

High Reproducibility Targeted Quantitation at Highest Multiplexing

MS/MS^{ALL} with SWATH™ Acquisition on the TripleTOF™ 5600+ System

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The goal of quantitative proteomics is to both identify and quantify a broad range of proteins and peptides. The extreme complexity and dynamic range of proteins in typical proteomic samples challenges traditional data dependent workflows by requiring very high speed MS/MS acquisition to reproducibly and deeply interrogate the sample. Data independent acquisition has been explored as an alternative strategy to increase the comprehensiveness of data acquisition². A data independent acquisition strategy in combination with the targeted data extraction using MS/MS spectral libraries provides a powerful workflow for targeted protein / peptide quantitation and will increase the reproducibility and comprehensiveness of data collection and processing.

The MS/MS speed and resolution of the TripleTOF™ 5600+ system have enabled a comprehensive data independent acquisition mode termed MS/MS^{ALL} with SWATH™ Acquisition¹. In this workflow, the Q1 quadrupole is stepped at ~25 Da increments across the mass range of interest, passing a 25 amu window through into the collision cell. The transmitted ions are



fragmented and the resulting fragments are analyzed in the TOF MS Analyzer at high resolution. This is done in a looped fashion in an LC compatible cycle time, such that MS/MS spectra have been acquired on every peptide in the sample. Because the fragment ions are collected at high resolution, high quality extracted ion chromatograms (XICs) can be generated post-acquisition to produce the MRM-like data^{1,2} (Figure 1). The utility of this workflow for highly multiplexed targeted quantification in plasma samples and other complex proteomes is examined here.

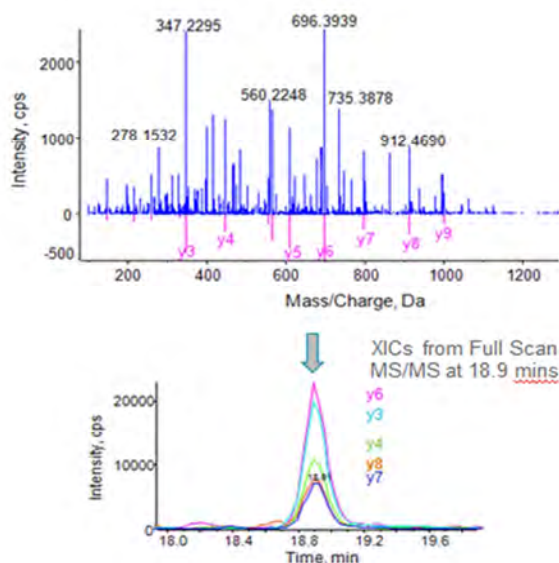


Figure 1. Quantitative Fragment Ion XICs from the Full Scan MS/MS collected during MS/MS^{ALL} with SWATH™ Acquisition. (Top) The full scan MS/MS of CRP peptide ES DTSYVSLK 2+ from 18.9 mins is shown in blue. The corresponding MS/MS fragment pattern obtained from the ion library is shown in pink. The most intense fragment ions based on the pink spectrum are extracted (bottom) at a width of 0.05 Da to generate MRM-like data for quantification.

Key Steps for Targeted Quantitation using MS/MS^{ALL} with SWATH™ Acquisition

- High quality, reproducibility LCMS using Eksigent nanoLC-Ultra® with cHiPLC®-nanoflex
- Data independent acquisition using the TripleTOF™ 5600+ System and MS/MS^{ALL} with SWATH™ Acquisition
 - Broad, uniform Q1 isolation windows
 - Generic acquisition method that can be used with all proteomes
 - High mass accuracy, high resolution MS/MS acquired at highest speed for high selectivity
- Post-acquisition extraction of large numbers of high resolution for sequence specific fragment ions of the targeted peptides and proteins to generate peak areas with high specificity.

Methods

Sample Preparation: Human plasma was depleted of the top 14 proteins using a MARS14 depletion cartridge. 20P sample was a mix of 20 standard proteins (Sigma), *E. coli* was a cell lysate (BioRad) and the cell sample was a lysate of human 293T cells. All samples were reduced, alkylated, and digested, providing a solution of ~1 µg/µL.

