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Novel approach to achieve high spectral quality without compromising identification for biopharma applications

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INTRODUCTION

Peptide mapping is the gold standard for in-depth characterization of biotherapeutics. LC-MS acquisition methods are optimized to provide good peptide identification and excellent sequence coverage. However, for some applications such as sequence variant and host cell protein analysis or the general identification of low level post-translational modifications, high quality MS2 is required for low intensity features. In these specific cases, the dilemma is that keeping high spectral quality requires sacrificing speed, and as a result, fewer features are identified. One strategy is to re-inject the sample several times, but the gain in identified features is often marginal because the same features are repeatedly targeted. In this study, we show how to achieve high spectral quality without sacrificing the number of identifications.

MATERIALS AND METHODS

Sample preparation: A 30-min trypsin digestion at 37 C, after reduction and alkylation and complete removal of guanidine from the digestion buffer by using BioSpin P-6 gel columns was performed (see reference for more details). The final concentration after digestion was approximately 0.9 ug/ul.

Samples: Cetuximab, pertuzumab and NIST mAb were digested. Cetuximab and pertuzumab were also stressed by storing them for 3 weeks at 40 C and digested.
Two types of sample were used in this study:

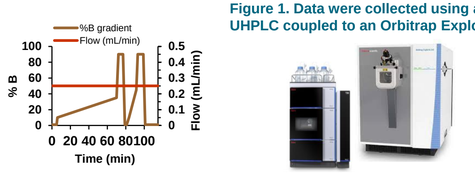
- *Stressed sample:* A 50/50 mix of cetuximab and pertuzumab stressed. 4ul of the stressed sample was used for each run.
- *Spiked sample:* A 50/50 mix of cetuximab and pertuzumab with NIST spiked at 1%. 8 uL of the spiked sample was used for each run.

Liquid Chromatograph: Thermo Scientific™ Vanquish™ Flex UHPLC

Columns: Thermo Scientific™ Acclaim™ column (C18, 250mm x 2.1 mm; 2.2 um).

Mobile phases: Buffer A: 0.1% Formic acid, in H_2O. Buffer B: 0.1% Formic acid, in CH_3CN

Figure 1. Data were collected using a Vanquish UHPLC coupled to an Orbitrap Exploris 240 MS.



Mass spectrometer: Thermo Scientific™ Orbitrap™ Exploris 240™ mass spectrometer.
Data were acquired in data dependent acquisition (DDA) mode with HCD fragmentation and an isolation window of 1.2 Da,

TopS (1.5s) DDA method	
MS1 OT res: 120K at 200 m/z	MS2 OT res: 15K at 200 m/z
AGC target MS1: 300%	AGC target MS2: 100%
MS1 Max Inject time: 200 ms	MS2 Max Inject time: 250 ms or 750 ms
m/z: 275-2000	Min Intensity threshold 2E4

Data acquisition: Thermo Scientific™ Xcalibur™ 4.7 with SII 1.6 and Orbitrap Exploris Series 4.2 SP2.

AcquireX™ Ab: After each ID run, MS2 parent ions are added to the exclusion list. For this study, an inclusion list was always automatically generated and used to trigger MS2 for subsequent ID runs. Three ID runs were acquired for each experiment.

Classic data acquisition: Each injection is independent and no information from one injection is transfer to the next injection. Triplicate injections were acquired for the classic data acquisition experiment.

Data Analysis: LC-MS raw files were processed with Thermo Scientific™ BioPharma Finder™ 5.1. The component detection settings are respectively 2000 and 20 for the MS noise level and the S/N threshold.
The following modifications were used: fixed: Carboxymethylation on C - variable: deamidation on N and Q, oxidation and double oxidation on M and W, glycation on K and isomerization on D.
For all experiments, BioPharma Finder results were filtered with the following criteria:
Must contains MS2, confidence score above 80 and mass error between =/- 5 ppm.
The average structural resolution (ASR) (fig. 6b and 8b) is a representation of the MS2 spectra quality. ASR = (Number of Amino Acids)/(Number of Bonds Found + 1).

RESULTS

Figure 2. Number of MS2 scans acquired and identifications for triplicate injections using a maximum fill time of 250 ms or 750 ms for the classic data acquisition.

