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Systematic evaluation of data-independent acquisition for sensitive and reproducible proteomics—a prototype design for a single injection assay

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Data-independent acquisition (DIA)-based proteomics has become increasingly complicated in recent years because of the vast number of workflows described, coupled with a lack of studies indicating a rational framework for selecting effective settings to use. To address this issue and provide a resource for the proteomics community, we compared 12 DIA methods that assay tryptic peptides using various mass-isolation windows. Our findings indicate that the most sensitive single injection LC-DIA method uses 6 *m/z* isolation windows to analyze the densely populated tryptic peptide range from 450 to 730 *m/z*, which allowed quantification of 4465 *Escherichia coli* peptides. In contrast, using the sequential windowed acquisition of all theoretical fragment-ions (SWATH) approach with 26 *m/z* isolation windows across the entire 400–1200 *m/z* range, allowed quantification of only 3309 peptides. This reduced sensitivity with 26 *m/z* windows is caused by an increase in co-eluting compounds with similar precursor values detected in the same tandem MS spectra, which lowers the signal-to-noise of peptide fragment-ion chromatograms and reduces the amount of low abundance peptides that can be quantified from 410 to 920 *m/z*. Above 920 *m/z*, more peptides were quantified with 26 *m/z* windows because of substantial peptide ¹³C isotope distributions that parse peptide ions into separate isolation windows. Because reproducible quantification has been a long-standing aim of quantitative proteomics, and is a so-called trait of DIA, we sought to determine whether precursor-level chromatograms used in some methods rather than their fragment-level counterparts have similar precision. Our data show that extracted fragment-ion chromatograms are the reason DIA provides superior reproducibility. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: DIA; SWATH; PacIFIC; Protalizer; label-free quantification

Introduction

Liquid chromatography interfaced to tandem mass spectrometers (LC-MS/MS) allows targeted and discovery-based identification of proteins, post-translational modifications and isoform variants.^[1] While peptide identification has become routine, mainly through the increased confidence from high-resolution mass spectrometers, more work remains to be done with regard to developing robust discovery-based quantitative proteomic platforms that are reproducible and broadly used in the proteomics community.

Among the MS acquisition methods that have been described, shotgun acquisition^[2] and selected reaction monitoring (SRM)^[3] have gained wide acceptance. In shotgun mode, a dynamic process is used to detect precursor-ions in real time, followed by ranking the most intense precursors, and typically fragmenting the 5–50 most abundant ions. The resulting tandem mass spectra allow peptides to be identified in a discovery-based manner by MS/MS database searching programs that match fragment ions observed from cleavage of the peptide backbone to peptide sequences in reference proteome databases.^[4] For protein quantification across samples in shotgun studies, the number of peptide spectra assigned to a protein is used for relative

quantitation,^[5] or the area under the curve (AUC) from precursor extracted-ion chromatograms (EICs) associated with peptides.^[6]

To measure a targeted list of proteins in a highly reproducible manner, SRM^[7,8] is the method of choice because of the elimination of various issues encountered in shotgun assays.^[9] Typically in SRM studies, a set of up to 100 peptides is measured, either to confirm protein differences identified in shotgun studies or to measure

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proteins related to a specific hypothesis. In SRM, a predetermined list of fragment ions for precursors corresponding to peptides of interest is acquired in a continuous cycle to generate multiple data points as each peptide elutes and is detected. Depending on the instrument used, both the precursor and specific fragment ions are entered for each peptide (e.g. triple quadrupole), or in the product-ion mode when full-scan tandem MS spectra are recorded the precursor information is input for acquisition, and the related fragment ions are extracted following LC-MS/MS analysis. Despite procedures to increase the number of peptides that can be measured in SRM studies by acquiring peptides at the approximate times they are expected to elute,^[10] the approach allows measuring far fewer proteins than shotgun-based methods that are intended to provide an unbiased analysis of samples.

An emerging acquisition approach termed data-independent acquisition (DIA) offers the ability to assay thousands of proteins in a rapid, discovery-oriented manner with the potential for superior quantification accuracy and precision because of the continuous collection of fragment-ion spectra over a preselected precursor range.^[11–16] Multiple variations of the DIA approach have been applied; for a review see Tate *et al.*^[17] For instance, in the MS^E method,^[12] high and low collision energy is used to fragment all precursors simultaneously from 400 to 1200 *m/z*. Whereas, in the sequential windowed acquisition of all theoretical fragment-ions (SWATH)^[13] procedure, 26 *m/z* windows are stepped through every ~3 s covering the 400–1200 *m/z* precursor range in a single analysis. Other methods such as precursor acquisition independent from ion count (PACIFIC)^[14] use isolation windows as small as 2.5 *m/z* that provide ideal sensitivity and specificity, but require many analyses to assay the 400–1400 precursor *m/z* range (e.g. in each sample injection a 15 *m/z* precursor range is analyzed). Although these extreme differences in isolation window sizes have been applied from 2.5 to 800 *m/z* in the PACIFIC and MS^E methods, respectively, systematic evaluation of this important parameter in various DIA methods and the precursor *m/z* ranges analyzed have not been reported.

Just as there is little consensus in the best approach to acquire DIA data, there also is a lack of standardization in the approaches used to identify peptides and their surrogate proteins. Using small isolation windows (e.g. 2.5 *m/z*), such as those applied in the PACIFIC method, unprocessed MS/MS spectra can be analyzed by MS/MS searching programs initially developed for shotgun proteomics.^[14] In another method, spectral libraries are used for identification whereby peptide fragment ions are extracted at normalized elution times based on shotgun analyses of the samples analyzed.^[13] A drawback to this approach is that additional shotgun runs are needed to generate the spectral library, and peptide standards are generally required to accurately normalize retention time coordinates between the spectral library and actual DIA analyses. Alternatively, spectra deconvolution approaches have been applied to separate co-eluting peptides transmitted through the same mass-isolation window followed by using MS/MS database searching programs for peptide and protein identification. To date, tandem spectra have been deconvoluted by the following procedures: (1) matching precursors detected with similar elution time that fall within the mass-isolation window of MS/MS spectra generated – thereby reassigning mass-isolation windows with vast numbers of possible precursor *m/z* values with accurate mass values^[18]; (2) eliminating fragment ions in MS/MS spectra that do not have similar chromatographic behavior (e.g. lift-off, touchdown or maximum elution time)^[19]; (3) matching fragment ions with similar elution profiles to precursors with similar elution time that fall within the

same isolation window^[20,21]; and (4) a two-stage identification procedure where (2) and (3) are applied and the search results from both rounds of identification are combined.^[22]

In this study, we assessed sequential windowed precursor acquisition with three isolation window sizes and various precursor ranges in trypsin digests from *Escherichia coli* on a Sciex 5600+ QTOF to provide an optimized prototype DIA method for maximizing sensitivity in single injection LC-DIA studies. Although our data were generated from analyzing *E. coli* tryptic peptides, the results presented from various DIA settings are expected to have general applicability to tryptic peptide samples derived from other species as suggested previously by Scherl *et al.*^[23] We also validate a spectral deconvolution approach for the direct identification of peptides without spectral libraries and benchmark DIA to shotgun sensitivity and assay reproducibility.

