Automating Biologics Signature Peptide Quantitation

SCIEX BioBA Solution Using the Biomek FX^P Laboratory Automated Workstation

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Key Challenges in Biologics Quantitation

- ELISA assays have a limited linear dynamic range and lack selectivity in some cases
- Signature peptide MRM quantitation offers high sensitivity, linear dynamic range and specificity but sample preparation is a multi-step process
- Several pipetting steps and incubations in the process make sample preparation a labor intensive process
- Achieving consistent results within a batch and between batches

Key Features of the SCIEX Automated BioBA Solution

- Complete solution for automating biologics immuno-affinity sample preparation (Figure 1)
  - BioBA kits provide all the reagents necessary from high capacity streptavidin beads to digestion enzyme
  - Biomek FX^P Workstation automates the entire process
- Process 96 samples at once
- Highly reproducible, a monoclonal antibody sample was isolated from plasma and digested with an overall %CV <10%
- Easy-to-use software interface for routine operation

Figure 1. Biomek FX^P Workstation, BioBA reagents kit and SCIEX 6500 QTRAP system.

Introduction

Protein based biotherapeutics are a growing component of pharmaceutical companies' drug pipelines. In order to support this growing class of new drug molecules, robust and reliable bioanalytical methods are required. The signature peptide approach is the most commonly used LCMS based strategy for protein quantitation due to its high sensitivity and specificity. When this strategy is combined with immuno-affinity sample preparation to concentrate the target analyte and reduce the matrix background, the sensitivity and selectivity of the technique is greatly expanded. There are, however, several steps in immuno-affinity sample preparation plus several incubation wait times that consume an analyst's valuable time. This creates a new bottleneck in sample preparation compared to traditional small molecule workflows. Added to this challenge is the fact that reproducible sample preparation is critical for delivering high quality pre-clinical and phase I-IV study results.

Magnetic beads offer several advantages for immuno-affinity workflows including: ease of handling, scalability, improved sample recovery, parallel processing of samples using a variety of magnetic stands and use in high-throughput formats with robotics. Using automation reduces the variability of multi-step sample preparations within a batch and between day-to-day preparations. It also reduces the labor required to process sample batches and liberates scientists to do other work required to deliver study results. In this note we demonstrate successful method transfer of the BioBA sample preparation protocol to the Biomek FX^P Workstation and show its ability to deliver robust results from real study samples.

Methods

Dosing Study

Four male Sprague-Dawley rats were given a sub-cutaneous dose of rituximab at 10 mg/kg and blood samples were collected at: predose, 0.5, 2, 6, 24 hr, 2, 3, 6, 8, 10, 14, 17, 21, 24 and 28 days and kept frozen. The samples were analyzed by QPS using a previously validated ELISA assay in the range of 100 to 10,000 ng/mL and samples were pre-diluted five- or ten-fold prior to analysis. The remainder of the samples was shipped to SCIEX in Concord for analysis by immuno-affinity LCMS.

Calibration standards were prepared in the range of 100 to 100,000 ng/mL and QC samples at 300, 3250 and 75,000 ng/mL. Samples from rats 1,2: Day 2, rat 3: 0.5 hr, Day 2,14, rat 4: 0.5 hr, Day 2,14 were diluted five-fold with blank rat plasma prior to analysis due to low sample volume. Twenty-five microliters of each standard, QC and study sample was then processed following the procedure outlined below using SILuMab (Sigma-Aldrich) internal standard, 1.0 µg/mL.

Automated Sample Preparation

The SCIEX BioBA sample preparation protocol shown in Figure 2 was automated for 96 samples on the Biomek FX^P Workstation. The Biomek FX^P Workstation is set up with a Peltier heater with deep well plate adapter, a MagnaBot® 96 Magnetic Separation Device and an orbital shaker. Pipetting techniques were optimized for all transfer steps to ensure accurate delivery of all reagents.

Figure 2. The SCIEX BioBA Automated Workflow and Biomek FX^P Method Launcher workspace.

