Rapid, Automated High-Throughput Trypsin Digest for Highly Reproducible Peptide Maps

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INTRODUCTION

The extended capabilities of LC/MS characterization for biotherapeutics has put increasing strain on throughput and efficiency. Many processes still depend on manual sample handling, such as proteolytic digestion, leading to bottlenecks that tie up valuable scientific resources. The added risk of variability is high from the inherent error in pipetting, digestion time, or amount of time each sample is exposed to reduction and alkylation reagents. Highly reproducible LCMS measurements of biopharmaceuticals may therefore be at risk from processes that are not reproducible. This is especially true of LCMS techniques which are increasingly able to pick up minute variations and may lead to undesired repeat analyses or additional QC procedures and costs. This work examines the automation of default workflows to address variability and improve analytical efficiency in the modern biopharmaceutical industry.

Figure 1. Beckman Biomek NXP Span-8 and AB Sciex TripleTOF® 6600 Mass Spectrometer

MATERIALS AND METHODS

Sample Preparation: In this study we programmed a Biomek NXP Span-8 Laboratory Automation Workstation (Beckman Coulter) operated by Biomek Software to perform rapid automatic trypsin digestion of 16 sample replicates of a representative monoclonal antibody (mAb). The Biomek was programmed to perform the Flash Digest (Perfinity Biosciences) in the Peltier-heater shaker ALP according to the instructions from the kit. All steps of the digest were performed in the Biomek except the final step: a laboratory microfuge was used to pellet the trypsin beads. **[NB: The method Is transferrable: inquire with the author if you'd like a copy of the file].**

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Chromatography: Samples were analyzed using an Agilent 1290 UPLC System and a BEH 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 55 ºC. Solvent A is 0.1% formic acid; solvent B is acetonitrile with 0.1% formic acid.

Mass Spectrometry: All 16 mAb digests were analyzed using a TripleTOF® 6600 system. An information dependent acquisition (IDA) LCMS/MS method was used for peptide identification. This IDA method consisted of a high resolution TOF MS survey scan followed by 20 MS/MS in a second with a minimum accumulation time of 50 msec.

Data Processing: IDA data were searched using BioPharmaView™ Software against the sequence of the antibody. Quantitative analysis was performed using MultiQuant™ software on a selection of high, medium and low abundance peptides...

BioMek Software Leverages Graphical Representations of Labware

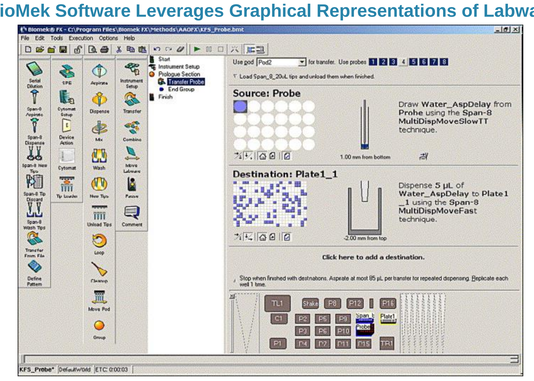


Figure 2. BioMek Software. *Using drag and drop virtual labware and modular methods, a digest protocol can be conceived and executed virtually before committing reagents and labware to the experiment.*

Total Ion Chromatograms Overlaid From 16 technical replicates

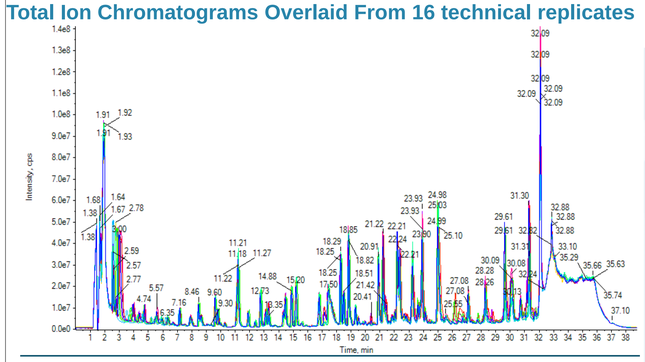


Figure 3. SWATH™ Scan Obtains MSMS data on all ions. *By stepping the mass range in 20 or 25 Da increments, fragment ion chromatograms of all observed ions are observed.*

