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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INTRODUCTION

In data independent acquisition (DIA), high resolution MS/MS is acquired across a mass range, using wide Q1 windows, which creates complex spectra from which fragment or chromatograms (XICs) can be extracted for quantitation. The most widely used of these techniques is MS/MS® with 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 SCIs. 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 functionality¹. Increasing signal can be obtained by using higher sample loads on optimised chromatography (Figure 3). Higher sample loads 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.

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.

MATERIALS AND METHODS

Sample Preparation: Yeast proteome samples were reduced, alkylated, and digested, providing a solution of ~1 µg/µL. iRT peptides (Biosearch, Zurich) were spiked 1/120 dilution of stock) for retention time calibration.

Chromatography: The samples were analysed using the ekspert® nanoLC 425 System with the chpLC® System operating in serial column mode (ExciTip). The samples were first loaded on the first chpLC column (75 µm x 15 mm ChromXP® C18 CL, 3 µm, 300 A) and washed for 30 min at 0.5 µL/min. Then, elution gradients of 0-30% acetonitrile (0.1% formic acid) in 60 or 120 mins were used to elute peptides off the first column and through the second nano chpLC column. Both columns were maintained at 35°C for retention time stability.

Max Spectrometry: Eluent from the column was sprayed using the nanoSpray® Source into a Triplet OFP™ 6600 system with Analyst® Software TF 1.7 (AB SCIEX). The SWATH™ acquisition methods were built using the Variable Window Calculator 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 (200–5000 m/z) was acquired in every cycle.

Data Processing: A spectral ion library for the yeast proteome was used to drive data processing. 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 MS/MS® with SWATH™ Acquisition Module 2.0 in PeakView Software 2.1. Peptide peak group detections were filtered at a 1% global FDR and replicates were analyzed using the SWATH Replicates Analyze Template®.

RESULTS

Figure 2. Extending Depth of Coverage

As proteomic samples are so complex, improving the signal/noise balance is always critical to coverage. From an MS independent acquisition perspective, we can decrease our chance of interferences through running longer gradients and running smaller Q1 windows, leveraging the variable window strategy (Figure 4). In terms of increasing signal, the extended dynamic range of the Triple TOF 6600 System can be leveraged and more sample can be loaded on the detector. Finally, use of external ion libraries for data interrogation are also key to getting the most out of every sample.

Figure 3. Higher Sample Loads And Longer Chromatography

Because of the TripleTOF 6600 system has an expanded dynamic range and can accommodate a much higher signal level, higher sample loads can be used during SWATH™ Acquisition. To take advantage of this, the serial chromatography workflow was employed, where sample was first loaded into a 15cm column running at 100 µL/min, and was 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.

Figure 4. 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 XICs. Using a high quantification requirement (20 CV cutoff, gray line), a 40% increase in peptide coverage was obtained. The load was increased from 1 to 3 µg on yeast 20% gain. A further 12% gain was obtained when the gradient length was increased to 120min from 60mins.

Figure 5. Further Increasing Window Sizes Provides More Quality Peptide Detection

The number of iRT standard peptides used to cover the mass range increased from 60 to 100 windows and the reproducibility curves were plotted. The number of confident peptide detections (~1%) with 20% CV is better further increased as the window size decreases (grey dotted line). (Bottom) The number of peptide detections with <1% FDR and <20% CV was plotted vs. increasing number of Q1 windows and 25% gain in peptides was observed. As the Triple TOF 6600 System supports up to 200 variable Q1 windows, we will explore going to even higher Q1 window numbers in future work.

Figure 6. Maintaining Quantitation with Increasing Depth of Coverage

The accumulation time per MS/MS is related 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 order dynamic range and the CV for the XIC peak area range was plotted vs. peak area across 5 order dynamic range and the CV for the XIC peak area range. Therefore, even though the quantitation rates are faster for the 100 variable window strategy, the quantitation has not degraded.

REFERENCES

1. Improved Data Quality Using Variable Q1 Window Widths in SWATH™ Acquisition - Data Independent Acquisition on the TripleTOF®6600 and 5600+ Systems. AB SCIEX Technical note 109201-01.
5. SWATH Replicates Template. Downloaded from www.absciex.com/SWATHReplicatesTemplate.

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MS™ with 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 combined with higher number of windows enables higher sample loads during SWATH acquisition experiments.

• The broad linear dynamic range (5 orders LOD) 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.

The depth of coverage and quality of quantitation obtained from these experiments will depend on the signal/noise of the generated SCIs. 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. Increasing signal can be obtained by using higher sample loads on optimised chromatography. Finally, use of external ion libraries for data interrogation are also key to getting the most out of every sample.