The protocol is divided into two workflows: capture and digestion. The software contains a dashboard workspace (Figure 2) where one of the two workflows is selected. When a method is launched the Guided Labware Setup (Figure 3) takes the user through the setting up of the deck step by step to ensure no labware is misplaced. The Guided Labware Setup also informs the user of the reagent volumes required for the batch. Once a method is launched the Milestone View informs the user of the current status of the labware as it moves through the workflow as well as the overall progress and time remaining for the entire method (Figure 3). When the Biomek Workstation is networked, this information can be viewed in a standard web browser, thereby allowing remote monitoring of the system.

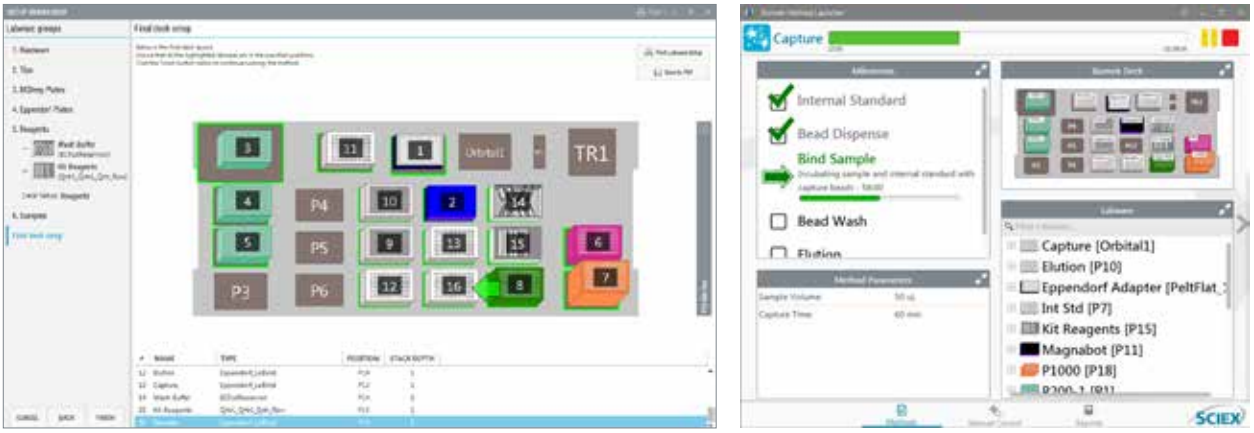

Figure 3. The Guided Labware Setup and Milestone View of the Biomek Method Launcher software for the BioBA solution.

In the capture workflow the deck of the Biomek FX^P Workstation is loaded with streptavidin magnetic beads conjugated with capture antibody, isotopically-labeled internal standard, bind/wash buffer, elution buffer and neutralization buffer. A sample plate containing 25 µL to 200 µL calibration standards, QC samples, blank and double blank controls and subject samples is first prepared and placed on the deck of the Biomek FX^P Workstation. First, the Biomek FX^P Workstation transfers 2X sample volume of internal standard (1.0 µg/mL SILuMAB) to the sample plate. 25 µL of the beads are added to the capture plate and the sample plus internal standard is transferred to the beads. After incubation for one hour the sample supernatant is removed from the beads and transferred to a storage plate. The beads are then rinsed three times with buffer. After washing is complete the beads are then incubated with 50 µL of elution buffer (0.1% TFA) for ten minutes. After elution is complete the acidic supernatant is transferred to a clean elution plate and neutralization buffer (500 mM ammonium bicarbonate) is added. The analyte in the elution plate is now ready for digestion.

