Automating Protein Digestion for Reproducible Proteomics

SCIEX Protein Digestion Automated Solution using Biomek NX^P Laboratory Automated Workstation

Christie Hunter[1], Qin Fu[2], Mike Kowalski[3], Jennifer E. Van Eyk[2]
[1]SCIEX, USA, [2]Cedars-Sinai Medical Center, USA, [3]Beckman Coulter, USA,

There are many steps in the process to generating high quality proteomics data on biological samples. There are now powerful MS workflows for quantitation (MRM and SWATH® acquisition) that enable highly reproducible quantitation on small or large numbers of samples. This creates a new bottleneck in sample preparation, the ability to reproducibly generate digested proteomic samples is critical for performing protein quantitation studies (Figure 1, top).

By using automation, the day-to-day variability of multi-step protocols such as protein denaturation, reduction, alkylation and digestion can be significantly reduced. Removing the labor intensiveness of MS sample preparation frees up scientists to do higher value work on other aspects of projects. Here, we adapted a reliable protein digestion protocol for use on the Biomek NX^P Span-8 Workstation[1]. This was coupled with an optimized protein preparation kit[2] that provides ready to use reagents for reproducibility and efficiency.

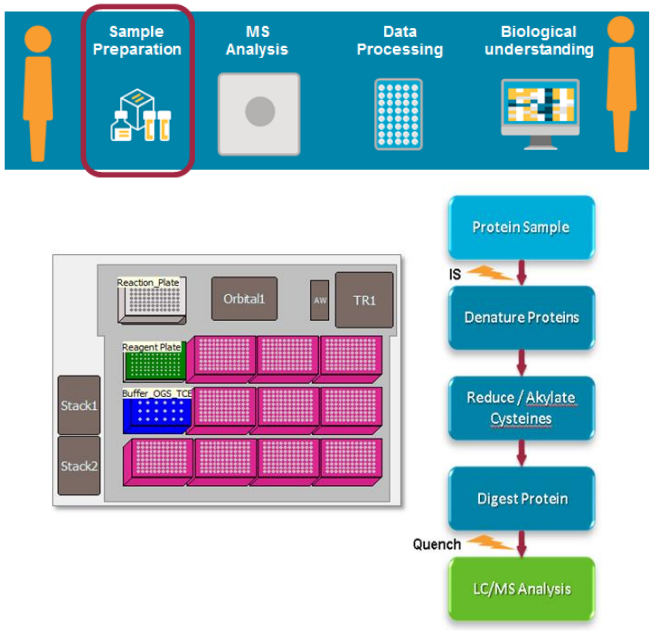

Figure 1. SCIEX Protein Digestion Automated Solution. Obtaining reproducible sample preparation is a key to success of large scale protein quantitation studies (top). The workflow of the SCIEX Protein Preparation kit (bottom right) has been automated on the Biomek NX^P Workstation for ease of generating reliable, reproducible protein digestion. Deck layout of the method (bottom left) illustrates how the whole digestion workflow for up to 96 samples can be performed in a single automation run.

Key Features of the SCIEX Protein Digestion Automated Solution

- Full solution for automated protein digestion of complex proteomics samples
  - Biomek NX^P Span-8 Workstation combines all the key aspects of automation into an economical solution
  - SCIEX Protein Preparation Kit provides all the reagents required from denaturation through to digestion
  - Optimized method reliably automates all of the workflow steps
- Digest from 8 to 96 samples automatically in a single automation run
- Obtain high reproducibility of digestion, in this work on plasma, ~80% of peptides monitored in the digestion replicate had overall CVs of <10% (Figure 6)

Methods

Sample Preparation: Up to 96 samples can be prepared at a time, using a method organized to run sets of 8 samples in column format. The general workflow (Figure 1) automates the SCIEX Protein Preparation Kit[2] on the Biomek NX[P] Workstation. Protein samples are processed by first heating the samples to 60°C in the presence of denaturant and reducing agent. Following heating, the protein's cysteine residues are blocked and the proteins are digested into peptides by adding trypsin and heating to 37°C for 3 hours. Test runs were performed using 24, 32 or 48 samples at a time.

Chromatography: Separation of the digest samples was performed on a nanoLC™ 425 System (SCIEX) operating in microflow mode using a 0.3x15 cm HaloPeptide column (Eksigent). A short gradient was used for rapid sample turn-around, 5-30% solvent B in 20 min (B: 95% ACN, 0.1% formic acid in H_2O) at 5 µL/min. Typically 3 µg of human plasma digest was injected onto the column for each run.

