Increasing Depth of Coverage in Data Independent Acquisition with Higher Sample Loads and Smaller Q1 Windows

SWATH® Acquisition on the TripleTOF® 6600 Systems

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In data independent acquisition (DIA), high resolution MS/MS is acquired across a mass range, using wide Q1 windows, which creates composite spectra from which fragment ion chromatograms (XICs) can be extracted for quantitation. The most widely used of these techniques is SWATH® Acquisition on the TripleTOF® Systems.

The depth of coverage and quality of quantitation obtained from these experiments will depend on the signal/noise of the generated XICs. Increasing specificity to decrease chemical noise can be achieved by using increasingly smaller Q1 windows, and varying the window width as a function of precursor density using the variable window functionality[1]. Increasing signal can be obtained by using higher sample loads on optimized chromatography. Higher signal must be accompanied by an increase in dynamic range of the mass spectrometer detection system to take full advantage and dig deeper into the sample.

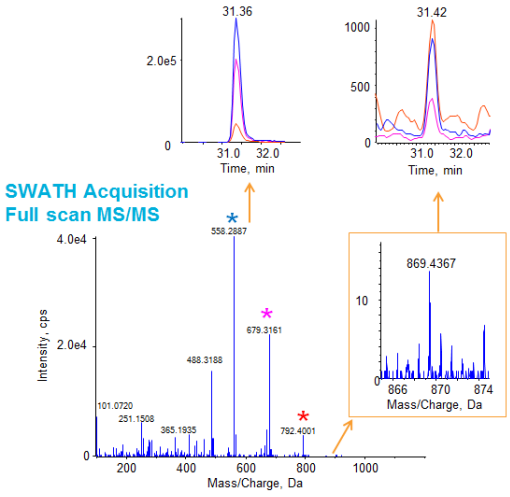

Figure 1. Importance of Dynamic Range. In SWATH Acquisition, fragment ion XICs are extracted from full scan MS/MS data post-acquisition and used for quantitation of peptides. The intra-scan dynamic range of the spectrum will determine how broad an abundance range accurate quantitation can be obtained. The wide dynamic range of the TripleTOF® 6600 System enables low abundant peptides and fragments (orange box) to be quantified in the presence of high abundant peptides, which further extends the power of SWATH acquisition for quantitation in complex biological samples.

Here, the combination of higher sample loads, smaller variable Q1 windows and expanded detector dynamic range of the TripleTOF® 6600 System will be explored as a strategy for increasing the depth of coverage of complex proteomic samples.

Key Advantages of the TripleTOF® 6600 System for SWATH® Acquisition

- **Increased dynamic range** on the TripleTOF® 6600 System
  - Greater than 5 orders linear dynamic range using an ADC detection system
  - Mass accuracy is maintained intra-scan across a wide abundance range of peptides
  - Increased workflow flexibility, enabling higher sample loads
- High sensitivity, resolution and speed of MS/MS acquisition allows a **large number of Q1 isolation windows** to be used in an LC time frame with no loss in quantitation quality
  - Up to 200 Q1 windows per cycle on TripleTOF 6600 system, with upper mass limit 2250 m/z
  - Narrower Q1 isolation windows provide improved data quality through increased specificity
  - Variable window acquisition method building[1] can be used to optimize SWATH acquisition for the proteome of interest
- Automatically design methods that use of a greater number of smaller variable sized Q1 windows in combination with higher sample loads on the TripleTOF 6600 System provided a 90% increase in peptide coverage of the proteomic sample.

Methods

Sample Preparation: Yeast proteome samples were reduced, alkylated, and digested, providing a solution of ~1 µg/µL. iRT peptides (Biognosis, Zurich) were spiked (1:20 dilution of stock) in for retention time calibration.

Chromatography: The samples were analyzed using the nanoLC™ 425 System with the cHiPLC® System operating in serial column mode[2] (SCIEX). The samples were first loaded on the first cHiPLC column (75 µm x 15 cm ChromXP™ C18-CL, 3 µm, 300 Å) and washed for 30 mins at 0.5 µL/min. Then, elution gradients of 5-30% acetonitrile (0.1% formic acid) in 60 or 120 mins were used to elute peptides off the first column and through the second nano cHiPLC column. Both columns were maintained at 35 ºC for retention time stability.

Mass Spectrometry: Eluent from the column was sprayed using the Nanospray® Source into a TripleTOF® 6600 system with Analyst® Software TF 1.7 (SCIEX). The SWATH® acquisition methods were built using the Variable Window Calculator[3] and the SWATH Acquisition method editor. A variety of window numbers and accumulation times were explored. Total cycle time was kept constant at 3.0 sec. Q1 mass range interrogated was 400-1250 m/z, and a TOF MS scan (200 msec) was acquired in every cycle.