At the end of the capture workflow the deck of the Biomek FX^P Workstation is then cleared of the capture reagents and digestion labware are placed on the deck. Digestion reagents (TCEP, IAM, anionic surfactant and trypsin/lys-C) are placed in 2 mL sample tubes and formic acid and water are placed in divided reservoirs. The digest reagents are stamped out into 96 v-bottom well plates using the Span-8 head and the 96-channel head is used to deliver the required volume to the elution plate. The digestion reagents are not stamped out until required in 'just-in-time' delivery fashion to minimize evaporation loss of the small volume reagents. Performing reagent delivery in this way and taking advantage of both the 96-channel and Span-8 heads also minimizes the amount of dead time in the method and synchronizes reagent addition across the 96 samples. The automated digestion workflow begins with addition of the reducing reagent (100 mM TCEP) and the elution plate is heated at 50°C for one hour. Next the alkylation reagent (100 mM IAM) is added to the elution plate and mixed for 30 minutes at room temperature. Next the anionic mass spec compatible surfactant is added followed by trypsin/lys-C and the elution plate is incubated for three hours at 37°C. At the end of the digestion 3 µL of formic acid is added to the elution plate to stop digestion. Lastly, 50 µL of water is added to the digested samples and 75 µL of the diluted samples are transferred to a clean 96-well LC injection plate.

Chromatography

Separation of the signature peptides of the digested samples was performed on a Shimadzu LC-20 system consisting of the following components: CBM-20A system controller, LC-20AD isocratic pumps (2), SIL-20AC autosampler, CTO-20AC column oven (50°C) using a Phenomenex 2.6 µm, Kinetex C18 Column, (50 x 2.1 mm). A short gradient was used (Table 1) and 5 µL of sample was injected onto the column.

Table 1. LC Conditions

Step	Total Time (min)	%B*	Flow Rate (µL/min)
1	0.00	10	400
2	4.00	40	400
3	4.25	95	400
4	5.50	95	400
5	5.60	10	400
6	6.30	10	400

Mobile Phase A: 0.1% formic acid in water (v/v)
*Mobile Phase B: 0.1% formic acid in acetonitrile (v/v)

Mass Spectrometry

The MRM analysis was performed on a SCIEX QTRAP 6500® system equipped with an IonDrive™ Turbo V source. The following source/gas parameters were used, IS 5500, CUR 25 psi, TEM 500 °C, GS1 85 psi, GS2 80 psi and CAD High. Table 2 lists the analyte MRM parameters used for signature peptide quant using a conserved signature peptide from the Fc region of rituximab and SIGMAMAB.

Table 2. MRM parameters

Q1	Q3	Dwell	DP	CE	CXP	Retention Time (min)	Peptide
560.1	708.8	25	60	22	28	2.1	Sig Peptide 1_1
560.1	615.7	25	60	23	15	2.1	Sig Peptide 1_2
562.9	713.3	25	50	23	28	2.1	Heavy Sig Peptide 1_1

Data Processing

After acquisition, data was imported into MultiQuant™ software for peak integration, calibration and calculation of unknown sample and QC concentrations.

Results

To test the reproducibility of the digestion protocol a plate was prepared containing samples of SILuLite (Sigma-Aldrich) antibody standard plus SILuMAB internal standard (3.6 µg/mL) in 50 mM ammonium bicarbonate buffer. Columns 1, 4, 6, 9 and 11 contained SILuLite at 540 ng/mL, columns 2, 5, 7, 10 and 12 contained SILuLite at 180 µg/mL and columns 3 and 8 contained blank buffer. Three universal signature peptide peak area ratios were monitored for reproducibility. The data in Figure 4 represent the DTLMISR universal signature peptide. The %CV across 40 wells of the 540 ng/mL sample was 5.9% and 4.4% for the 180 µg/mL sample. Two other universal peptide MRM area ratios were monitored (data not shown) and the %CVs were 8.2% and 5.4% for the low concentration sample and 5.2% and 4.1% for the high concentration sample. There was no signature peptide MRM response from the blank samples in columns 3 and 8 in the middle of the plate indicating there was no cross contamination of sample during the liquid handling steps.