Experimental

Preparation of *E. coli* lysates tryptic digests

E. coli strain BL21-DE3 was grown in 1000 ml of Luria broth (LB) overnight and centrifuged at 4000 ×*g* for 10 min to pellet the cells. The supernatant was decanted, and the cells were frozen at –80 °C. The following day the cell pellet was resuspended in 25 ml of a lysis buffer containing Lysozyme (Sigma L6876 at 0.5 mg/ml), 50 mM Tris HCl, 10 mM DTT, 1 mM EDTA, Roche complete protease inhibitor (11697498001, one tablet for 50 ml) and sigma phosphatase inhibitor cocktail II (P5726, 500 μl for 50 ml). The cell suspension was probe sonicated on ice for 5–8 s and allowed to cool down. This process was repeated for 8 cycles, then the sample was sonicated for 30 min in a water bath at room temperature (RT). The lysate was centrifuged at 15 000 ×*g* for 30 min, and the supernatant was removed. Protein quantification was performed using a colorimetric assay based on dye-metal complexation and monitoring absorbance at 660 nm using a Pierce BCA kit. The *E. coli* lysate was separated into 1-mg aliquots and stored at –80 °C. A 1-mg aliquot of the lysate was removed from the –80 °C freezer and separated into five 200-μg aliquots for running on a 1D SDS Page gel. The sample volume was reduced by speed vac, and the volume of the lysate was brought up to 40 μl with 4× Invitrogen LDS buffer and 10× reducing agent (NP0009). The sample was loaded on a 1.5-mm, 4–12% Bis-Tris Invitrogen NuPage gel (NP0335BOX) and electrophoresed in MOPS buffer (NP0001) until the sample ran 1.5 cm into the gel. Molecular weight markers (Thermo spectra 26623) were run between the samples to indicate the protein-containing region of the gel. The gel was fixed in 50% ethanol/10% acetic acid overnight at RT, then washed in 30% ethanol for 10 min followed by two 10-min washes in MilliQ water (MilliQ Gradient system) and finally scanned on an Epson V700 scanner to record an image of the gel. The lanes were cut out of the gel, cut into small (~2 mm) squares and were subjected to in-gel tryptic digestion and subsequent recovery of peptides as described previously.^[24] The *E. coli* peptides from each lane were reconstituted at a concentration of 0.2 μg/μl, combined and stored at –80 °C.

Nano liquid chromatography coupled to electrospray tandem mass spectrometry (nLC-ESI-MS/MS)

nLC-ESI-MS/MS analyses were performed on a 5600+ QTOF mass spectrometer (Sciex, Toronto, On, Canada) interfaced to an Eksigent (Dublin, CA) nanoLC.ultra nanoflow system. Each acquisition

method was analyzed in technical triplicate. An amount corresponding to 500 ng of total protein was loaded (via an Eksigent nanoLC.as-2 autosampler) onto an IntegraFrit Trap Column (outer diameter of 360 μm , inner diameter of 100 and 25- μm packed bed) from New Objective, Inc. (Woburn, MA) at 2 $\mu\text{l}/\text{min}$ in formic acid/ H_2O 0.1/99.9 (v/v) for 15 min to desalt and concentrate the samples. For the chromatographic separation of peptides, the trap column was switched to align with the analytical column, Acclaim PepMap100 (inner diameter of 75 μm , length of 15 cm, C18 particle sizes of 3 μm and pore sizes of 100 Å) from Dionex-Thermo Fisher Scientific (Sunnyvale, CA). The peptides were eluted using a variable mobile phase (MP) gradient from 95% phase A (formic acid/ H_2O 0.1/99.9, v/v) to 40% phase B (formic acid/acetonitrile 0.1/99.9, v/v) for 70 min, from 40% phase B to 85% phase B for 5 min and then keeping the same MP composition for five additional min at 300 nl/min. The nLC effluent was ionized and sprayed into the mass spectrometer using NANOSpray® III Source (Sciex). Ion source gas 1 (GS1), ion source gas 2 (GS2) and curtain gas (CUR) were respectively kept at 8, 0 and 35 vendor specified arbitrary units. The mass spectrometer method was operated in positive ion mode, and the interface heater temperature and ion spray voltage were kept at 150 °C, and at 2.6 kV respectively. The data was recorded using Analyst-TF (version 1.7) software.

We used 12 DIA methods to compare various isolation windows and precursor m/z range results. The details of each of these methods are provided below and in Table 1. Method 1) the mass spectrometer method was set to go through 1757 cycles for 99 min, where each cycle performed one TOF-MS scan type (0.25-s accumulation time, from the 410.0 to 690.0 precursor m/z range) followed by 56 sequential DIA windows of 6 Daltons each across the precursor range. Note that the Analyst software automatically added 1 Dalton to each DIA window to provide overlap between adjacent isolation windows; thus an input of 5 Da in the method set up window results in an overlapping 6-Da collection window width (e.g. 410–416 m/z , then 415–421 m/z , followed by 420–426 m/z , etc.). Within the DIA windows a charge state of +2, high sensitivity mode and rolling collision energy with a collision energy spread (CES) of 15 V was selected. Methods 2–4) these are the same as method 1 except that the TOF-MS scan range was 550 to 830 m/z , 690.0 to 970.0 m/z and 970.0 to 1250.0 m/z , respectively. Method 5) the mass spectrometer method was set to go through 1358 cycles, where each cycle performed one TOF-MS scan type (0.25 s accumulation time, from 550.0 to 750.0 precursor m/z) followed by 40

sequential DIA windows of 6 Daltons each. Method 6) the mass spectrometer method was set to go through 1523 cycles, where each cycle performed one TOF-MS scan type (0.25-s accumulation time, from 410.0 to 550.0 precursor m/z range) followed by 70 sequential DIA windows of 3 Daltons each. Methods 7–11) these are the same as method 6 except that the TOF-MS scan range and 3 Dalton DIA isolation windows acquired data from 550.0 to 690.0 m/z , 690.0 to 830.0 m/z , 830.0 to 970.0 m/z , 970.0 to 1110.0 m/z and 1110.0 to 1250.0 m/z , respectively. Method 12) the mass spectrometer method was set to go through 1742 cycles, where each cycle performed one TOF-MS scan type (0.10-s accumulation time, across the 400.0 to 1200.0 precursor m/z range) followed by 32 sequential DIA windows of 26 Daltons each.

In shotgun mode the mass spectrometer was set to perform one TOF-MS scan type (0.25-s accumulation time, in a 350 to 1600 m/z window) followed by 50 information-dependent acquisition (IDA)-mode MS/MS-scans on the most intense candidate ions having a minimum intensity of 150 counts. Each MS/MS scan was operated under vendor specified high-sensitivity mode with an accumulation time of 0.05 s and a mass tolerance of 100 ppm. Precursor ions selected for MS/MS scans were excluded for 30 s to reduce the occurrence of redundant peptide sequencing.

