# Investigating Biological Variation in Human Hepatocytes of Phase I and II drug metabolism enzymes

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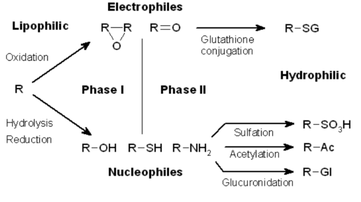
## INTRODUCTION

MS/MS$^{ALL}$ with SWATH™ Acquisition is a data independent workflow, performed on the TripleTOF® 5600+ system, that is of great interest today as it provides a higher level of reproducibility and comprehensiveness in proteomics data [1]. In a SWATH acquisition experiment, a wide Q1 isolation window is stepped through the precursor mass range, transmitting multiple analytes into the collision cell. The transmitted ions from each step are fragmented and a composite MS/MS spectrum is measured in the TOF MS Analyzer at high speed and high resolution. Post-acquisition, the peptides of interest are quantified by generating fragment ion extracted ion chromatograms (XICs) and measuring their peak areas.

For drug development, measurement of drug metabolizing enzymes responsible for phase I and II bio-transformations (**Figure 1**) is a fundamental aspect of assessing drug-drug interactions, and evaluating drug safety and efficacy. Targeted quantitation using multiple-reaction-monitoring (MRM has been successfully applied to quantitatively profile these enzymes in liver hepatocytes or microsomes. However, an MRM experiment normally focuses on a limited set of selected proteins for quantitation and requires significant upfront assay development work. In this work, the MS/MS$^{ALL}$ with SWATH™ Acquisition method was used analyze large numbers of proteins and multiple enzyme families involved in drug metabolism.

**Figure 1. Phases of Detoxification.** Drug metabolism is the biochemical modification of xenobiotics or drugs using a specialized enzymatic system in the liver. These metabolic pathways modify the chemical structure of the foreign compounds to detoxify and prepare for excretion. © Wikipedia.




## MATERIALS AND METHODS

***Sample Preparation:*** Tissue cells (0.625 million) were resuspended in 1ml extraction buffer from ProteoExtract Native Membrane Protein Extraction Kit (EMD, Billerica, MA) with protease inhibitor. The cells were then lysed by for 10 min at 4 ºC. The lysate was mixed with 250uL 100mM $NH_4HCO_3$ /3.6% DOC buffer and shaken for 20minutes. Reduction agent (50µL 100 mM DTT) was added and incubated for 10 min at 95 ºC to disrupt disulfide bonds, followed by alkylation of free sulfhydryl groups with 50µL of 5mM iodoacetamide at room temperature in the dark for 30 min with continuous shaking. Extracted proteins were digested with trypsin (1:50) at 37 ºC for 18 hrs. The digestion was stopped with addition of 0.2% formic acid/$H_2O$ solution, then vortexed and centrifuged at 10,000 g for 5 min. The supernatant was transferred to a new eppendorf tube and dried down in speed vacuum for 3 hrs at 50 ºC.

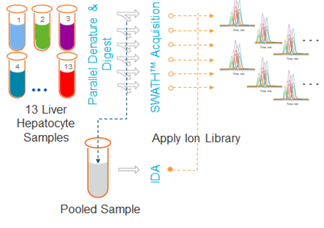
***Chromatography:*** Tryptic digests were separated using an Eksigent ekspert™ nanoLC 425 system with a cHiPLC® column (75 µm x 50 mm, 300 Å pore size ChromXP™) running a flow rate of 300 nL/min. The gradient was 90 mins as follows: 55 % B for 2 min, from 55 % B to 85 % B in 8 min, from 85% B to 98 % B in 8 min, 98% B for 2 min, from 98 % B to 55 % B in 2 min, and 55 % B for 5 min. (10 µL sample) Mobile phase A consisted of $H_2O$ and 0.1% formic acid, and mobile phase B consisted of acetonitrile and 0.1% formic acid. The column oven was operated at 35 ˚C. Sample injection volume was 10 µL.