Data Processing: A spectral ion library for the yeast proteome was used to drive data processing[4]. iRT standard peptides were used for automatic retention time calibration of the different LC gradients with the ion library retention times. All SWATH acquisition data were processed using the SWATH Acquisition MicroApp 2.0 in PeakView® Software. Peptide peak group detections were filtered at a 1% global FDR and replicates were analyzed using the SWATH Replicates Analysis Template[5].

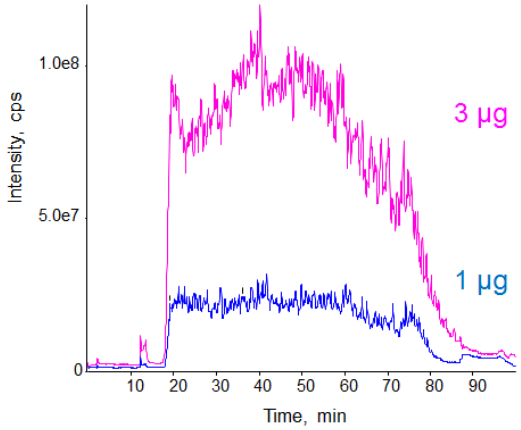

Figure 2. Higher Sample Loads And Longer Column Chromatography. Because the TripleTOF® 6600 system has an expanded dynamic range and can accommodate a much higher signal level, higher sample loadings can be used during SWATH Acquisition. To take advantage of this, the serial column workflow was employed, where sample was first loaded onto a 15cm column (acting as a trap), desalted, and then eluted onto a second 15cm column to yield a 30 cm column for higher peak capacity. The load was increased from 1 µg (blue) to 3 µg (pink) and chromatographic peak shape and robustness was maintained.

Increasing Peptide Detection with Increasing Sample Load

A spectral ion library with peptides representing proteins for the entire yeast proteome was used to interrogate the SWATH Acquisition experiments. iRT peptides were added for retention time alignment during processing. Replicate analyses were also done for each experimental condition such that peak area variation could be measured (% CV). Six fragment ion XICs for each peptide were extracted and then scored. The peptides were then filtered at 1% FDR from the different SWATH acquisition experiments and reproducibility of the data assessed.

The serial column workflow was used to increase the loading capacity of the experiment (Figure 2) and to also gain from an extended column length of 30cm column length. The baseline reference experiment was a typical 1 µg sample load and a 60 min gradient on the 30cm column, using 60 variable windows as explored in previous work[1]. Increasing the sample load to 3 µg provided a 31% increase in peptide detections (< 1% FDR and <20% CV were used as the figures of merit). Next the gradient length was extended to 120 minutes and a further 22% increase in peptide detections was obtained (Figure 3). The extended dynamic range of the TripleTOF® 6600 system allows for higher sample load providing this significant increase in sample coverage.

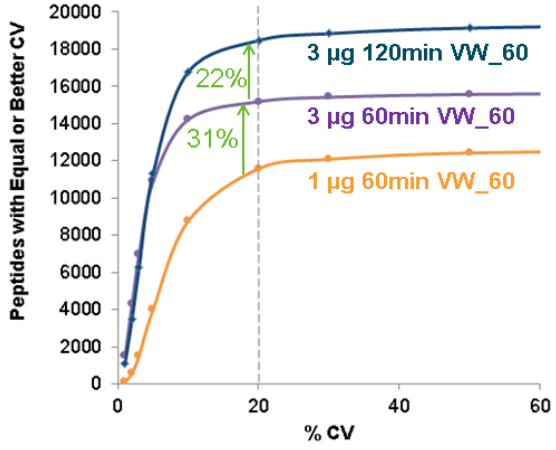

Figure 3. Higher Sample Loads and Longer Gradients Provide Significant Increases in Peptide Coverage. Five replicate analyses were performed with varied sample load and gradient length. Peptides with high detection confidence (1% FDR cutoff) were analyzed for reproducibility and plotted according to cumulative %CV. Using a high quantitation requirement (20% CV cutoff, gray line), a 40% increase in peptide coverage was obtained when the load was increased from 1 to 3 µg yeast on column. A further 12% gain was obtained when the gradient length was extended to 120min from 60mins.