Chromatography: The samples were analyzed using the Eksigent nanoLC-Ultra[®] 2D System combined with the cHiPLC[®]-nanoflex system in Trap-Elute mode. The samples were first loaded on the cHiPLC trap (200 µm x 500 µm ChromXP C18-CL, 3 µm, 300 Å) and washed for 10 mins at 4 µL/min. Then, an elution gradient of 5-35% acetonitrile (0.1% formic acid) in 30 – 90 mins (depending on proteome complexity) was used on a nano cHiPLC column (75 µm x 15 cm ChromXP C18-CL, 3 µm, 300 Å). Trap and column were maintained at 30 °C for retention time stability. Replicate injections were run to assess reproducibility of ~500 ng of sample on column.

Mass Spectrometry: Eluant from the column was analyzed using the Nanospray[®] Source on a TripleTOF[™] 5600 system (AB SCIEX). The acquisition method was an MS/MS^{ALL} with SWATH[™] Acquisition method, where Q1 was scanned from 400-1000 m/z and MS/MS was acquired from 100-1500 m/z. Q1 transmission window was 25 Da wide and 24 steps were used with a 100 msec accumulation time on each for a total cycle time of 2.5 sec.

Data Processing: A protein identification experiment was performed on each of the proteomes and searched with ProteinPilot[®] Software to create a spectral library of the proteins and peptides in the sample. The group file was loaded into the MS/MS^{ALL} with SWATH[™] Acquisition MicroApp in PeakView[®] Software and extracted ion chromatograms were generated for fragment ions from all targeted peptides and the peak areas were integrated. Reproducibility analysis was done using SWATH Processing Template.

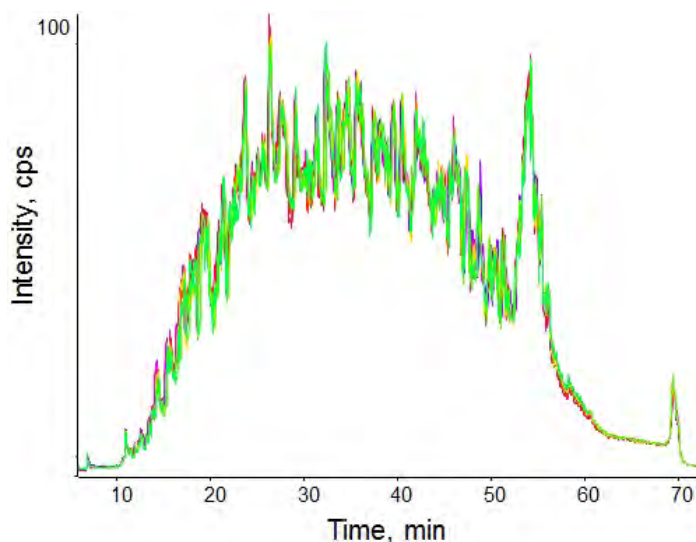


Figure 2. Replicate Injections of Depleted Plasma using nanoLC and MS/MS^{ALL} with SWATH[™] Acquisition. High reproducibility is a key component of targeted quantitation experiments. The Eksigent nanoLC-Ultra[®] with the cHiPLC[®]-nanoflex provides high quality, reproducible separations.

Reproducibility of Quantitation in Depleted Plasma

To assess the utility of this technique for quantitative profiling of proteins in plasma, the reproducibility of raw peak areas across ten replicate injections was analyzed (Figure 2). First, an information dependent acquisition was performed on the plasma sample and processed with ProteinPilot[®] Software. The group file was used as the ion library to drive the targeted data analysis during the SWATH[™] Acquisition processing (Figure 2).