***Mass Spectrometry:*** Eluant from the column was sprayed using the NanoSpray® Source into a TripleTOF® 5600+ system (AB SCIEX). Data were acquired using an MS/MS$^{ALL}$ with SWATH™ acquisition method with a Q1 window size of 25 Da and a mass range of 400-1000 m/z (cycle time 2.5 sec). Information dependent acquisition (IDA) experiment was performed on the pooled sample to obtain peptide identifications to generate ion library. Thirteen individual hepatocytes samples (labeled 098, 091, DAD, I2G, IDE, KMI, MRS, NQT, RML, ROE, SED, VCM, YAA) were analyzed in triplicate by SWATH acquisition (Experiment Design). Protein/peptide data were loaded into Skyline for MRM assay development and samples were also run in triplicate by MRM on the QTRAP® 6500 System..

***Data Processing:*** The pooled sample was analyzed with ProteinPilot™ Software 4.5 beta to create a spectral library of proteins and peptides in the sample. SWATH Acquisition data were processed using the SWATH™ Acquisition MicroApp 1.0 in PeakView® Software. Only proteins that were identified at a 1% global FDR were used in SWATH acquisition processing. Fragment ion XICs were summed to obtain peptide peak areas, and the areas for multiple peptides per protein were summed to obtain protein areas. Statistical analysis including principal component analysis (PCA) and t-tests were conducted with MarkerView™ Software 1.2. Data analysis of the MRM data was performed using MultiQuant™ Software 2.1.

### *Experiment Design*

The experiment design is shown in the right figure. The pooled sample was analyzed using an IDA experiment in order to generate a spectral ion library. The individual samples were analyzed in triplicate using SWATH™ Acquisition. Resulting data was then analysis using targeted data extraction as guided by the ion library.



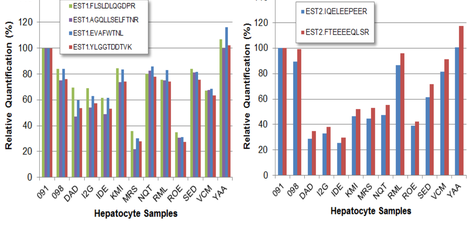

## Results

### *High Quality Quantification on Large Number of Proteins*

Pooled samples were used to generate an ion spectral library containing more than 2000 proteins. From the SWATH™ acquisition, 1987 proteins, in average, were quantifiable across the 13 samples. Highly reproducible results were created for target enzymes of interest, such as 19 CYP proteins, 12 UGT proteins, 7 GST proteins, etc. The reproducibility was assessed via technical replicated by injecting same sample three times. The relative quantitation was carried out on multiple identified drug metabolizing enzymes. The quantitative profiling potentially helps to discover the expression variations of these enzymes, as well as their correlation/anti-correlation properties among the population. The systematically understanding of their relation is essential for drug development.

**Figure 2** shows the relative comparison of the two phase II metabolism enzymes (liver carboxylase EST1 and EST2) across 13 samples. For comparison, the peptide signal was normalized against sample 091. A good correlation of quantitative differences was observed across multiple peptides of each of the two proteins, demonstrating that good reliability was observed in the SWATH™ Acquisition data.

**Figure 2. Reproducible Quantitation of Liver Proteins.** Quantitation for individual proteins of interest can be easily extracted from the dataset. Shown here are two different esterases, EST1 - liver carboxylesterase 1 and EST2 - cocaine esterase. There is very good agreement between the multiple peptides per protein highlighting the reproducibility of quantitation.




Because of the comprehensive nature of SWATH acquisition, it overcomes some common limitations existing in the MRM based targeted methods, such as limited multiplexing capabilities and the fact that only targeted analytes will be detected and quantified. Unlike a targeted method, SWATH acquisition creates a permanent record of MS and MS/MS spectra of all detectable species in the sample, which can be revisited *in silico* without the need for further data acquisition. This offers an opportunity to ask more questions of the study as new information arises.

