursors or resulting low scanning frequency can be avoided by increase the sample load or switch gradient programs to “refill” the candidates of interest.

The here described methodology is using dynamic handling of exclusion and inclusion of precursors and related charge members, an automatic adaption of injected sample volume and an ion inject time control to stay at a high sequenceing speed with a maximized number of selected precursors.

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Results

API

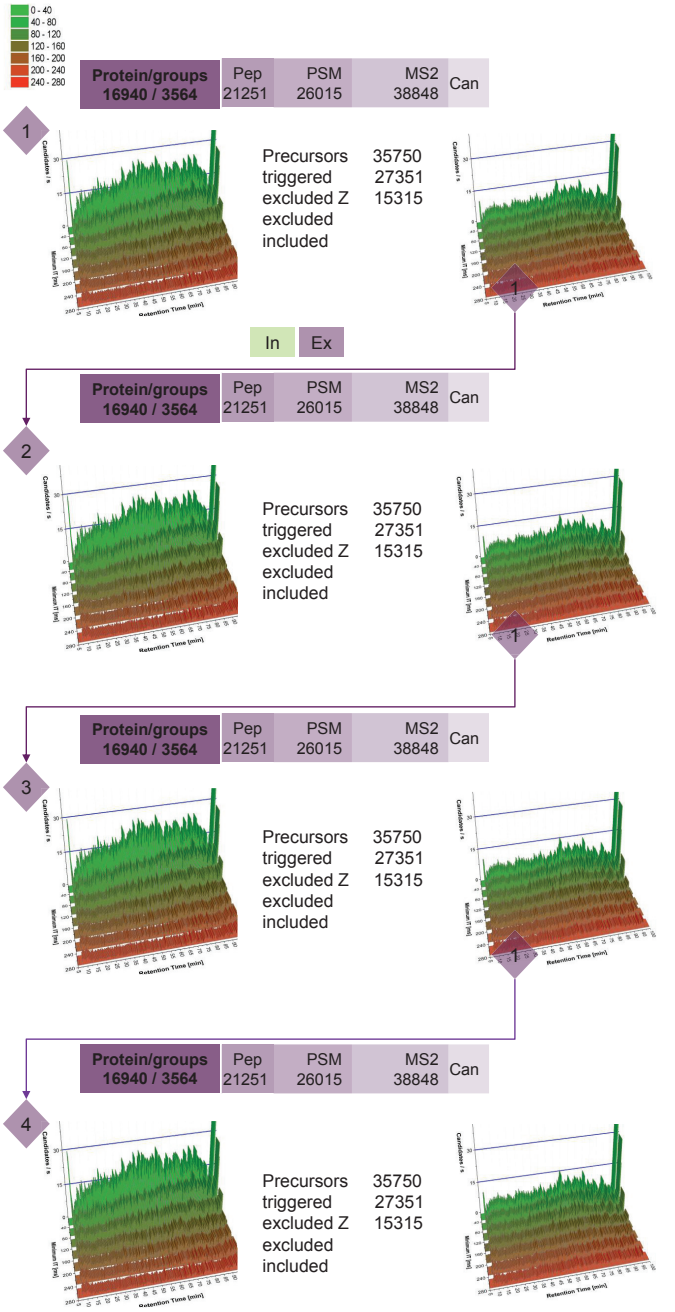

FIGURE 3. Extracted Precursors for a variation of load and gradient.

Inter Run Logic: Gradient, Load and maxIT

To avoid a decreasing sequencing speed and redundant data generation, the method parameter of following iteration steps can be adapted based on the data scenario of the current iteration. The distribution of the remaining precursor, after removing the already triggered ones, will indicate, if the IT band to run in "parallel mode" is still providing enough candidates over time (Figure 4a). If not, either the sample load or the maxIT can be increased to "refill" the IT bands or accessible precursors. An automation of the next LC injection can handled via the Thermo Scientific™ Xcalibur™ Development Kit (XDK) to define LC method and sample volume.

When observing indicatons of high ion suppression effects with decreased dynamic range or coalescense effects at high signal to noise ratios, the sample load should be decreased.

Based on this feedback, also gradient program optimization for an equal distribution of precursors over time can be approached.

Intra Run Logic: parallel inject optimization

The transient length of the Full MS scan can be used to allow longer injection times for precursors located in a lower IT band.