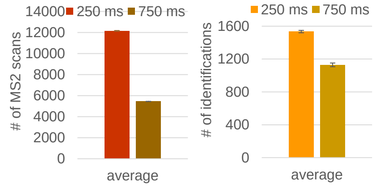
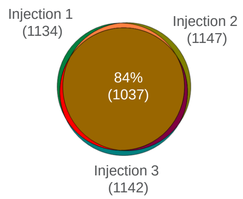
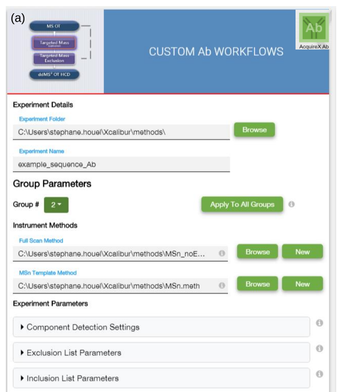


Figure 3. Venn diagram of identified peptides between the 3 injections (Fig. 2b) using a maximum fill time of 750 ms.



One strategy to increase the identification of low-level peptides is to accumulate more ions before fragmentation to collect better MS2 spectra. This can be achieved by increasing the maximum fill time. One caveat, with this strategy is that the number of MS2 scans decreases significantly (fig.2a), impacting the total number of identified peptides (Fig. 2b). Even by re-injecting the same sample, the improvement is limited due to the fact the same features are repeatedly targeted so the same peptides are identified (fig. 3). Another approach is to automatically remove the targeted peptides for the subsequent injections by adding them to an exclusion list. This strategy is at the core of AcquireX.

Figure 4. Overview of AcquireX Ab.



In figure 4a, the AcquireX Ab settings are displayed. Per group, the user can define the acquisition methods, the component detection settings and the inclusion and exclusion parameters.
The settings for the component detection are the MS Noise Level and S/N threshold (same as BioPharma Finder) and the user can define the start and end time for the component detection. The component detection settings for the inclusion and exclusion lists are independent.
The exclusion and inclusion lists parameters are filters or parameters applied to the lists after component detection or between ID runs when MS2 are transferred to the exclusion list.
In figure 4b, the AcquireX Ab workflow editor is displayed. For each group, the user can add one exclusion and/or inclusion sample and up to 30 ID runs. An exclusion and/or an inclusion from a group can be reused in subsequent groups. A maximum of 25 groups can be added in one sequence. Groups can be added, modified and deleted before submission.

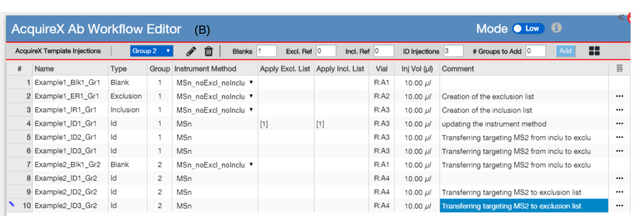
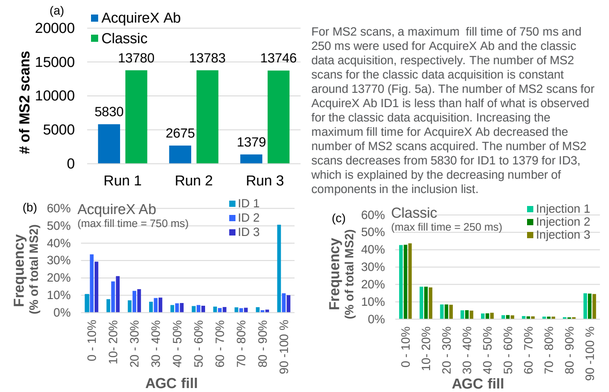


Figure 5. Number of MS2 (a) and % AGC fill distribution for the stressed sample for AcquireX Ab (b) and the classic data acquisition (c).



For MS2 scans, a maximum fill time of 750 ms and 250 ms were used for AcquireX Ab and the classic data acquisition, respectively. The number of MS2 scans for the classic data acquisition is constant around 13770 (Fig. 5a). The number of MS2 scans for AcquireX Ab ID1 is less than half of what is observed for the classic data acquisition. Increasing the maximum fill time for AcquireX Ab decreased the number of MS2 scans acquired. The number of MS2 scans decreases from 5830 for ID1 to 1379 for ID3, which is explained by the decreasing number of components in the inclusion list.

For the classic data acquisition, more than 40% of the time the AGC fill is less than 10% of the AGC target. On the other hand, for AcquireX Ab ID1, 50% of the time the AGC fill is between 90 and 100% of the AGC target, which should result in higher quality MS2. For AcquireX Ab ID2 and ID3, lower intensity components are targeted resulting in lower AGC fill, but the proportion of AGC fill between 20 and 50% is still significantly higher than the ones for the classic data acquisition.

Figure 6. Number of identified peptides with modifications presented as a cumulative profile (a) and the best average structural resolution (ASR) (b) for three AcquireX Ab IDs and triplicate injections for the classic DDA.