DIA data analysis parameters

Protilizer DIA software (Vulcan Analytical, Birmingham, AL) was used to analyze every DIA file with settings previously described for Sciex 5600 QTOFs.^[22] The Swiss-Prot *E. coli* database downloaded 17 March 2015 was used as the reference proteome for all MS/MS searches. A precursor and fragment-ion tolerance for QTOF instrumentation was used for the Protilizer spectral-library free identification algorithm.^[22] A multistage spectra deconvolution approach was applied as previously described, except only fragment ions where the intensity was $\geq 70\%$ of the scan containing the most intense value were retained to associate fragment ions that are more likely derived from the same peptide based on the chromatographic elution profile. The $\geq 70\%$ fragment-ion deconvolution setting was used because this allowed the most proteins to be quantified in three different DIA methods evaluated in Supplemental Fig. 1. Potential modifications included in the searches were phosphorylation at S, T and Y residues, N-terminal acetylation, N-terminal loss of ammonia at C residues and pyroglutamic acid at N-terminal E and Q residues. Carbamidomethylation of C residues was searched as a fixed modification. The maximum valid protein and peptide expectation score from the X! Tandem search engine used for peptide and protein identification on reconstructed spectra was set to 0.005.

For DIA quantification by Protilizer the maximum number of b and y series fragment-ion transitions was set to nine excluding those with m/z values below 300 and not containing at least 10% of the relative intensity of the strongest fragment ion assigned to a peptide. A minimum of five fragment ions were required for a peptide to be quantified (except where indicated otherwise in Fig. 7). In datasets where a minimum of seven consistent fragment ions were not detected for the same peptide ion in each of the three files compared in a triplicate analysis, the algorithm identified the file with the largest sum fragment-ion AUC and extracted up to seven of these in the other files using normalized retention time coordinates based on peptides detected by the Protilizer algorithm in all the files in a dataset.

Table 1. Multiple DIA method precursor range and mass-isolation window sizes evaluated

DIA method	Precursor range (m/z)	Number of sequential DIA windows	Isolation width (m/z)	Duty cycle (s)
1	410–690	56	6	3.1
2	550–830	56	6	3.1
3	690–970	56	6	3.1
4	970–1250	56	6	3.1
5	550–750	40	6	4.3
6	410–550	70	3	3.9
7	550–690	70	3	3.9
8	690–830	70	3	3.9
9	830–970	70	3	3.9
10	970–1110	70	3	3.9
11	1110–1250	70	3	3.9
12	400–1200	32	26	3.4

Shotgun identification and quantification via MS1 EICs

An unpublished version of the Protalizer tool for shotgun data analysis was applied in this study. Peptide and protein identifications were made using the X! Tandem Sledgehammer search engine (version 2013.09.01.1) against the forward and reverse *E. coli* Swiss-Prot database used in the DIA analyses with a 50-ppm precursor and fragment-ion mass tolerance. Potential and fixed modifications searched were the same as those described for the DIA data analysis. A maximum of two trypsin miscleavages were included in the analysis and protein and peptide maximum valid expectation scores were set to 0.005. Only 'top hit' peptides were further analyzed by the pipeline. Redundant peptide identifications with the same charge and modification state were eliminated for downstream analysis in each file except for one scan corresponding to the largest sum fragment-ion intensity according to X! Tandem.

MS1 peaks corresponding to peptides identified by the MS/MS search were extracted with the OpenMS feature finder centroid tool (version 1.11.1).^[25] Default settings were used with the following exceptions: a mass trace m/z tolerance of 0.05, mass trace min spectra of 4, isotopic pattern charge low 2 and high 3, isotopic pattern m/z tolerance of 0.05, seed min score of 0.5, feature overall min score of 0.5, feature min isotope fit of 0.5 and feature min trace score of 0.5. Peptide ions identified were matched to MS survey scan peaks using a tolerance of $\pm 0.025 m/z$, identical charge and retention time within ± 15 s from the MS/MS scan the peptide was sequenced.

A similar procedure for data normalization with endogenous reference peptides was applied as previously described for the DIA tool.^[22] However, instead of using fragment-ion AUC sum values corresponding to peptides with the most consistent abundance and similar elution time as normalization factors for each peptide quantified, MS1 EIC peptide intensity values were applied.

Retention time alignment was applied to enable feature detection of peptides not identified by MS/MS in every sample. Peptide elution times were normalized in each file based on the difference in elution time for each sample peptide to reference peptide(s) in files a peptide was identified by X! Tandem and matched to an MS1 EIC. This elution time difference was then used to predict the

retention time range in which precursor ions were extracted in files where a peptide was not initially identified. A retention time extraction window of ± 15 s was used during this step to match precursor EICs across all files. Peptide and protein relative abundance were calculated as described previously for the DIA platform.^[22]

Availability of raw data and software result files

All .wiff raw files and results generated by Protalizer data processing software were deposited in the ProteomeXchange Consortium^[26] via the PRIDE partner repository^[27] with the dataset identifier PXD002688.

Results and discussion

Overview of DIA and shotgun proteomics

In DIA methods where sequential precursor m/z ranges are acquired,^[13] the mass spectrometer is set to scan MS/MS fragment-ion spectra from multiple isolation windows that sequentially step through a precursor mass range in a duty cycle of several seconds (Fig. 1A). In contrast, the shotgun mode collects data by determining precursors with the strongest intensities and charge states typical of peptides ($2-3^+$) followed by fragmenting these precursors with small mass-isolation windows (Fig. 1B). In the remaining sections below we characterize various settings including the size of mass-isolation windows and the precursor ranges analyzed that have important implications in generating DIA results and benchmark identification and quantification metrics to shotgun results.

Evaluation of 3, 6 and 26 m/z mass-isolation windows

We designed multiple DIA methods shown in Table 1 to assess mass-isolation window size in a comprehensive manner across the entire tryptic peptide precursor m/z range. Each approach used an isolation window of 3, 6 or 26 m/z and had a duty cycle ranging from 3.4 to 4.3 s to provide well-defined peak shapes amenable for accurate quantification via fragment EICs. With this duty cycle, each

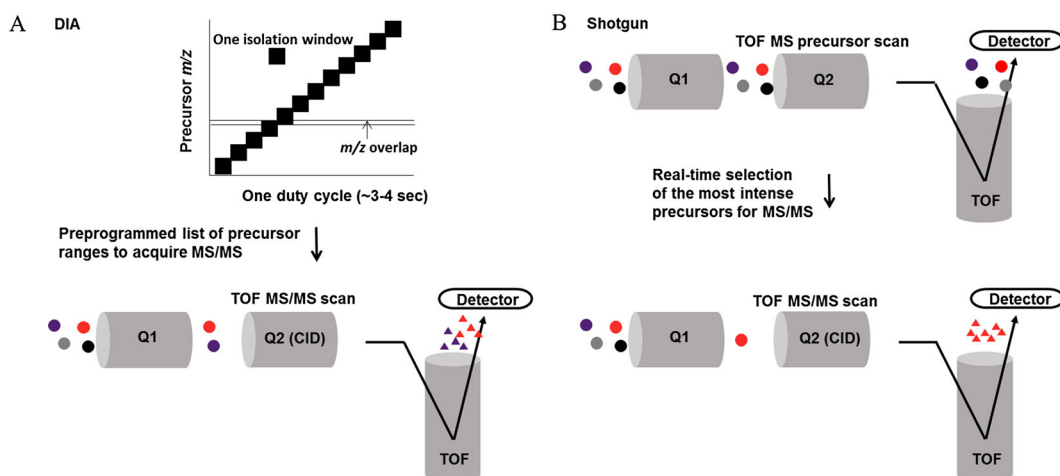


Figure 1. Schematic of sequential precursor DIA mode compared to shotgun-based data collection. Tryptic peptides generated from cell culture, tissue biopsies or bodily fluids are subjected to accurate mass analysis using LC-QTOF (as shown) or other high-resolution mass spectrometers (e.g. Q-Exactive). A) In the DIA mode, a list of precursors is targeted for fragmentation that falls within a desired range to be covered. For instance, 26 m/z mass-isolation windows that step in increasing increments of 25 m/z in 32 separate MS/MS spectra that analyze the 400–1200 precursor m/z range. B) For shotgun acquisition, precursor scans are used to detect the strongest signals eluting into the instrument at a specific time. Usually, 5–50 of the most intense ions are then selected for TOF MS/MS scans.