### *Mining Data for Interesting Protein Expression Patterns*

Since quantitative profile (low and high biological variation) of phase I and II enzymes are needed to facilitate drug development, we also systematically investigated the expression of them. Correlation/anti-correlation of protein expression can be discovered after PCA analysis (**Figure 3**). In the results, we found multiple proteins were correlated, such as CYP3A4 and CYP3A5, which are two major phase I drug metabolizing enzymes in cytochrome P450 superfamily. Their sequence overlap is >80%. The quantitation was carried out by using characteristic peptides acquired in the SWATH data. **Figure 4** shows strong correlation of their expression across 13 samples. Previously published data also demonstrated the "coordinator" feature of these two proteins [2]. Besides them, we also found the correlation between UDP glucuronosyltransferases (UGT) 2B7 and UGT2B15, and between CYP3A4 and UGT1A6 (**Figure 5**).

**Figure 3. Analyzing Global Protein Expression Differences across the Samples.** After the data extraction was performed on the SWATH acquisition data, the protein areas were loaded into MarkerView Software for statistical analysis. PCA was performed to obtain the Scores and Loadings plots (top). This enables the easy visualization of the proteins that are showing the highest degree of change across the multiple samples. As an example, the protein expression differences across the different liver hepatocyte samples for the two cytochrome P450 proteins, CYP2C8 and CYP 2A6, are shown.

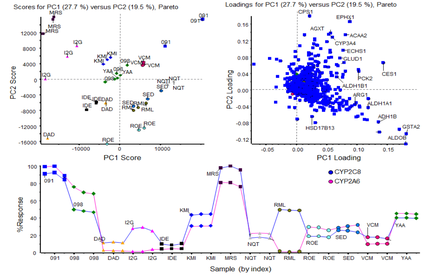
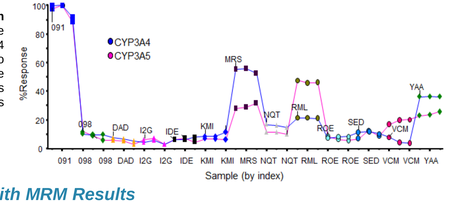



**Figure 4. Correlated Protein Expression.** Three respective characteristic peptides of 3A4 and 3A5 were summed to represent protein intensity. The absolute signal intensities were used to compare across the samples.




### *Good Correlation with MRM Results*

After quantitative discovery with SWATH acquisition data, a set of peptides can be selected for future verification with MRM approach for better sensitivity and throughput. The Skyline software was used to assist developing the MRM assay. **Figure 6** shows comparable results between MRM assay generated with QTRAP 6500 system and the SWATH acquisition data.

**Figure 5. Selected Protein Profile.** The figure was plotted in the MarkerView software. PCA helps find correlated and anti-correlated behaviors.

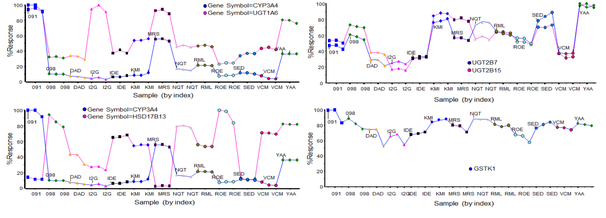
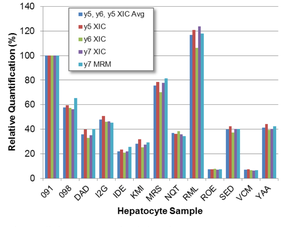



**Figure 6. Comparable Results Between MRM and SWATH Acquisition Data.** Selected transitions of YLPNPALQR, a tryptic peptide from CYP1A2, were plotted based on the data from SWATH and MRM. The bar height is normalized intensity of each transition against sample 091.




## CONCLUSIONS

- SWATH™ Acquisition was easy to setup and required very little to no method development.
- SWATH™ Acquisition provides comprehensive quantitate data (minimal missing information).
- PCA, the statistical analysis, makes data interpretation easier.
- Multiple drug metabolizing enzymes were identified and quantitatively profiled in a single assay
- SWATH Acquisition experiment delivered comparable quantitation results to the MRM experiment.

## 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.Lin Y. S. et al., Mol. Pharmacol. 62: 162-172, 2002

## TRADEMARKS/LICENSING