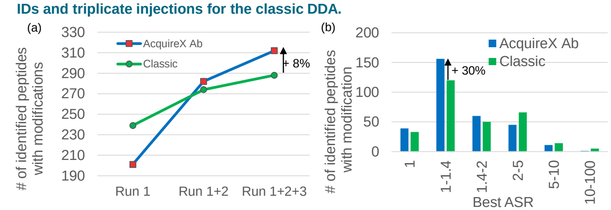
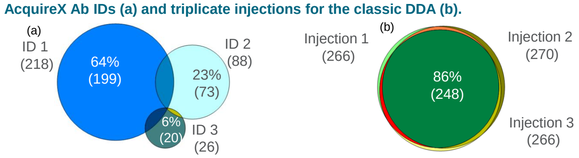
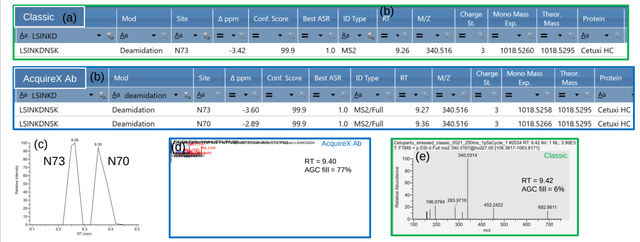


Figure 7. Venn Diagram for the identified peptides with modifications after three AcquireX Ab IDs (a) and triplicate injections for the classic DDA (b).



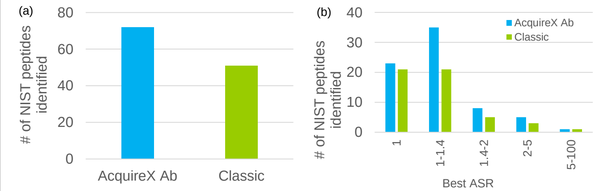
In this experiment, AcquireX Ab was tested with the stressed sample, and the overall goal is to identify high confidence peptides with modifications. More peptides containing modifications are identified after one run with the classic data acquisition than with AcquireX Ab (Fig. 6a). However, after three runs, an eight percent increase in identified peptides with modification is observed using AcquireX Ab. The average structural resolution (ASR) is a representation of the MS2 quality. An ASR of 1 means a fragment ion is observed between every amino acid. Using a longer maximum fill time for AcquireX Ab is reflected by a significant increase of identified peptide containing modifications with low ASR. As an example, a 30% increase is observed for ASRs between 1 and 1.4. Using a longer maximum fill time often results in a lower number of identified peptides but this can be avoided with AcquireX Ab because most of the identified peptides are unique to each ID run (Fig. 7a). Ninety three percent of identified peptides are only observed in 1 ID run using AcquireX Ab (Fig. 7a) while 86% are common to all three injections with the classic data acquisition (Fig. 7b).

Figure 8. Results for deamidated peptides at N70 and N73 on the heavy chain of cetuximab for three AcquireX Ab IDs (a) and classic DDA triplicate injections (b) for the stressed sample. Extracted ion chromatogram (c) and spectra for deamidated peptide at N70 (d and e).



Two deamidated peptide at asparagine 70 and 73 are observed at 9.26 min and 9.35 min (Fig. 7c). The deamidation at N73 is identified in both data sets but the deamidation at N70 is only identified, with an ASR of 1, using AcquireX Ab. A MS2 spectra was also collected for the correct m/z but the quality was too low (Fig. 7e) to be identified.

Figure 9. Number of NIST peptides identified and distribution of ASR for three AcquireX Ab IDs and classic DDA triplicate injections for the spiked sample.



NIST was spiked at 1% in a 50/50 mix of cetuximab/pertuzumab. Significantly more NIST peptides were identified using AcquireX Ab than the classic data acquisition (Fig. 8a). More peptides identified with AcquireX Ab have low ASR so the extra identified peptides have high spectral quality.

CONCLUSIONS

- AcquireX Ab provides alternative option to acquire data. Inclusion and exclusion lists can be created automatically, and these lists are updated after each ID run.
- The custom workflow in AcquireX Ab provides flexibility on experimental design. In the same sequence you can use exclusion and/or inclusion lists but you can also acquire ID runs without it. You can "reuse" an exclusion and/or list from a previous group in the same sequence.
- For the stressed sample, more peptides with modifications were identified using AcquireX Ab with higher maximum fill time. The spectral quality evaluated using the average structural resolution (ASR) is higher with AcquireX Ab.
- AcquireX Ab can be applied and beneficial for other modalities (digested mRNA for example) or for other fragmentation techniques that need more time for MS2 scans.

REFERENCE

Millán-Martín, S., Jakes, C., Carillo, S. et al. Comprehensive multi-attribute method workflow for biotherapeutic characterization and current good manufacturing practices testing. Nat Protoc 18, 1056–1089 (2023)

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