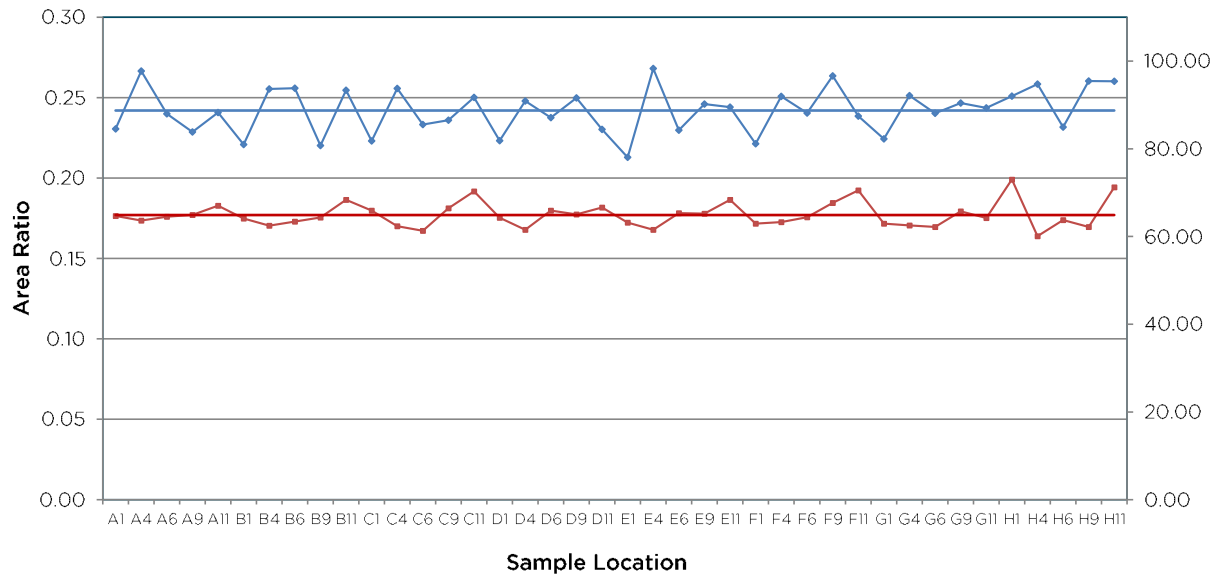

Figure 4. Digestion reproducibility as measured (peak area ratio of DTLMISR/DTLMISR*) from a neat 180 µg/mL sample (red) and neat 540 ng/mL sample (blue).

With the reproducibility of the digestion protocol established we moved to test the reproducibility of the entire workflow. A large QC sample of rituximab 3.25 µg/mL was prepared in rat plasma and 50 µL was aliquoted in 94 wells of a 96-well plate. The data in figure 5 represent the peak area ratio of a universal peptide and its heavy labelled internal standard. The %CV across all wells of the extracted plasma sample was 8.7%.