Total Ion Chromatograms From 16 Technical Replicates: Zoom-in

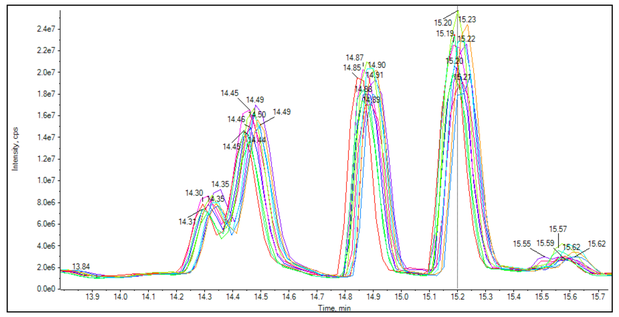


Figure 4. Zoom-In to see overlay detail. *Reproducible relative abundance from 16 technical replicates showing a miniscule variation over a 2-minute segment of the Total Ion Chromatogram (TIC).*

Peptide Map Replicates Analyzed With BioPharmaView™ 1.0 Software

Figure 5. Peptide Map and Extracted Ion Chromatogram (XIC) Analyses. *The Sequence of The mAb was digested in-silico with trypsin and searched with 0-3 deamidations per chain, 0-3 Oxidized methionines per chain, and with G0, G1, G2, as well as their fucosylated counterparts assigned to the correct amino acid in the heavy chain. The cysteine alkylating agent was MMTS. Sequence coverage was very high for all 16 runs. The individual light and heavy chain sequence coverage for all 16 runs is featured in Table 1.*

BioPharmaView™ Software Sequence Coverage View

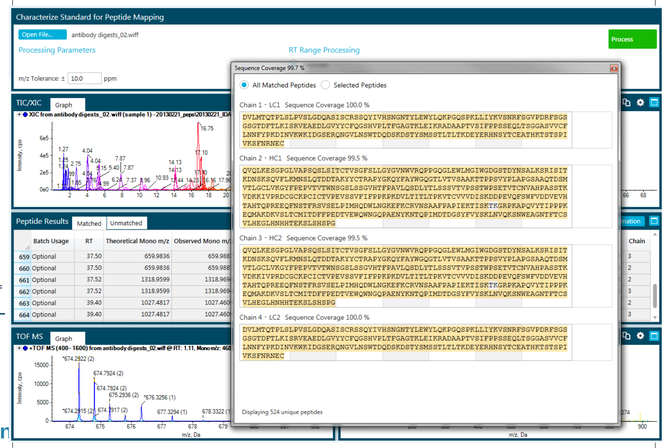


Figure 6. Peptide Map and XIC Analyses. *In this view we have clicked the "View Sequence" button seen in the software screen shot in figure 5. NB: the chromatogram section behind the sequence coverage, the mode has been switched from TIC view to show XICs of all, individual, matched peptides. The sequence coverage data for each of the 16 digests is organized into Table 1. 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)*

Peptide Mapping Qualitative Reproducibility

	Light Chain		Heavy Chain
#	% Coverage	#	% Coverage
1	100	1	100
2	100	2	99.1
3	100	3	100
4	100	4	100
5	100	5	99.1
6	100	6	98.9
7	100	7	99.6
8	100	8	98.4
9	100	9	99.6
10	100	10	98.4
11	100	11	98.4
12	100	12	98.4
13	100	13	98.4
14	100	14	99.6
15	100	15	98.4
16	100	16	100