3 m/z mass-isolation window approach measured a 140 precursor m/z range and required six injections to analyze the mass range from 410 to 1250 m/z . Whereas, the 6 m/z isolation width methods analyzed a 280 m/z range and required three injections to assay the 410–1250 m/z precursor range. These 3 and 6 m/z isolation window approaches were benchmarked to 26 m/z SWATH windows initially applied by Gillet *et al.* that analyze an 800 m/z range in a single LC-DIA analysis.^[13] All of the data collected were analyzed using Protalizer, a fully automated software platform that performs spectra deconvolution, MS/MS database searching for peptide identification, as well as label-free quantification of peptides and proteins across samples with fragment EICs.^[22] Figure 2 shows the number of proteins and peptides identified and quantified with each of the DIA methods compared to the results from a shotgun analysis with the same LC-MS/MS system and tryptic digest. Although more proteins and peptides were identified by shotgun acquisition than any of the DIA methods tested (1046 proteins vs 922), the number of quantifiable proteins using 6 m/z mass-isolation windows from 550 to 830 precursor m/z allowed the most proteins to be assayed in a single injection (897 compared to 894 proteins via shotgun). The lowered rate of converting identified peptides into those capable of being quantified with shotgun acquisition has been previously reported.^[25] In this dataset, 62.7% of the shotgun sequenced peptides were matched to MS1 EICs. In contrast, 94.2% of peptides detected by the MS/MS database searches were able to be matched to a minimum of five fragment ions in the most sensitive DIA method using 6 m/z isolation windows to analyze the 550–830 precursor m/z range.

A noteworthy finding in our results is 26 m/z mass-isolation windows that analyze the largest precursor range in a single injection from 400 to 1200 m/z , identified only 630 proteins and quantified 600, whereas the DIA assay from 550 to 830 precursor m/z using 6 m/z windows both identified and quantified ~50% more proteins. We speculated that the 26 m/z isolation window method effectively captures less useful information than methods that analyze only the very densely populated tryptic peptide range with smaller isolation windows because more specific isolation of peptides allows detection and quantification of lower abundance peptides. To determine if peptides detected with narrower isolation windows, but not with 26 m/z windows generally had smaller intensities, we compared the sum fragment EIC intensities of peptides that were not detected

with the 26 m/z isolation window method but were with the two other approaches in Fig. 3A. This data shows that the peptides not detected with 26 m/z windows generally had small intensities. We then speculated that the reduced sensitivity observed with 26 m/z isolation windows may be because of an increase in co-eluting peptides in the same mass-isolation window causing a decrease in the signal-to-noise of fragment ions and detection of peptides with small intensities. To test this, we determined the pool of peptides detected with each of the 3, 6 and 26 m/z isolation window DIA methods. A total of 2307 of the same peptides detected with all three isolation windows and signal-to-noise histograms are shown in Fig. 3B. Among the peptides detected with each method, the 3 m/z isolation windows had an average signal-to-noise of 42, the 6 m/z wide windows had a 28 average signal-to-noise and the 26 m/z isolation windows had an average signal-to-noise of 16. Thus, indicating an inverse relationship between isolation window size and signal-to noise in DIA assays of complex peptide mixtures. We also evaluated another possible explanation for reduced sensitivity with 26 m/z windows – the detection of peptides near the upper and lower precursor m/z regions of each isolation window is not ideal because of quadrupole transmission imperfections or artifacts from the MS/MS database search. To investigate this, we compared the distribution of identified peptide precursor m/z values within two separate 26 m/z isolation windows to see if these were skewed to the center of windows, indicating a non-random distribution of peptide precursor values within each window. The 549–575 and 574–600 precursor m/z isolation windows are shown in Supplemental Fig. 2 and indicated that peptides were identified with a near random distribution, suggesting that quadrupole transmission and the MS/MS database search were not factors causing the sensitivity reduction with 26 m/z isolation windows. In conclusion, our data indicate that the decreased sensitivity with 26 m/z isolation windows is because of reduced signal-to-noise from co-eluting compounds that inhibit the detection of peptides with small intensities. A similar observation shown in Fig. 3A comparing 3 and 6 m/z isolation windows was also apparent – 3 m/z isolation windows allows quantification of more low intensity peptides and has increased signal-to-noise across the same 2307 peptides as shown in Fig. 3B.

To test if 50-ms dwell times had a negative impact on sensitivity, we included a longer dwell time method of 100 ms that analyzed

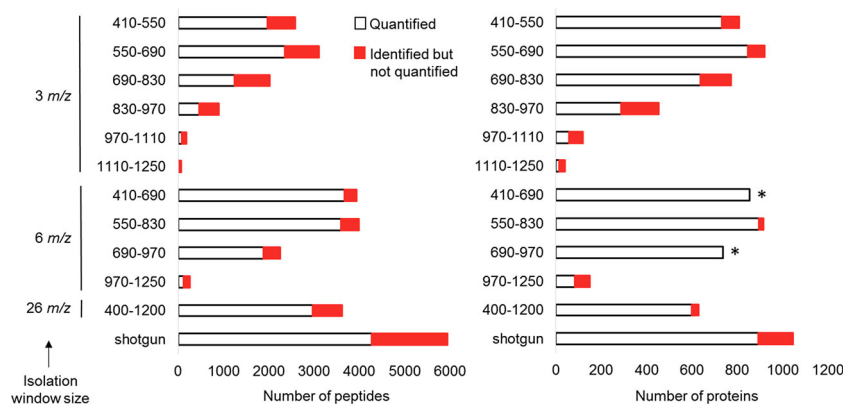


Figure 2. Number of peptides and proteins measured by shotgun acquisition and DIA methods that apply fixed isolation widths across preselected m/z ranges. An amount corresponding to 500 ng of *E. coli* total lysate trypsin digests was analyzed in technical triplicate for each method shown with a 70-min reverse-phase gradient connected online to a Sciex 5600+ QTOF. Data shown are peptides and proteins quantified in a minimum of two out of three replicates by fragment EICs in the DIA analyses and MS1 EICs in the shotgun data. The number of identified but not quantified values shown is the difference between the average number of identified peptides and proteins to those quantified by the Protalizer DIA software tool. The (*) shown at two of the protein results were methods where ~20 more proteins were able to be quantified than the average detected per file by matching fragment EICs with normalized elution time across replicates.