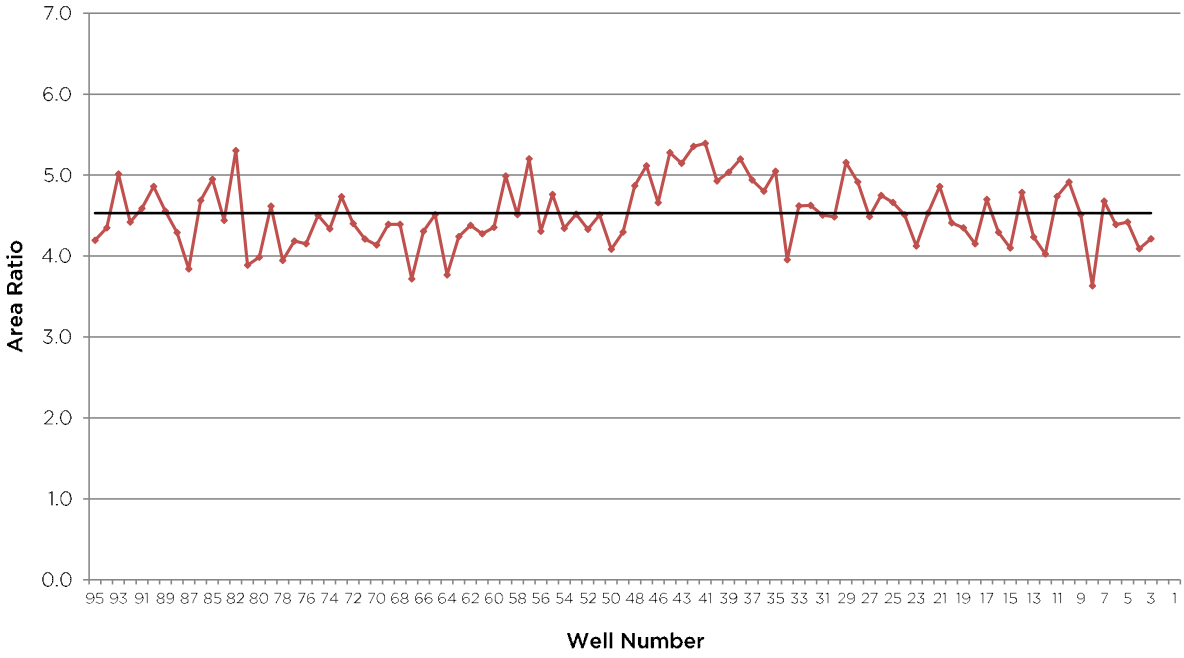

Figure 5. Reproducibility of the automated BioBA protocol as measured from the signature peptide peak area ratio of a single 3.25 µg/mL rituximab plasma sample aliquoted to 94 wells of a 96-deep well plate.

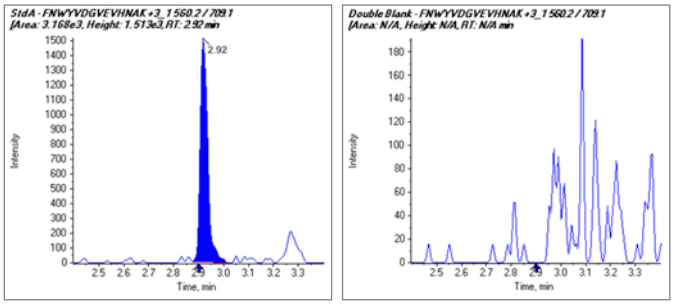

Figure 6. Example chromatograms of the LLOQ standard and double blank sample processed using the automated BioBA protocol.

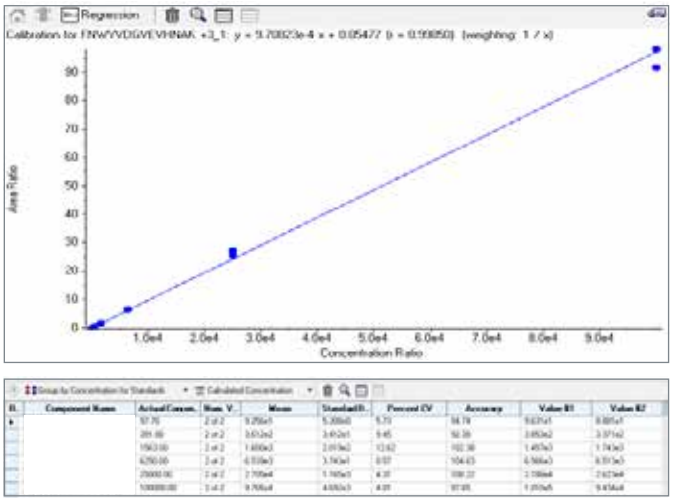

Figure 7. The calibration curve and calibration curve statistics from the rat study sample batch processed by automation.

