Automated High Throughput Trypsin Digests for Highly Reproducible Peptide Map Analysis

The Beckman Coulter Biomek NX^P Span-8 Workstation and the SCIEX TripleTOF® 6600 System for Routine Characterization of Biotherapeutics

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The extended capabilities of LC-MS characterization for biotherapeutics have put increasing strain on throughput and efficiency. Many processes still depend on manual sample handling, and proteolytic digestions that tie up valuable scientific resources. The added risk of variability in pipetting, digestion time, or the time each sample is exposed to reagents is high. Highly reproducible LC-MS measurements of biopharmaceuticals may therefore be at risk from processes that are not reproducible, independent of the analytical technique. LC-MS techniques are increasingly able to pick up small variations and may lead to undesired repeat analyses or additional QC procedures and costs. Automation of default standard workflows can address variability and improve analytical efficiency in the modern biopharmaceutical industry.

In this study, digestion replicates are performed on a standard monoclonal antibody to demonstrate the reproducibility of the workflow.

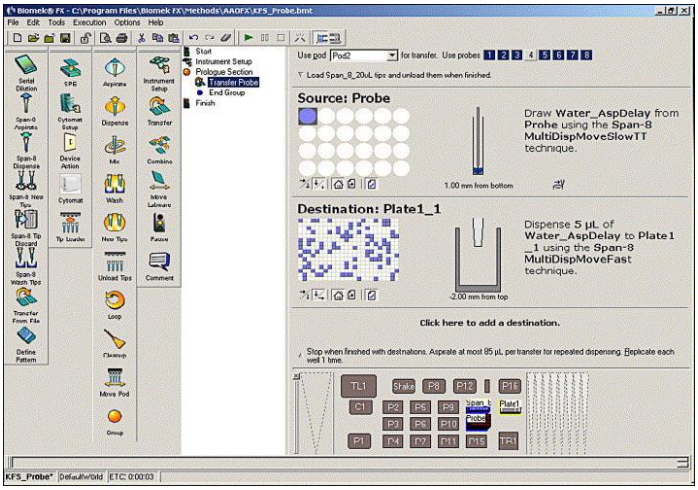

Figure 1: Biomek Method Provides Full Automation of the Protein Digestion Workflow. Biomek software uses graphical representations for easy method development / editing and for monitoring workflow progress.

Key Features of the SCIEX Protein Digestion Automated Solution

- Full solution for automated protein digestion of monoclonal antibodies (mAb) and other biotherapeutics
  - 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
- Automated and streamlines sample preparation to increase analytical throughput.
  - Digest from 8 to 96 samples automatically in a single automation run
- Increases efficiency and reduces time and money costs associated with human error in sample prep and pipetting measurements.

  BioPharmaView™ Software and MultiQuant™ Software for simplified data processing

Materials and Methods

Sample Preparation: A Biomek NX^P Span-8 Laboratory Automation Workstation (Beckman Coulter)[1] was used to perform rapid automatic trypsin digestion of 16 sample replicates of a representative monoclonal antibody (mAb). The Biomek was programmed to use N-octyl-glucoside (OGS) as a denaturant, tris-(2-caroxyethyl)-phosphine (TCEP) as a reducing agent, and methyl methane-thiosulfonate (MMTS) as the alkylating reagent. All reagents were from the SCIEX Protein Preparation Kit[2] and were used according to the instructions in the kit. A Peltier-heater shaker ALP was used primarily for shaking incubations at different temperatures.

Chromatography: Samples were analyzed using an ExionLC™ System (SCIEX) and a CSH c18 column (Waters, 130Å, 1.7 µm, 2.1 mm X 100 mm). Elution gradients of 5-35% B at 250 µL/min in 30 min were run with the column at 60 ºC. Solvent A was 0.1% formic acid; solvent B was acetonitrile with 0.1% formic acid.

Mass Spectrometry: All 16 mAb digests were analyzed using a TripleTOF® 6600 system. An information dependent acquisition (IDA) LC-MS/MS method was used consisting of a high resolution TOF MS survey scan followed by 20 MS/MS scans with a minimum accumulation time of 50 msec.

Data Processing: IDA data were searched using BioPharmaView™ Software using the sequence of the mAb, digested in-silico with trypsin and searched with 0-4 deamidations and 0-4 oxidized methionines per chain, and with G0, G1, G2 ± fucose assigned to the correct amino acid in the heavy chain. Quantitation was performed using MultiQuant™ Software on a set of high, medium, and low abundance peptides.

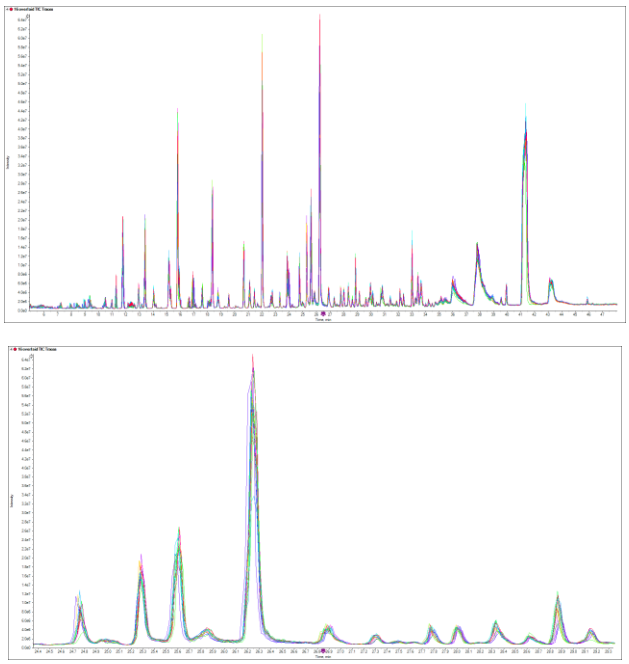

Figure 2: Chromatographic Overlay of 16 Replicates Digestions. Overlays of the Total Ion Chromatograms (TIC, top) and the expanded time region between 24.4 and 29.3 minutes (bottom) are shown to highlight the high reproducibility of the digestions.

Figure 3: Rapid Data Review of Digestions using BioPharmaView™ Software. Results for the digestion of SiluLight mAb is shown, highlighting the TIC of the resulting peptides, an Extracted Ion Chromatogram (XIC) of a selected peptide as well as the MS and MS/MS spectra.

Figure 4: BioPharmaView Software Results for mAb Peptide Mapping Experiment. The protein sequence coverage and XIC's for each of the matched peptides are shown, as well as the MS and MS/MS evidence.

Digestion Reproducibility

The reproducibility of the trypsin digestion was assessed visually with the chromatograms, as well as qualitatively and quantitatively using BioPharmaView™ and MultiQuant™ Software. The top of Figure 2 shows the chromatographic overlay of the 16 mAb digests, while the bottom shows an expanded region between 24 and 29 minutes. As shown in the figure, very good digestion as well as chromatographic reproducibility was obtained with only a small variation observed throughout the entire run.

Peptide Mapping Reproducibility

BioPharmaView Software was used to map the peptides to the mAb sequence. Sequence coverage was very high for all 16 runs. Figure 3 shows the Peptide Map and Extracted Ion Chromatogram (XIC) analysis for the doubly-charged ion eluting at 34.7 minutes with m/z 698.3857. Also shown are the TOF MS and MS/MS spectra for this ion. The middle pane shows the peptide sequence match for this peptide with 0.7 ppm mass accuracy. The time estimate for performing this analysis compared to a manual handling process and manual peptide mapping is approximately 8 person-weeks (compared to 2 days).

Table 1. Reproducibility of Peptide Mapping Results Across 16 Digest Replicates.

Digestion Replicate	% Sequence Coverage Light Chain	% Sequence Coverage Heavy Chain
1	*100*	98.9
2	*100*	99.3
3	*100*	100
4	*97.8*	96.8
5	*100*	99.1
6	*100*	96.8
7	*100*	97.8
8	*100*	96.7
9	*97.3*	96.8
10	*96.8*	98.0
11	*100*	100
12	*100*	95.8
13	*97.3*	94
14	*100*	97.6
15	*100*	99.3
16	*100*	96.4

The sequence coverage data for each of the 16 replicate digests is shown in Table 1. As shown in the table, highly reproducible coverage maps are obtained from all 16 replicates. Data for all different types of peptides were highly similar. The small variation in Heavy chain sequence coverage was entirely attributable to one dipeptide and one tetramer peptide with m/z that were too low to be detected by the method (not within the scan range), but were sometimes detected as a low-level missed cleavage peptide.

The digest reproducibility was also assessed quantitatively using MultiQuant™ Software. In this case, it was the peak area of the XIC from three different peptides across all 16 digest replicates (Table 2). These three peptides were chosen as they represent high abundance (Row 1), medium abundance (Row 2, chosen from the middle of the list of all peptides sorted by XIC area), and a low abundance peptide (Row 3). The raw XIC data from all 16 replicates of the low abundance peptide is shown in Figure 5.

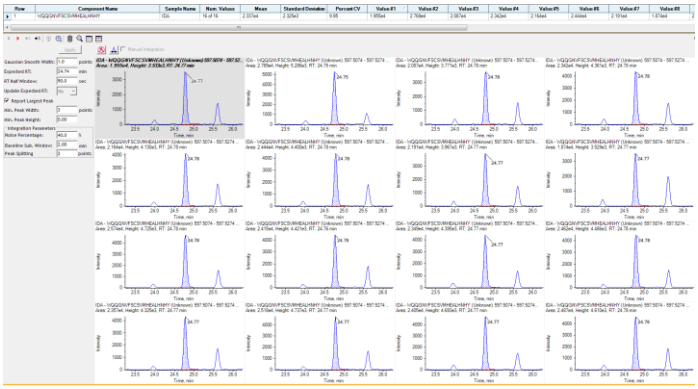

Figure 5: Good Reproducibility was Achieved on Even the Low Abundance Peptides. XIC data for all 16 replicates is displayed for a low abundant peptide. A %CV of 9.95 was obtained for the 16 peak areas.

Conclusions

The automated digestion protocol has been demonstrated here to be highly reproducible, reducing the error associated with human error in sample prep and pipetting measurements.

Qualitatively each of 16 digests had 96.8% or higher coverage of the light chain and 94% or higher coverage of the heavy chain with slight variation in detection of very small peptides that were below the acquisition m/z range in the mass spectrometry IDA method. Quantitatively the chromatographic peak areas from low, medium and high abundance peptides were within 10% CV for the entire workflow from digestion through data processing.

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 Preparation Kit (SCIEX P/N 4445247) and TPCK-treated trypsin (SCIEX P/N 4445250).

Table 2: MultiQuant™ Software Analysis of 16 Digest Replicates for Three Example Peptides.

Component Name	# of Values	Mean	Standard Deviation	% CV
ALPAPIEK	*16*	*1.67 E7*	*1.17 E6*	*6.97*
WQQGNVFSCSVMHEALHNHY	*16*	*1.19 E6*	*2.33 E3*	*9.95*
YVDGVEVHNAK	*16*	*1.06 E5*	*7.73 E3*	*6.37*

AB Sciex is doing business as SCIEX.

Document number: RUO-MKT-02-6976-C

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