Narrower Isolation Windows through Variable Window Acquisition Provides Increased Specificity

Next, the impact of increasing the number of Q1 windows, and thereby decreasing the precursor ion isolation width, was explored for its impact on peptide detection and quantitation quality. This was done at the higher sample load to fully take advantage of the enhanced peptide signal.

As the number of Q1 windows was increased from 60 to 80 to 100 windows and the width of the isolation windows was narrowed accordingly, the number of peptides that could be detected (<1% FDR and <20% CV) increased (Figure 4, top). This decrease in window size resulted in a further 20% increase in peptide coverage (Figure 4, bottom). This is mostly likely due to an improvement in specificity and signal/noise obtained from the reduced complexity of the MS/MS data from each SWATH® window across the whole mass range. The higher specificity will improve the peak group scoring process as the XIC data will have much improved S/N and less chance of interferences that could confound peak group detection. Improved S/N will also improve consistency of peak integration and therefore quantitation quality. Further work will be done to explore how much farther we can refine this technique and utilize the 200 window maximum of the TripleTOF® 6600 System.

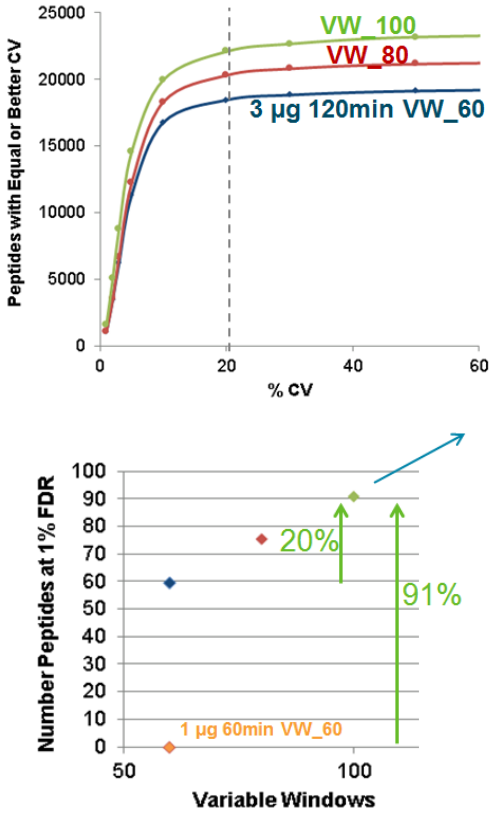

Figure 4. Further Increasing Window Sizes Provides More Quality Peptide Detection. (Top) The number of windows used to cover the mass range was increased from 60 to 100 windows and the reproducibility curves were plotted. The number of confident peptide detections (<1% FDR) with 20% CV or better further increased as the window size decreases (gray dotted line). (Bottom) The number of peptide detections with <1% FDR and <20% CV was plotted vs. increasing number of Q1 windows and 20% gain in peptides was observed.

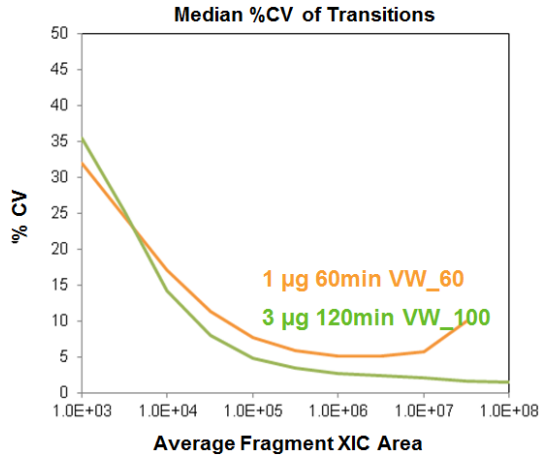

Figure 5. Maintaining Quantitation Quality with Increasing Depth of Coverage. The accumulation time per MS/MS is reduced as the number of Q1 window steps is increased to ensure an optimal cycle time for LC/MS quantitation. Therefore, assessing the reproducibility of the XICs as a function of peak area for the confidently detected peptides is important to monitor. The median CV for the XIC peak areas in a particular peak area range was measured and plotted vs. peak area across 5 orders dynamic range and the CV curves for the two experimental conditions look very similar. Therefore, even though the acquisition rates are faster for the 100 variable window strategy, the quantitation has not degraded.

Maintaining High Quantitative Reproducibility

As the number of windows increases, the accumulation time used per window must decrease to maintain the optimal cycle time. As this could have an impact on quantitation, especially on the lower abundant peptides, it is important to monitor the impact on %CV as a function of XIC peak area for the various acquisition strategies. As seen in Figure 5, the median CV for the XIC peak areas in a particular peak area range was measured and plotted vs. peak area across 5 orders dynamic range and the results show a very similar performance between the experimental strategies.

Therefore, the accumulation time could be reduced from 37 msec per MS/MS for the 60 variable window experiment to 30 msec per MS/MS for the 100 variable window experiment with no major reduction in quantitative quality.

Data Completeness

One reason why researchers move from data dependent acquisition to targeted quantitation with MRM when doing larger study sizes is because of the 'missing values problem' inherent in data dependent acquisition strategies. For the key peptides and proteins of interest, MRM provides a measurement for each targeted analyte in almost every sample across very large numbers of samples, across a broad intensity regime (Figure 6) providing data completeness in the study. However, the multiplexing capacity is limited to a few 100 proteins as opposed to the 1000s of proteins identified using conventional data dependent acquisition (DDA) strategies.

But with SWATH® Acquisition (DIA), MS/MS is collected on all masses all the time, and peptide data is analyzed in a targeted fashion from this comprehensive dataset. Therefore, much better data completeness, approaching that of MRM, is achieved but with much higher multiplexing.

In this study, we wanted to ensure that the additional peptide detections obtained as we expanded our coverage still provided the desired high data completeness (maintain a low number of 'missing values'). In Figure 6, the data completeness across the 5 replicates for the 1% FDR fragment ions and peptides were plotted vs. number of windows. Therefore more peptides are added to the dataset with high quantitative quality and high data completeness.

Conclusions

SWATH Acquisition combined with the expanded dynamic range of the TripleTOF® 6600 system and the variable window acquisition strategy enables much deeper coverage of the proteomic sample. Here a 90% gain was achieved in peptides detected with high confidence and quantified with good reproducibility.

- Variable window SWATH Acquisition[1] combined with higher number of windows greatly improves the specificity of the SWATH acquisition experiment.
- The broad linear dynamic range (> 5 orders LDR) of the TripleTOF 6600 system enables higher sample loads during SWATH acquisition.
- Combining more optimized and smaller SWATH windows with increased signal due to higher sample loads provided a 90% gain in peptide coverage.

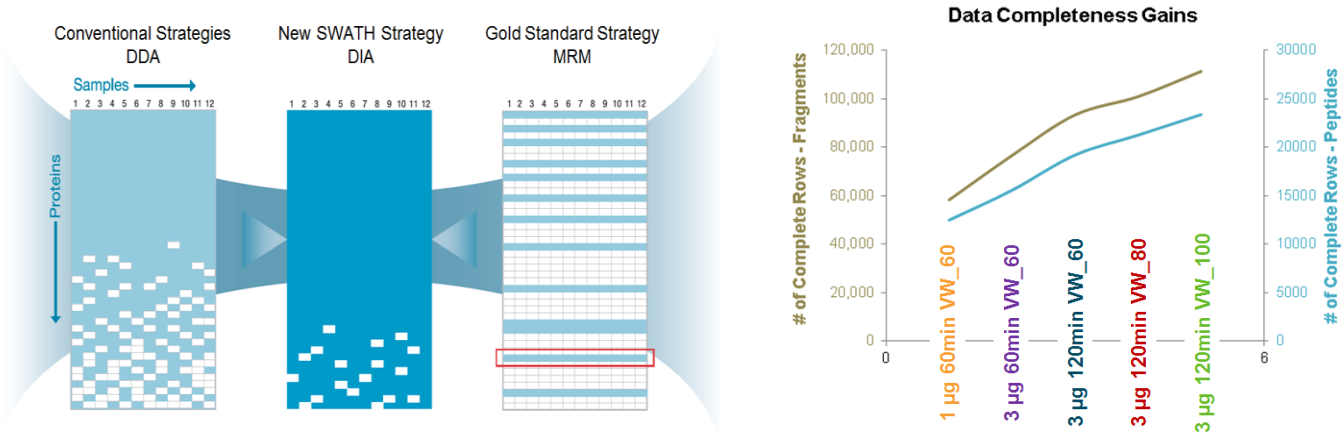

Figure 6. Assessing Data Completeness. (Top) SWATH Acquisition provides the high depth of coverage of conventional data dependent (DDA) workflows but with the data completeness and quality of quantitation of the targeted MRM workflows. (Bottom) Analysis was done to confirm that the increased peptide coverage obtained with the increased sample load and decreased window size provided more fragment (brown) and more peptide (blue) coverage with 5 out of 5 measurements (complete rows, high data completeness).

References

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