Mass Spectrometry: The MRM analysis was performed on a QTRAP® 6500 system (SCIEX) equipped with an IonDrive™ Turbo V Source. For the microflow experiments, the 25 µm I.D. electrospray probe (SCIEX) was used. SWATH® Acquisition was performed using a TripleTOF® 5600 system equipped with a DuoSpray™ source (SCIEX) and a 25 µm I.D. electrospray probe (SCIEX).

Data Processing: MRM acquisition methods were built using Skyline software for a range of peptides from many proteins, with the goal of creating an assay that measured global digestion quality. After acquisition, data was imported into MultiQuant™ Software for peak integration. Peak areas were exported and results analyzed using Excel. For SWATH® Acquisition, ion libraries were generated from ProteinPilot™ Software searches on plasma IDA data. Ion libraries were imported into SWATH® 2.0 Software for processing of SWATH Acquisition data. Peak areas were exported and results analyzed using Excel.

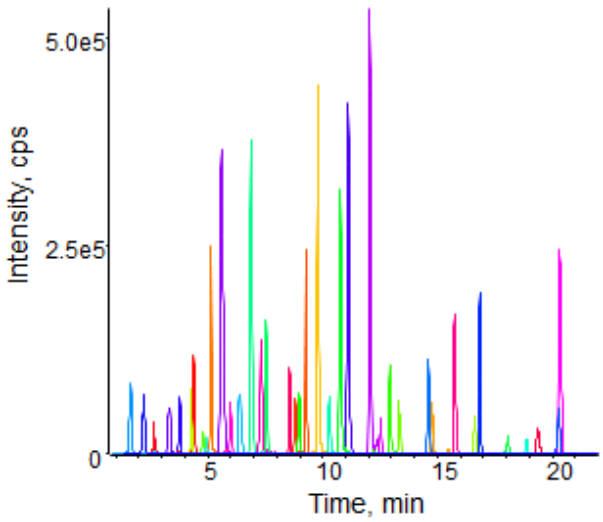

Figure 2. Microflow LC on the QTRAP® 6500 System for Rapid Assessment of Digestion Reproducibility. Using flow rates of 5 µL/min, digestion quality could be rapidly assessed. 150 peptides from plasma proteins were used in this set of tests.

Assessing the Overall Performance of the Digestion Protocol

First, the quality of the protein digestion workflow was assessed by performing a careful manual digestion on 24 aliquots of plasma. These digests were analyzed using SWATH® acquisition on the TripleTOF® 5600 System using a microflow LC method to look at the overall digestion performance on a large range of peptides. ~1-3 µg of total protein was loaded on column (Figure 2). Run times of ~30 minutes per sample were used to enable the fast assessment of many digestion replicates.

Very good reproducibility was obtained (Figure 3, purple trace) with ~90% of peptides monitored providing digestion reproducibility %CVs of <10%. Three technical replicates were also run on single digestions to separate out the LC/MS reproducibility of the assay (Figure 3, green trace). Digestion added an additional 2-3% of the coefficient of variance (%CV) to the experiment.

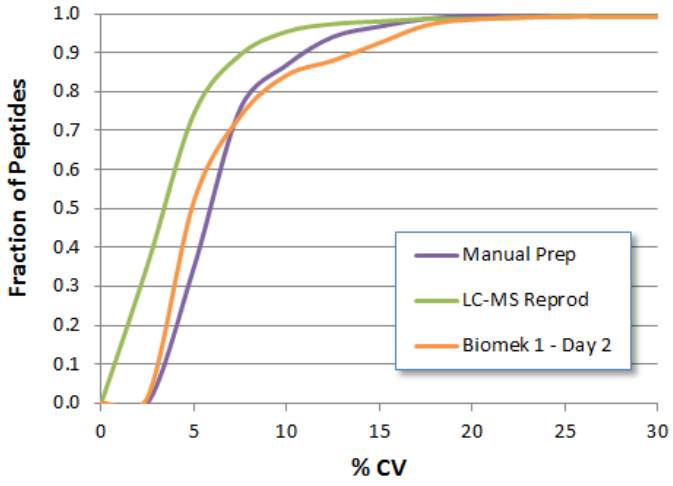

Figure 3. Cumulative %CV Plots to Assess Digestion Replicate Reproducibility. Manual sample preparation using kit provided very high digestion reproducibility on 24 digestion replicates (purple trace, 124 peptides) run by microLC on the QTRAP® 6500 System. Technical replicates of a single digestion were performed to assess LC-MS reproducibility (green trace). After optimization of the automation protocol, 32 digestion replicates were performed with the automation system (orange trace, 133 peptides) which provided equivalent reproducibility to a very careful manual preparation.