Over 750 peptides to 150 proteins were quantified in a single injection. ~Six fragment ions per peptide and up to 6 peptides per protein were extracted at a width of 0.05 Da to generate ~4500 high resolution XICs (Figure 3, top). Very high reproducibility of XIC peak areas was observed, approaching the reproducibility of MRM targeted quantitation. Over 80% of raw XICs had peak areas with lower than 20% CVs (Figure 3, middle). The distribution of XIC peak areas spanned ~4 orders (Figure 3, bottom). A small portion of lower abundance XICs had higher variance as expected (red line) but the vast majority of fragment XICs were very reproducible.

Reproducibility in Higher Complexity Proteomes

To understand the impact of increasing complexity on the reproducibility of SWATH™ Acquisition, LCMS replicates were performed on more complex proteomes. Again, the fragment ion XICs were extracted and raw XIC peak areas were analyzed. The numbers of proteins and peptides analyzed in each sample are shown in Figure 4 (top). The data independent acquisition strategy differs from MRM targeted quantitation as all ions are collected and then extracted post-acquisition. This allows significantly higher multiplexing to be obtained while maintaining high quality quantification.

Using 20% CV as a point of comparison, the highest reproducibility is observed for the simplest matrix. As the complexity of the proteomes increase and a much larger number of XICs are extracted, the overall reproducibility of the total XIC population does degrade a small amount. In the Cell lysate example, ~80% of the raw peak areas had a variance across analytical replicates of less than 20% CV. This is from an experiment where almost 1500 proteins and over 5300 peptides were analyzed with 31500 high resolution XICs. This level of reproducibility on such a high level of multiplexing illustrates the power of the technique for targeted quantitation.

Extraction Summary	20P	DepPlas	Ecoli	Cell
Total # of Proteins	50	150	796	1494
Total # of Peptides	236	756	2533	5303
Total # of Ions	1174	4536	15198	31523

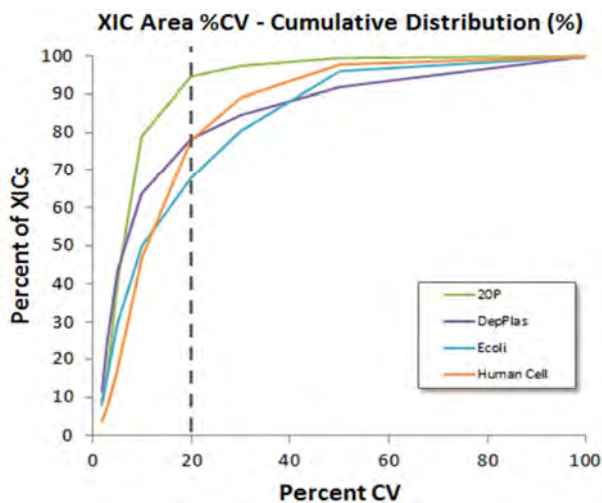


Figure 4. Reproducibility Across Increasingly Complex Proteomes. The curves show the cumulative reproducibility curves, the gray line highlights the number of fragment ion XICs that have reproducibility better than 20% across the replicates. The table (top) shows the number of fragment ions, peptides and proteins quantified in each of the curves, highlighting the tremendous degree of multiplexing possible in the analysis, while still maintaining good reproducibility.

Extraction Summary	
Total # of Proteins	150
Total # of Peptides	756
Total # of Ions	4536

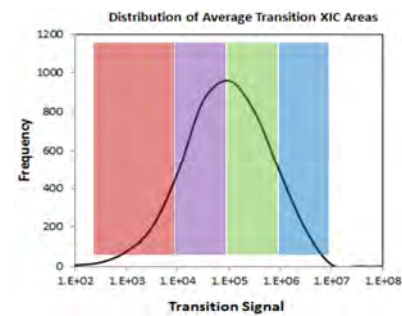
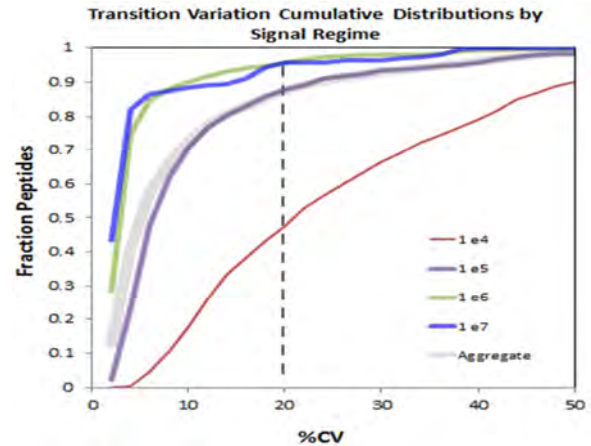