Inter Run Logic: Dynamic charge exclusion

The selection of different charge states of the same precursor is leading to a lower Peptide to PSM ratio. For the first run of an iteration set the dynamic exclusion of all charge members of selected percursors is helping to avoid the fragmentation of multiple charge states and focusing on the most abundant. (see TABLE 2.)

Ex Inter Run Logic: Static Exclusion

To avoid redundant data, all precursors with a acquired MS2 scan with sufficiently spectral quality should be excluded, as well, as all of their existing charge members. To avoid ClusterTop hopping, due to statistical variations, a set of up to three most abundant isotopes is added to the exclusion list. The timing of the exclusion is set to an extended time span, as the recognition is done on isotope pattern to compensate automatically for any retention time shifts. This opens the possibility to switch gradient programs and load at the same time, if no further candidates are available for the initial one.

In Inter Run Logic: Static Inclusion

As the maximum ion flux of untriggered precursors is known from previous iteration steps a spectral intensity based filter can be applied in a current run.

A selection of the candidate will be rejected, until a base flux level is reached. Thus, it is possible to focus on higher flux regions of the precursors and reduce the required inject time.

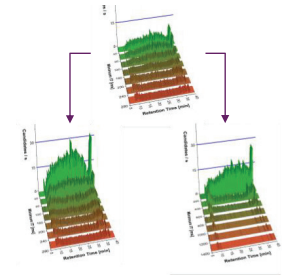

FIGURE 4. Adatption of (a) Sample Load or (b) MaxIT to "refill" accessible precursors

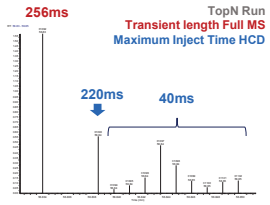

FIGURE 5. Adatption of sample

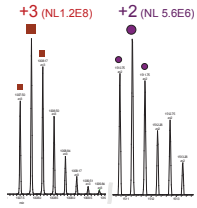

FIGURE 6. Three most abundant isotopes of each charge member

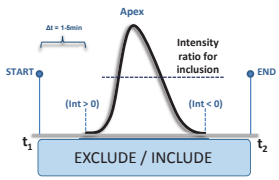

FIGURE 7. Three most abundant isotopes of each charge member

		In	Ex
mz	z	start	end
1510.7572	2	40.637	44.759
1511.2557	2	40.637	44.759
1511.7563	2	40.637	44.759
1007.5027	3	40.637	44.759
1007.8365	3	40.637	44.759
1008.1704	3	40.637	44.759

TABLE 1. Inlcusion / Exclusion entries for top three isotopes of each charge member of a analyte.

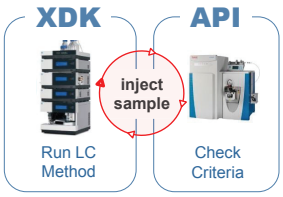

FIGURE 8. Adatption of (a) Sample Load or (b) MaxIT to "refill" accessible precursors

Gradient [min]	Load [ng]	Pre	IT ≤ 40ms	IT ≤ 80ms
30	**200**	56076	25282	11381
	600	58518	38216	10828
	1000	65267	49934	6780
60	**200**	93289	24402	15691
	600	102190	48294	20708
	1000	105000	61199	18312
90	**200**	126126	23951	17263
	600	135281	50619	27556
	1000	139242	62342	32424

TABLE 1. Inlcusion / Exclusion entries for top three isotopes of each charge member of a analyte.

Retention time shifts due to e.g. sample load or e.g. temperature shifts can be compensated as the isotope pattern is taken into account. (see Figure 9.)

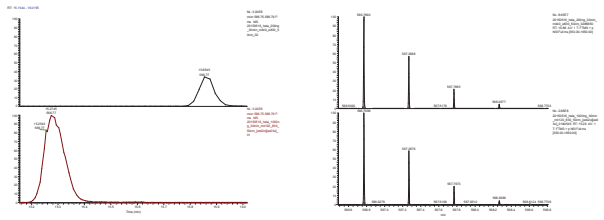

FIGURE 9. Elution profile of precursor 566.77 at 30min gradient with (top) 200ng and (bottom) 1µg load. Delta time of 30s will be compensated and exclusion of the already fragmented precursor stays valid.

The number of coexcluded charge members varies up to 5 with minimum of 2 isotopes makes a maximum total number of correlated isotopes per precuors of ten. The corresponding distribution can be seen in Figure 10. (a).

Precursors that have been already monitored in previous runs, but yet not being triggered, can be rejected from being selected, until a minimum of the expected ion flux is reached (minApexFilter). A benefitial reduction of inject time distribution can be seen in Figure 10 (c) with the distribution of the reached ratio of minApexFilter (Figure 10. (b).

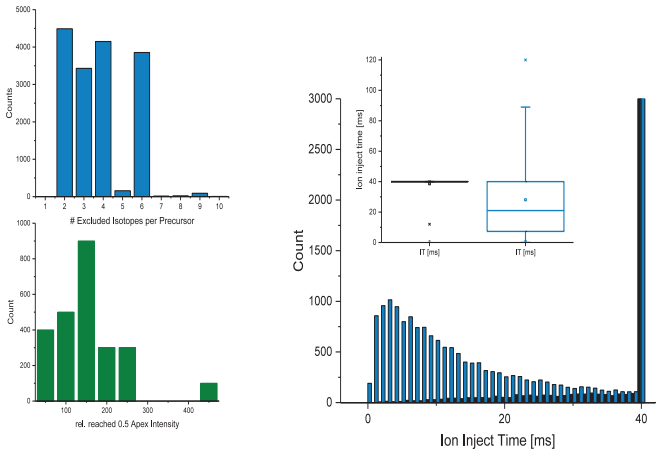

FIGURE 10. (a) Distribution of excluded #Isotopes per precursor, (b) relative reached flux compared to 50% of the apex intensity observed for the same precursor in a previous run. (c) Inject time (IT) distribution with reduced amount, when waiting for selection until min. 50% of previously (run) observed flux is reached (see b).

Methods

A HeLa Protein Digest Standard 0.5 µg/µL was gratefully obtained from Matthias Mann's lab (MPI Martinsried), diluted in HPLC grade H_2O (Thermo Fisher Scientific) to a final concentration of 0.2 µg/µL.

Mass Spectrometry

Thermo Scientific ™ Q Exactive ™ HF Mass Spectrometer

Liquid Chromatography

LC Stack: Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano system equipped v
nano pump NCS-3500 and autosampler WPS-3000TPL
Mobile Phases: A: 0.1 % FA in water; B: 0.1 % FA in Acetonitrile (Fisher Chemicals)
Gradients: 8–30 % B in of 30, 45, 60, or 90 min
Flow Rate: 250 nL/min
Trapping Column: Thermo Scientific™ Acclaim™ PepMap™100 µCartridge Column C18
300 µm × 0.5 cm, 5 µm, 100 Å
Separation Column: Acclaim PepMap C18, 75 µm × 50 cm, 2 µm, 100 Å

MS Method Parameter:

[Full MS only] Resolution: 120K, AGC Target: 3e6, maximum IT: 20ms
[Top15] FullMS only settings, ddMS2: Resolution 15K, AGC Target: 1e5,
maximum IT: 40ms / 80ms, Underfill ratio: 8% (AGC Target 8E3), Isolation width: 1.4 m/z
Peptide match: preferred, Dynamic exclusion: 30s

Data Analysis

All TopN runs have been processed with Thermo Scientific™ Proteome Discoverer™ Software 2.0 search engine SEQUEST® HT against IPI fasta database human v3.87. Exclusion and inclusion lists have been generated by a prototype software and partly imported in the Exactive Series instrument TopN method or directly linked to the method

Conclusion

Monitoring the time and flux distribution of available precursors over LC-MS/MS runs can be used to adapt sample loads for iterative LC-MS/MS runs to stay at a high sequenceing speed with a maximized number of selectable precursors.

Alternatively or in combination with a variation of the maximum inject time for data dependent MS2 scans, significant increase of protein and peptide IDs can be achieved, even with a reduced amount of required sample.

An extended exclusion strategy of related charge members and isotopes avoids redundant data acquisition, while including precursors can be used to control specific selection strategy for lower inject time band precursors and focus on the higher flux region to reduce the required inject time.

References

1. Chen, H.; Rejtar, T.; Andreev, V.; Moskovets, E.; Karger, B. L. *Anal. Chem.* **2005,** *77,* 7816–7825.
2. "Customized Real-Time Control of Benchtop Orbitrap MS", *Poster ASMS 2013* Kühn A, Grosse-Coosmann F, Rietpietsch T, Hauschild JP, Stratton T, Tham K, Bailey D, Malek R, Kellmann M, Henrich C, Lange O, Wieghaus A, Horning S, Makarov A.

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