Automating the Digestion Protocol

Next, the protocol was adapted to the Biomek NX^{P} automation system. Effort was made to optimize the pipetting techniques to the various liquid types to ensure accurate delivery of reagents. On-deck shakers were used to ensure high quality mixing after each addition. Once all steps of the protocol were optimized, digestion replicates were again performed (24, 32 or 48 wells per automation run) and analyzed, this time using a *Scheduled* MRM™ Algorithm method on the QTRAP® 6500 system. The reproducibility across 32 wells is shown in Figure 3 (orange trace) and was found to be similar to a carefully performed manual preparation of the same protocol. A number of cysteine containing peptides were monitored to ensure the alkylation step was robust, similar reproducibility of cysteine peptides were seen as the non-modified peptides (data not shown).

A range of peptides were included in the MRM assay so the digestion protocol could be optimized for overall high performance. Many peptides to specific proteins showed very high reproducibility with % CVs ~ 2-3% across the 32 wells (Figure 4). Here, 3 peptides to Alpha-1-B glycoprotein (A1BG) were monitored and very high digestion reproducibility was observed (NGVAQEPVHLDSPAIK - 3.6%, HQFLLTGDTQGR – 2.6%, LLELTGPK – 3.5%).

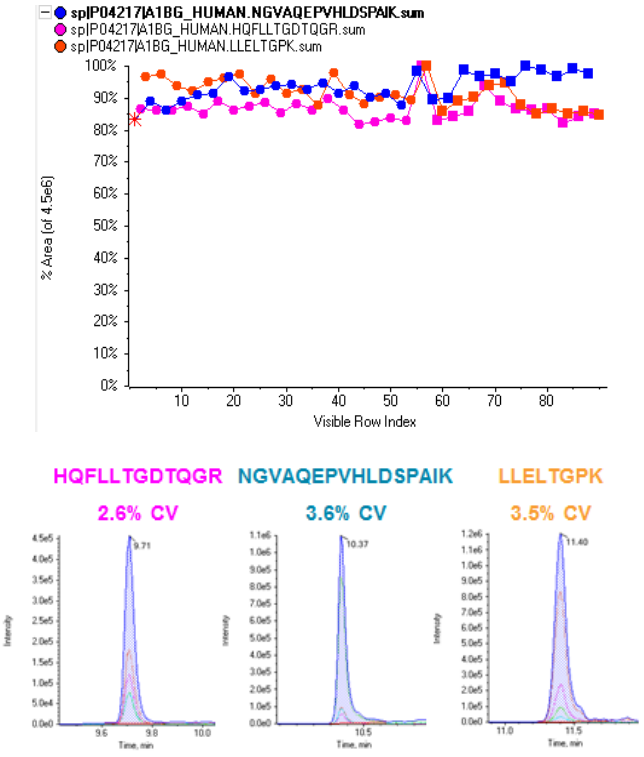

Figure 4. Digestion Reproducibility of Peptides from A1BG. Three peptides to Alpha-1-B glycoprotein were monitored across the 32 wells; top plot shows the relative areas. The % CV for each peptide was computed, and is displayed along with the typical peak shape in the bottom plot.

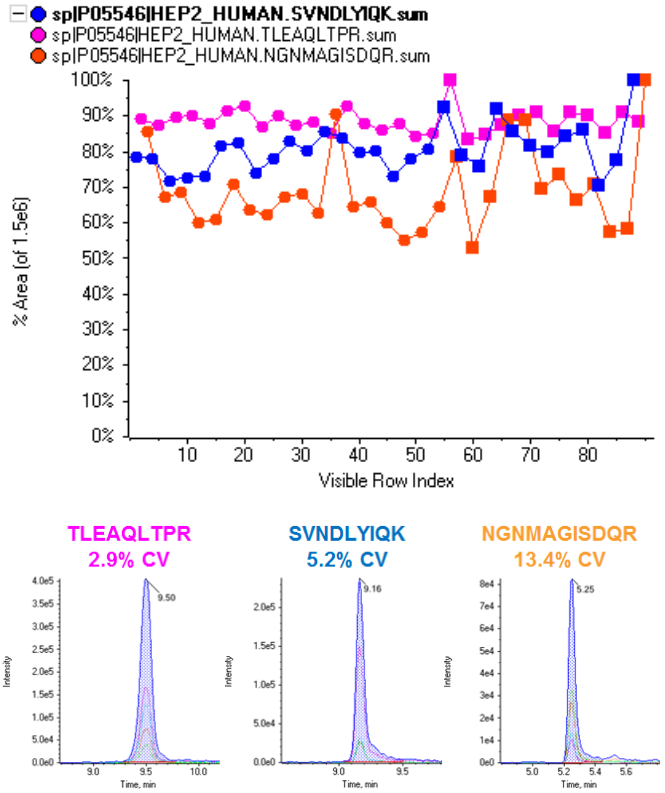

Figure 5. Assessing Digestion Variability of Peptides within a Single Protein. Three peptides to Heparin were monitored across the 32 wells; top plot shows the relative areas. The % CV for each peptide was computed, and is displayed along with the typical peak shape in the bottom plot. Analyzing reproducibility is good practice when choosing peptides to monitor in a quantitative MRM assay.