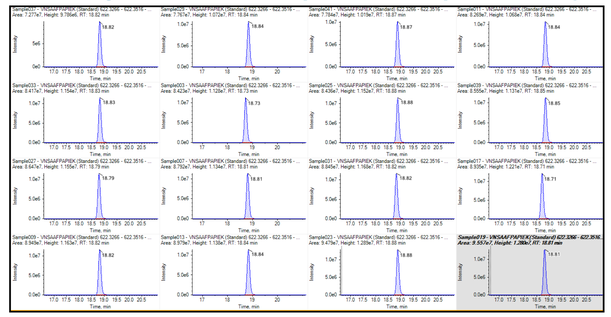
Table 1. Peptide Map Qualitative Data for all 16 replicates. *Highly reproducible coverage maps from all 16 replicates. Data for all different types of peptides was highly similar. The small variation in Heavy chain sequence coverage was entirely attributable to one dipeptide and one tetramer whose m/z were too low to be detected by the method, but were sometimes detected as a low-level missed cleavage peptide.*

MultiQuant™ Software Analysis of 16 Digest Replicates.

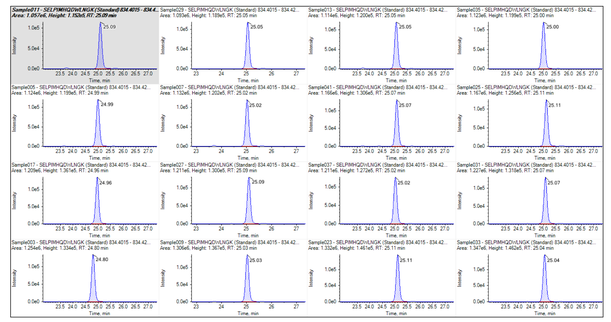
Row	Component Name	Num. of Values	Mean	Standard Deviation	Percent CV
1	VNSAAFPAPIEK	16	8.57E+07	6.04E+06	7.04
2	PIMHQDWLNGK	16	3.14E+05	2.87E+04	9.14
3	SELPIMHQDWLNGK	16	1.19E+06	8.61E+04	7.22

Table 2. MultiQuant™ Software Analysis. *For any type of MS based chromatographic quantitation, MultiQuant™ Software will automatically integrate, calculate peak areas, Mean, Standard Deviation and Percent CV for any selected group. The Software can also be validated for 21CFR11 compliance. In this case, it was the peak area of the XIC from each of the above peptides across all 16 digest replicates. These three peptides were chosen as they represent high abundance (Row 1), medium abundance (Row 3, chosen from the middle of the list of all peptides sorted by XIC area), and a low abundance peptide (Row 2). The raw XIC data from all 16 replicates of each of the three peptides is shown in Figure 7.*

High Abundance Peptide XIC Reproducibility



Medium Abundance Peptide XIC Reproducibility



Low Abundance Peptide XIC Reproducibility

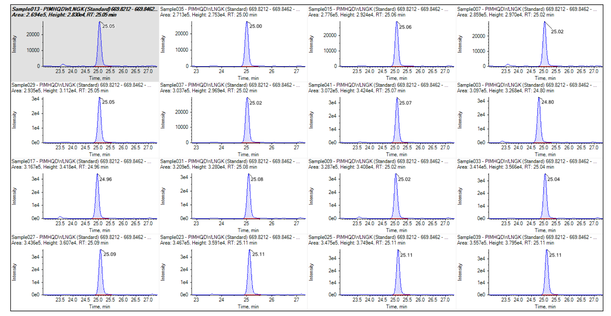


Figure 7. MultiQuant™ Software Analysis. *The raw XIC data from all 16 digest replicates of each of three peptides is shown. MultiQuant is able to calculate the peak areas automatically.*

RESULTS

Qualitatively each of the 16 digests had 100% coverage of the light chain and 98.4% or higher coverage of the heavy chain with very slight variation in detection of very small peptides that were below the observed *m/z* range in the mass spectrometry IDA method. Quantitatively: chromatographic peak area from extracted ion chromatograms of low, medium and High abundance peptides were all within 10% CV.

CONCLUSIONS

Automated digestion and data analysis with the toolkit shown provide robust and reproducible relative intensity results to benefit an organization in a number of ways:

- Streamlines peptide mapping sample preparation.
- Reduce time and money costs associated with human error in sample prep and pipetting measurements.
- Automated sample prep increases your analytical throughput.
- Increase scientist efficiency and reduce costs and errors associated with manual pipetting.
- Saves time for scientists to do science.