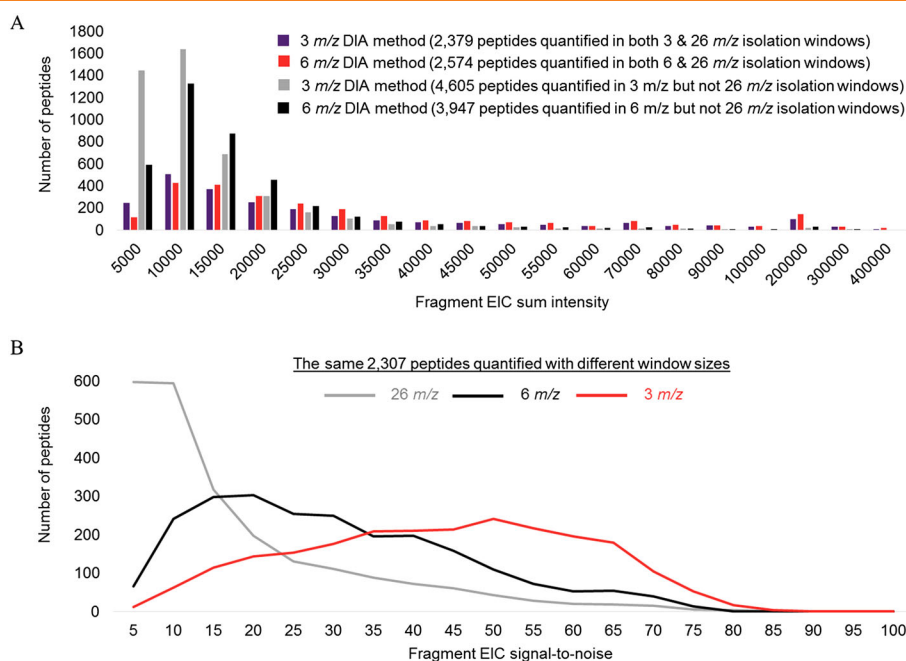


Figure 3. Smaller isolation windows allow quantifying more of the low abundance peptide population by increasing the signal-to-noise. A) Histograms showing the number of peptides quantified in a minimum of two out of three replicates with an average fragment EIC sum intensity within each bin shown (5000 shows data from 0 to 5000, 10 000 shows the number of peptides from 5001 to 10 000, etc.). Approximately 1% of the total peptides compared were omitted with intensities ranging from 400 000 to 1 300 000 to conserve space. B) Comparison of the average fragment EIC signal-to-noise across the same peptides quantified in a minimum of two out of three replicates using 3, 6 and 26 m/z isolation windows.

peptides with precursor m/z values from 550 to 750. From comparing the number of peptides identified with this method, to a similar method acquiring peptides from 550 to 830 precursor m/z that also used 6 m/z isolation windows but with a 50 ms dwell time, each method had nearly an identical number of peptide identifications in the 550–750 precursor m/z range (data not shown). Thus, indicating the 50-ms dwell time used in this study did not compromise sensitivity with the 5600+ QTOF instrument.

Combining 3 and 6 m/z isolation window results across the 410–1250 precursor m/z range

For studies that have extensive access to instrumentation and sufficient starting material to permit multiple injections per sample analyzed, we determined the number of proteins and peptides that can be identified in any one of three technical replicates and quantified in the 410–1250 precursor m/z range in multiple injections. Using 3 m/z isolation windows in six separate injections allowed identification of 1360 proteins and quantification of 1133 proteins from the detection of 14 277 peptides and quantification of 7004 peptides. In contrast, with 6 m/z isolation windows in three separate injections, we identified 1049 proteins and quantified 1017 proteins, whereas 9277 peptides were identified and 6537 were able to be quantified.

Spectral library-free DIA peptide identification by spectra deconvolution

Because of using larger mass-isolation windows in DIA mode compared to shotgun and SRM acquisition, highly complex MS/MS spectra are generated that contain fragment ions from multiple precursors. Thus, making identification with MS/MS database search engines designed to match each fragment-ion spectrum to a single peptide-ion nontrivial. In order to reduce this issue in

DIA studies, spectra deconvolution approaches are applied to simplify MS/MS spectra based on the principle that fragment ions originating from the same precursor should have the same elution profile.^[19–22] Additionally, the large precursor m/z range for each mass-isolation window in many DIA methods results in a vast number of potential peptide-ion matches in eukaryotic and prokaryotic proteomes that fall within the isolation window. In order to narrow the precursor m/z search space, several deconvolution methods allow replacing the large uncertainty in the precursor isolation window with a detected precursor that has similar elution characteristics to fragment ions in the same isolation window.^[20–22]

To assess the effect of DIA spectra deconvolution, we compared the number of peptide identifications using the Protalizer deconvolution approach^[22] to unprocessed raw spectra. Figure 4 shows with 3 m/z isolation windows, an average of 16% more peptides were able to be identified in demultiplexed spectra from combining data from six precursor m/z ranges from 410 to 1250. Whereas deconvolution of data with 6 m/z isolation windows resulted in an average of 67% more peptides detected than searching raw spectra, and the difference was even more drastic for 26 m/z isolation windows where an increase of 129% was observed for deconvoluted *versus* raw DIA spectra. Supplemental Fig. 3 shows the average number of peptide and protein identified with spectra deconvolution and searching raw spectra from each of the 12 DIA methods evaluated in the study and indicates that spectra deconvolution increases the average number of proteins detected by 23%, 33% and 45%, respectively, for the 3, 6 and 26 m/z isolation window methods. Taken together, our data indicate that deconvoluting spectra is critical for maximizing peptide and protein detection sensitivity, especially for methods using large mass-isolation windows where there is a greater likelihood of multiple precursors eluting at the same time through an isolation window. In addition to increasing sensitivity, spectral deconvolution reduced false discovery rates (FDRs) in the 6 m/z mass-isolation window

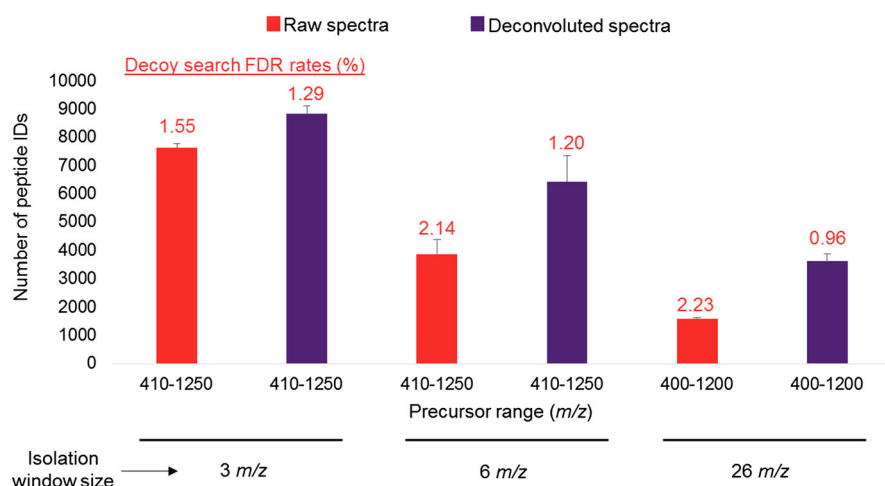


Figure 4. DIA spectra deconvolution enhances sensitivity and reduces false discovery rates. Data shown are the average number of identifications in *E. coli* lysate tryptic peptides analyzed in triplicate. Results from 26 *m/z* isolation windows across 400–1200 precursor *m/z*, the identifications obtained from 6 *m/z* isolation windows combined from three separate precursor range from 410 to 590 *m/z*, 590 to 970 *m/z* and 970 to 1250 *m/z*, and the identifications from 3 *m/z* isolation windows combined from six precursor range 410 to 550 *m/z*, 550 to 690 *m/z*, 690 to 830 *m/z*, 830 to 970 *m/z*, 970 to 1110 *m/z* and 1110 to 1250 *m/z*. MS/MS database search parameters were kept the same for the raw spectra searches to those with reconstructed spectra, except the precursor tolerance for raw spectra was set to \pm half of the total mass-isolation window size (e.g. 26 *m/z* windows were searched with a precursor tolerance of \pm 13 Da). Error bars are standard deviation. The false discovery MS/MS spectra matching rate was determined from the number of peptides detected by the reversed decoy database search divided by the total number of peptides detected, and the data shown are the average from three replicates.

methods by an average of 44% and lowered the FDRs in the 26 *m/z* window method by 57%. Moreover, in the six separate precursor ranges analyzed with 3 *m/z* isolation windows, the overall FDR average was decreased by an average of 34%.

A representative example of two peptides that co-elute in the same 26 *m/z* mass-isolation window is presented in Fig. 5. Fragment EICs of WILDHVEGSR and EHIPVLVYGPK are shown in Fig. 5A–B, where the former peptide has an \sim 4-fold stronger intensity than the latter peptide with overlapping elution time. As a result, the EHIPVLVYGPK peptide is only able to be detected by an MS/MS database search engine with deconvolution of the raw tandem spectra, whereas the stronger WILDHVEGSR peptide is able to be identified with or without spectra deconvolution. The dominating signal strengths of WILDHVEGSR b and y fragment ions are apparent in the raw MS/MS spectrum in Fig. 5C that allow the spectrum to be matched specifically to the peptide sequence from the MS/MS database search. In contrast, fragment ions belonging to the lower abundance EHIPVLVYGPK peptide are not readily apparent in the raw spectra shown in Fig. 5C, but are in the deconvoluted spectra shown in Fig. 5D where EHIPVLVYGPK is able to be confidently assigned to the reconstructed spectra.

DIA quantification reproducibility

Although identifying peptides and proteins is a critical part of any DIA study, detecting meaningful differences across biological conditions compared requires reliable quantification. Thus, we assessed whether peptides and proteins detectable by shotgun acquisition and quantified by MS1 EICs have similar quantitative precision to the most sensitive 6 *m/z* mass-isolation window DIA assay tested in this study from 550 to 830 precursor *m/z*. Our results comparing peptides in technical replicates shown in Fig. 6A indicate that DIA yielded more reproducible measurements than the shotgun MS1-intensity-based peptide quantification. Specifically, the MS1 intensity-based shotgun results had 465 of 4092 total peptides quantified (11.3%) with a \geq 30% fold-change difference across two

technical replicates. In contrast, the DIA method applying 6 *m/z* isolation windows had 200 of 3522 total peptides quantified (5.6%) with a \geq 30% fold-change difference across two technical replicates – approximately half the number of peptides with poor reproducibility using a 30% fold-change cutoff. Moreover, histograms of the coefficient of variation at the protein level in Fig. 6B indicate the DIA method has a fourfold reduction in peptides quantified with a \geq 20% or greater coefficient of variation (28 vs 119 proteins).

Because our data indicate that DIA fragment EIC quantification is more reproducible than shotgun-based MS1 quantification, and recent studies show that SWATH fragment EICs have improved quantification in complex peptide digests,^[21,28] we determined whether fragment EIC measurements in our dataset are inherently more precise than MS1 EICs. Figure 6C shows across 2091 of the same peptide ions that could be matched to MS1 and fragment EICs, the variation in replicates quantified by MS1 EICs had more differences than fragment EICs. In addition, histograms of the coefficient of variation for precursor and fragment peptide EIC quantification are shown in Fig. 6D, which indicated that the fragment EIC approach had a 6.8% average coefficient of variation and the precursor EIC method had an 11.9% average coefficient of variation. Thus, not only does our data support that the conclusion DIA is more reproducible than shotgun-MS1 EIC measurements, we also demonstrated that peptide quantification by fragment EICs is the reason for this improvement.

Impact of the number of fragment ions and baseline subtraction on reproducibility

Limits on the number of fragment ions used for deriving relative peptide quantification values across samples can be applied that emphasize sensitivity, measurement quality or a balance between these metrics. To provide an example of this, Fig. 7 shows technical replicates of peptides measured with the 550–830 precursor *m/z* DIA assay using 6 *m/z* mass-isolation windows with three fragment

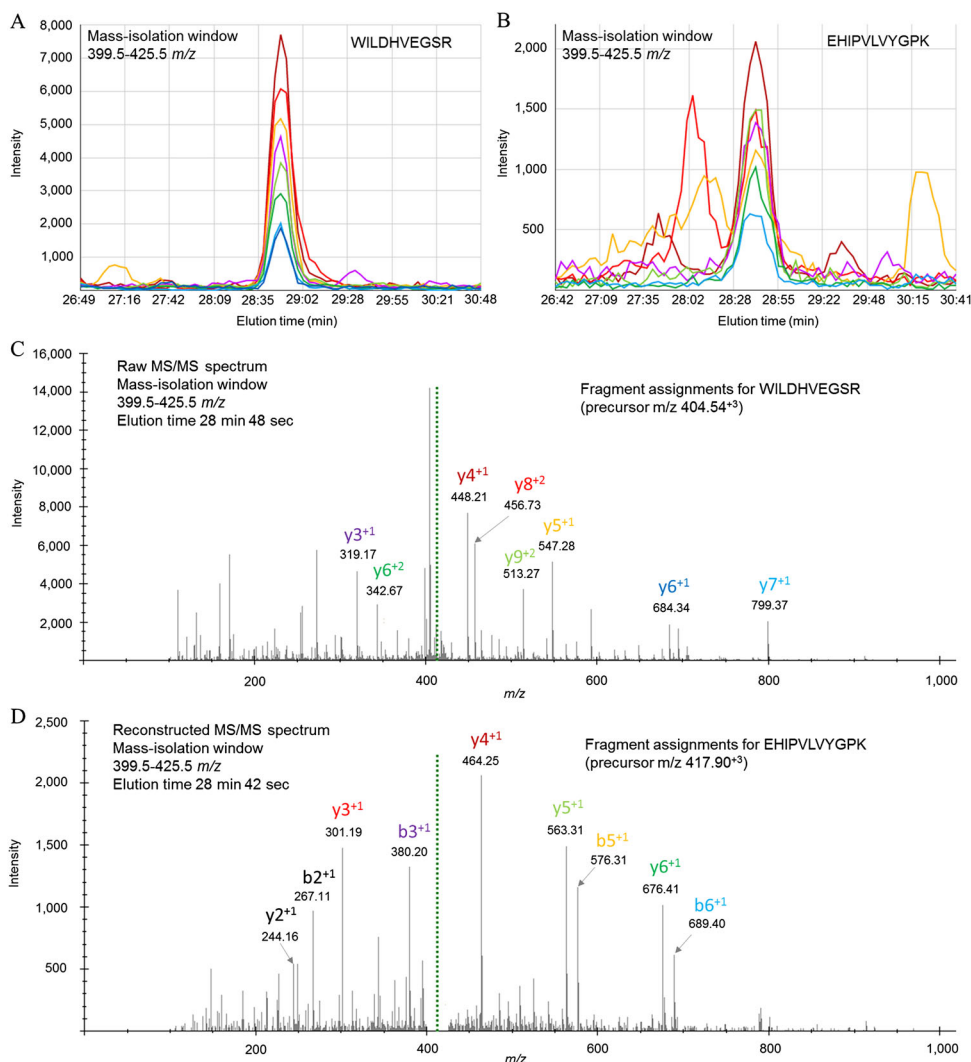


Figure 5. Example of detecting co-eluting peptides in the same MS/MS spectra. Data shown are from tryptic peptide digests of *E. coli* cell lysate analyzed with 26 m/z mass-isolation windows. A–B) Fragmentation chromatograms of WILDHVEGSR and EHIPVLVYGPK generated from the Protalizer DIA software platform. The colors of fragment-ion traces correspond to b and y -ions labeled in the raw MS/MS spectrum shown in panel (C) for WILDHVEGSR and the deconvoluted and reconstructed MS/MS spectrum of EHIPVLVYGPK in panel (D). Note that fragment ions that fall within the mass-isolation window from 399.5 to 425.5 m/z in the reconstructed spectra generated by Protalizer are eliminated to remove non-dissociated compounds from MS/MS spectra. The black labeled fragment ions in panel (D) are shown but were not used by the DIA software for quantification because of having a small m/z or intensity. MS/MS spectra shown in (CD) were visualized with ProteoWizard. Green dotted lines mark the center of the mass-isolation window.

ions compared to the same data analyzed with a minimum of five fragment ions. Also included in these comparisons were various amounts of baseline subtraction, although the amount of baseline noise that needs to be subtracted varies across different instrument platforms because of differences in the relative intensity scales and whether or not the on-board instrument software has already applied a baseline filter. Our data indicate using a moderate baseline subtraction to eliminate low signal-to-noise fragment ions that balances the number of peptides that can be quantified *versus* assay reproducibility allows 3522 peptides to be quantified with a minimum of five fragment ions per peptide. In contrast, 3919 peptides requiring a minimum of three fragment ions detected were able to be quantified. However, the number of peptides quantified with a coefficient of variation above 20% was 167 when a minimum of five fragment ions were used for peptide quantification and a moderate baseline subtraction compared to 281 peptides with three

fragment ions. Thus, our results indicate that these data processing parameters should be carefully evaluated in DIA assays and more conservative settings that require a minimum of five fragment ions for peptide quantification combined with a substantial baseline noise subtraction have the most favorable reproducibility.

Prototype for the most sensitive single-injection DIA method

A comprehensive analysis of tryptic peptides across the entire mass range shown in Fig. 8 indicates the most information-rich precursor region in tryptic peptide digests is from approximately 450 to 730 m/z . Of the total 6537 peptides quantified by fragment EICs in a minimum of two out of three replicates with 6 m/z isolation windows ranging from 410 to 1250 precursor m/z , 4465 (68%) were in the 450–730 precursor m/z range. Although this exact 280 m/z range was not analyzed in this study, the 6 m/z isolation windows

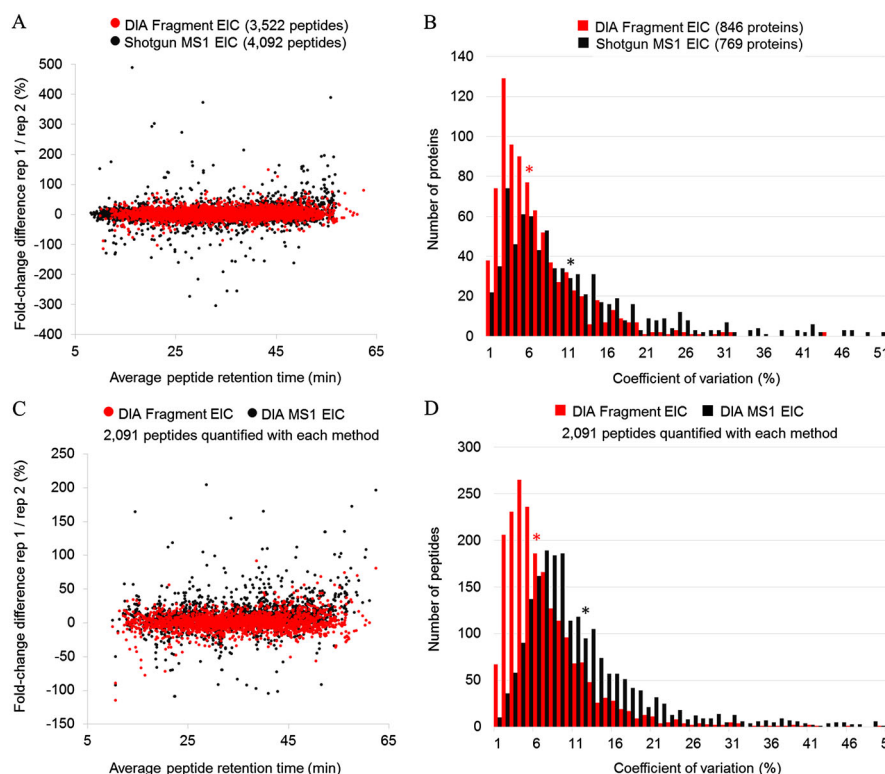


Figure 6. Reproducibility of fragment-ion quantification compared to MS1-intensity measurements. A) Data for each acquisition approach were technical replicates of *E. coli* lysate tryptic digest collected on a 5600+ Sciex QTOF mass spectrometer that were detected in all three technical replicate analyses and marked use for quantitation by the Protalizer software pipeline. The DIA data shown is the 6 m/z isolation window method that analyzed the 550–830 precursor m/z range. The percent fold-change difference was calculated by determining which replicate had a larger relative abundance. Then the replicate with larger abundance was input into the following formula as ‘replicate 1’ [(replicate 1 peptide abundance/replicate 2 peptide abundance) \times 100% – 100%]. To show the absolute error in both replicates the calculated percent fold-change values from relative peptide abundance values greater in replicate 2 than replicate 1 were multiplied by -1 . Two outlier values in the shotgun data with % fold-differences of -2138% and 798% across the replicates were removed to allow better visualization of the data. B) Histograms of the protein coefficient of variation for proteins quantified in each of three technical replicates. The calculated coefficient of variation for each percent value shown was rounded up to the nearest integer value to create the plot, and approximately 1% of the total peptides are not shown with coefficient greater than 51%. C–D) Comparison of the same peptides and DIA replicates quantified by fragment EICs or MS1 EICs. Data in panel (C) was calculated as described in panel (A). D) Peptide histograms of the coefficient of variation were calculated for peptides detected in all three replicates as described in panel (B). Asterisks in panels (B and D) indicate the average percent coefficient of variation for each method.