Figure 3. Reproducibility of Fragment Ion XICs across 10 Replicate Injections of Depleted Plasma. The reproducibility of the raw XIC peak areas across 10 replicate injections were analyzed both in total and by peak area range (middle pane). Over 80% of the raw peak area data has variance below 20% CV (gray line). As expected, smaller peaks showed increased variance across the experiment (red line) but in total, the reproducibility of the experiment was very high. The observed distribution of XIC peak areas is shown (bottom pane).

Multiple XICs are generated for each peptide, so the peak areas can be summed together to obtain peptide level data. Minimal data refinement was performed in this experiment, only XICs that showed variance greater than 60% were removed from the dataset. The remaining peak areas were summed and the reproducibility at the peptide was assessed (Figure 5). High reproducibility at the peptide level for all proteomes was observed. Additional data refinement could provide further improvements.

Conclusions

- The data independent acquisition strategy, MS/MS^{ALL} with SWATH™ Acquisition, provides a comprehensive analysis of complex proteomes, providing full scan high resolution MS/MS on all peptides eluting off the column, within the detection dynamic range.
- All samples are collected using the same generic acquisition method, then analyzed post-acquisition for the peptide targets of interest. The datafile is therefore an archive of MS/MS of all analytes which enable the retrospective *in silico* interrogation for any targets after acquisition.
- Depleted plasma and a range of other proteomes were analyzed using replicate analysis to understand the quantitative performance of this technique.
- High reproducibility of quantitation across replicates was observed, even in the most complex proteomes
- High resolution MS/MS provides higher resolution XICs with reduced chance of interferences, providing quantitative performance comparable to triple quadrupole quantitation.

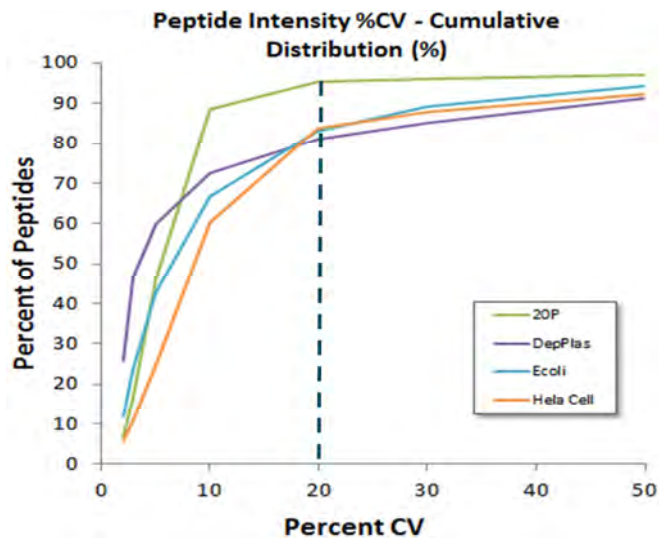


Figure 5. Reproducibility of Peptide Areas across Replicate Injections of Complex Proteomes. Multiple fragment ion XICs were summed to give peptide peak areas. The same XICs were used per peptide across all replicates. Fragment ion XICs that had higher than 30% CV were filtered out of the dataset before summing. This provides a high quality peptide level dataset for all proteomes.

References

1. MS/MS^{ALL} with SWATH™ Acquisition - Comprehensive Quantification with Qualitative Confirmation using the TripleTOF™ 5600 System. AB SCIEX Technical note 3330111-03.
2. Gillet LC *et al* (2012) *Mol. Cell. Prot.* E-pub.

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