Finally, to demonstrate the power and utility of the automated protocol, the validity of the method was tested on real study samples, not just QC samples. A study was commissioned with QPS to dose animals with rituximab. Samples were first analyzed by QPS using an ELISA method with a range of 100 to 10,000 ng/mL and study samples were pre-diluted prior to analysis. After analysis by ELISA, samples were shipped to SCIEX for analysis using a universal signature peptide MRM.

Figure 6 shows the peaks from the LLOQ standard and double blank sample. The signal to noise ratio was ~95, indicating excellent sensitivity. Figure 7 shows the calibration curve and statistics from the sample batch. Curve values ranged in accuracy from 92.4-108% of expected values and %CVs ranging from 0.8-12.6%. The calibration curve showed excellent linearity as evidenced by an r value of 0.9985. Figure 8 shows the average sample concentration (4 rats) at each time point as measured using both techniques. The data from the rat dosing study show excellent agreement (<15%) between the two analytical techniques and shows that the immuno-affinity LCMS assay provides equivalent results to the ELISA in this case while the IA-LCMS assay had the advantage of a wider linear dynamic range and required no sample pre-dilution. Although not explored in this study the LCMS method can be used to do simultaneous quantitation of antibody catabolism from the same sample set.

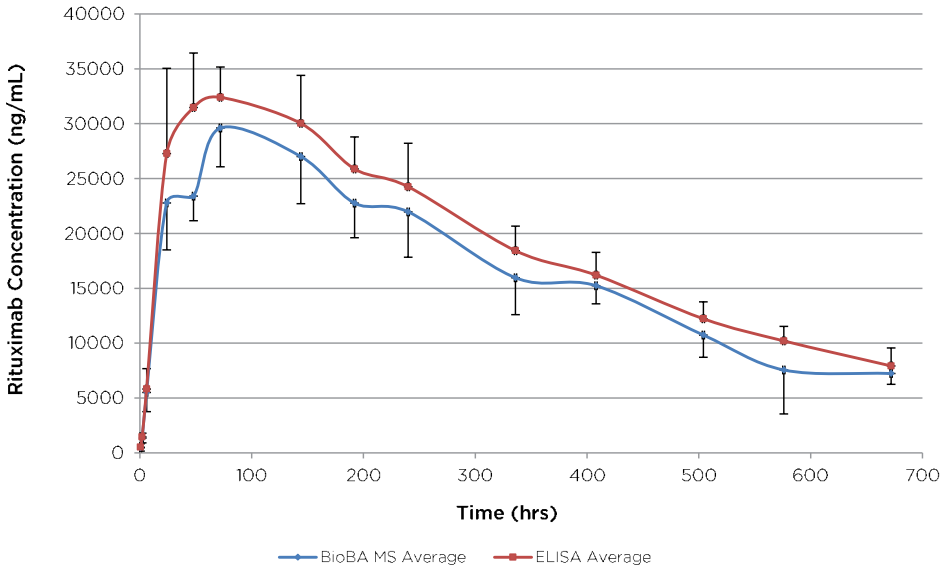

Figure 8. Average rituximab sample concentration (4 rats) as measured by an ELISA assay (QPS) and the BioBA automated sample processing protocol.

Summary

Fully automated sample preparation is critical to reducing bottlenecks in immuno-affinity sample preparation workflows for signature peptide quantitation. Not only does automation improve the consistency of multi-step protocols by reducing the day-to-day or user-to-user variability of sample preparation, but it enables scientists to focus on the critical aspects of study design and analysis.

Here, a fully automated solution has been developed for immuno-affinity sample preparation and signature peptide quantification and been successfully demonstrated for a monoclonal antibody therapeutic in real dose samples. Excellent sensitivity, accuracy and precision were achieved by the assay. The results of real dose samples analyzed by two different techniques, ELISA and immuno-affinity LCMS analysis, agreed to within 15% which demonstrates the automated BioBA solution to be a robust and accurate solution for mAb quantitation. The BioBA solution including the Biomek FX^{P} automated protocol and ready to use consumables from BioBA kits will increase productivity and accelerate biologics bioanalysis.

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