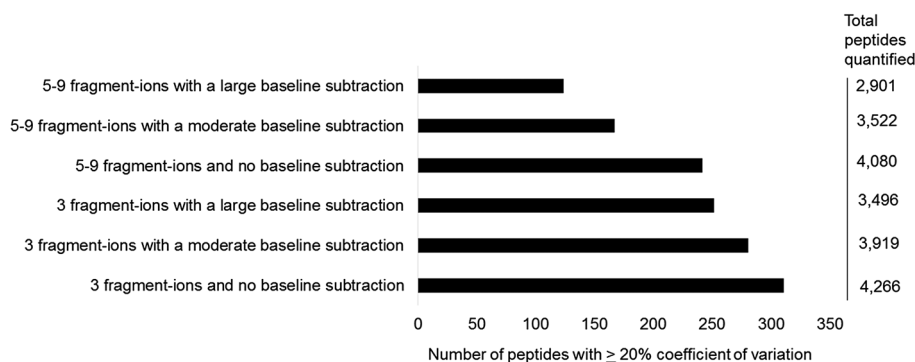


Figure 7. Increasing the minimum number of fragment ions and baseline subtraction used for peptide relative quantification increases assay precision. Data shown are from technical triplicate *E. coli* lysate tryptic peptides analyzed with the 550–830 precursor m/z DIA method with 6 m/z isolation windows. All peptides measured were quantified in all three replicates. The baseline subtraction AUC from each peptide fragment ion for the ‘moderate’ and ‘large’ subtractions were 200 and 400, respectively.

used to analyze the 410–690 and 690–830 precursor m/z ranges shown in Table 1 provide proof-of-principle for this optimized assay for future studies.

The data shown in Fig. 8 also indicates that the less-specific, 26 m/z mass-isolation windows actually have enhanced sensitivity

relative to the more narrow 3 and 6 m/z isolation window methods above 920 m/z . This finding suggests that the decrease in precursor-ion density in this range,^[23] coupled with the substantial carbon isotope envelopes in these large peptides, allows 26 m/z windows to quantify more peptides because the 3 and

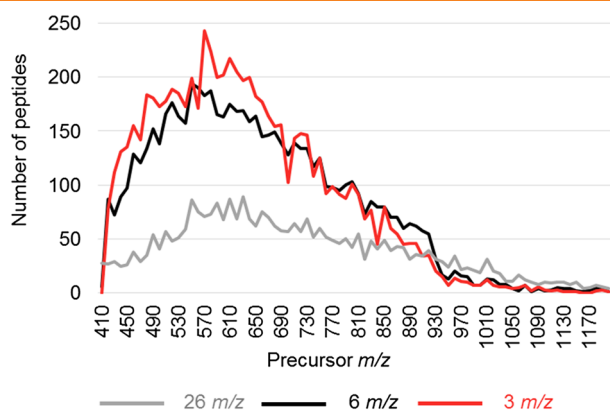


Figure 8. Tryptic peptide digests have the most quantifiable peptides in the 450–730 precursor m/z range. The number of peptides quantified by fragment EICs in a minimum of two out of three replicates for each 10 m/z precursor range bin from 410 to 1200 m/z . The number of peptides for the 26 m/z window method were generated from a single injection covering 400–1200 m/z , the 6 m/z window approach from three injections from 410 to 690, 690 to 970 and 970 to 1250 m/z and six injections for the 3 m/z isolation window approach from 410 to 550, 550 to 690, 690 to 830, 830 to 970, 970 to 1110 and 1110 to 1250 m/z .

6 m/z isolation widths disperse these large peptides into multiple isolation windows causing a reduction in fragment-ion intensity.

Conclusions and outlook

We have described an improved single injection LC-DIA method that differs from the initial SWATH approach^[13] by targeting the most dense peptide precursor m/z range in complex tryptic peptide digests in order to obtain the maximum amount of information possible from a single injection DIA approach. Our findings show that applying 6 m/z isolation windows to assay the 450–730 precursor m/z range, rather than 26 m/z windows across the 400–1200 precursor m/z range, increases the signal-to-noise of peptide fragment EICs by an average of 37% and number of peptides quantified by 35%. Although we emphasized the most effective isolation windows and mass ranges for the most sensitive single injection LC-DIA analysis in order to minimize the amount of sample and instrumentation required for practical reasons, our data also suggests a rational basis for DIA experimental designs when samples can be reinjected multiple times. Specifically, our data showed a substantial signal-to-noise difference between 3, 6 and 26 m/z window methods indicating an inverse relationship between isolation window size and signal-to-noise consistent with the premise underlying the high specificity and quantitative accuracy of targeted SRM.^[7,8] An interesting exception to the signal-to-noise and window size relationship was observed for the small population of peptides with precursor values greater than 920 m/z . These large peptides have substantial ¹³C isotope distributions causing them to have a diminished signal that is distributed over as much as a 3 m/z range for peptides in a 2+ charge state. Thus, causing the peptide to fall into multiple DIA isolation windows when using only 3 and 6 m/z isolation widths. Not only does this suggest DIA assays that analyze peptides with precursor m/z values greater than 920 should use isolation windows greater than 6 m/z to maximize sensitivity, it also indicates that targeted SRM assays would benefit by considering this effect to reduce false negative peptide detection rates.

Because multiple DIA methods have been described that apply MS1 EICs for relative peptide quantification,^[12,15,16] we sought to

ascertain if these DIA approaches could be expected to have better quantitative performance than shotgun-based proteomics. Comparisons of the peptide quantification reproducibility across technical replicates in the same DIA analyses and group of 2091 peptides indicated the fragment-based EIC approach had approximately half the amount of variation compared to MS1 EIC quantification, suggesting the improved precision of DIA is a product of fragment EICs. This has implications for DIA approaches that apply MS1 EIC-based quantification, because our data suggests that DIA MS1 EICs have similar quantitative reproducibility to findings obtained with MS1 EICs in shotgun analyses. Furthermore, this finding is also relevant for recently described DIA methods that utilize varying isolation window widths based on precursor-ion densities that are not held constant across specific precursor m/z ranges and samples analyzed – because such assays rely on MS1 EICs for relative peptide quantification.^[29] While the quantitative comparison of MS1 EICs compared to fragment EICs and QTOF instrument parameters in this study provides a framework for implementing DIA, future studies on other instruments such as quadrupole-Orbitraps are needed to ensure the findings are relevant across different mass spectrometers.

Altogether, our findings indicate that the LC-DIA methods described offer better reproducibility than shotgun-based proteomics and allow more peptides and proteins to be quantified with the same instrument and LC conditions. This practical resource for DIA assay settings described in this study, combined with the recent availability of DIA software,^[21,22] should facilitate access to DIA workflows for proteomics laboratories seeking more reliable quantitative findings in future proteomic studies.

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