Digestion Reproducibility Assessment Important for Assay Development

Many of the peptides monitored across the digestion replicates had very high reproducibility (%CV <10%). But for some proteins it was noticed that some peptides had higher variability than other peptides from the same protein (Figure 5), in both the manual and automated digestion results. Digestion reproducibility assessment is an important step to perform when choosing key peptides to monitor from target proteins when developing an MRM assay. The example shown here in Figure 5 is multiple peptides to Heparin from one of the automation runs. Two peptides show quite good reproducibility (TLEAQLTPR – 2.9%, SVNDLYIQK – 5.2%), however 1 peptide shows higher variance (NGNMAGISDQR – 13.4%). Using automation to create digestion replicates simplifies this step of assay development and enables the selection of high performing peptides.

Transferability of Automation Method

The advantage of automation is that similar reproducibility can be expected on every sample, every day. It is not subject to the variability that can happen in manual preparation due to different researchers, same researcher on different days with different time pressures. Automation also makes the transfer of protocols between laboratories more reliable. To confirm these assertions, the same automation method was tested on two different Biomek automation systems located in two different laboratories. The method was repeated on multiple days to ensure that the same reproducibility can be obtained again and again (Figure 6). In lab 2, 24 digestion replicates were analyzed using high flow LC on a QTRAP® 6500 System, monitoring a set of 117 peptides. Very similar cumulative reproducibility curves were observed. In this study, more than 80% of peptides monitored had reproducibility better than 10% CV, proving that the automation method and protocol was very robust. This provides a high number of peptides per protein with very high reproducibility to select for quantitative monitoring in both SWATH® Acquisition studies or targeted MRM assays. Use of heavy labeled internal standards in an assay could further improve this reproducibility.

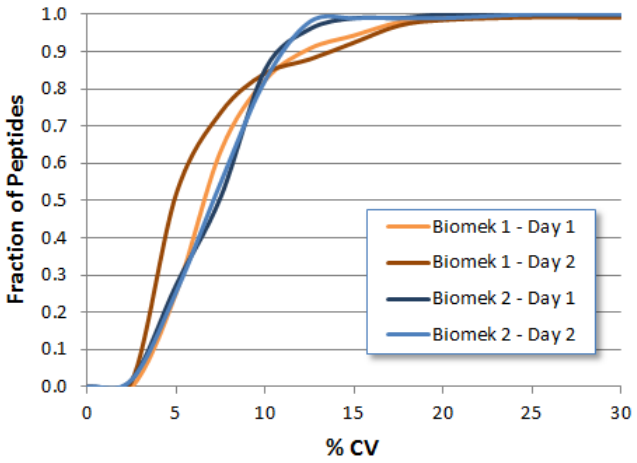

Figure 6. Inter-day and Inter-lab Reproducibility of Digestion. Same automation method was run in two different labs on similarly configured Biomek NX^P systems. Using the same sample and MRM assay run on two QTRAP 6500 systems, digestion reproducibility experiments were performed on multiple days. Assessing the peptide peak areas within each experiment across the 24 – 48 replicates, very similar reproducibility curves were obtained. More than 80% of peptides monitored had overall workflow reproducibility better than 10% CV in all 4 runs.

Summary

Automation of sample preparation is critical when performing large scale quantitative proteomics experiments. A sample preparation solution that is consistent on multiple days, even in multiple labs, will improve our ability to perform these important, more statistically powered studies. Reducing the variability of each step in the workflow is also important, enabling smaller biological changes to be quantified with more confidence, on small or large sample sets.

Here, an automation solution for the protein digestion portion of sample preparation has been developed to provide high reproducibility on small or large numbers of samples. For the plasma samples tested in this study, very high digestion reproducibility was obtained, with more than 80% of peptides monitored having reproducibility better than 10% CV (Figure 3). This reproducibility was then demonstrated on multiple automation workstations on multiple days (Figure 6). This work demonstrates a reproducible automated solution for protein digestion, useful for both smaller proteomic studies within a lab and also larger scale proteomics studies across labs.

References

1. For more information on the Biomek NX^P Span-8 liquid handling system, please see https://www.beckmancoulter.com/wsrportal/wsr/research-and-discovery/products-and-services/research-automation/index.htm
2. SCIEX Protein Digestion Automated Solution consists of the Biomek NX^P Span-8 liquid handling system along with the SCIEX Protein Preparation Kit (SCIEX P/N 4445247) and TPCK-treated trypsin (SCIEX P/N 4445250).

Document number: RUO-MKT-02-2364-A

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices