



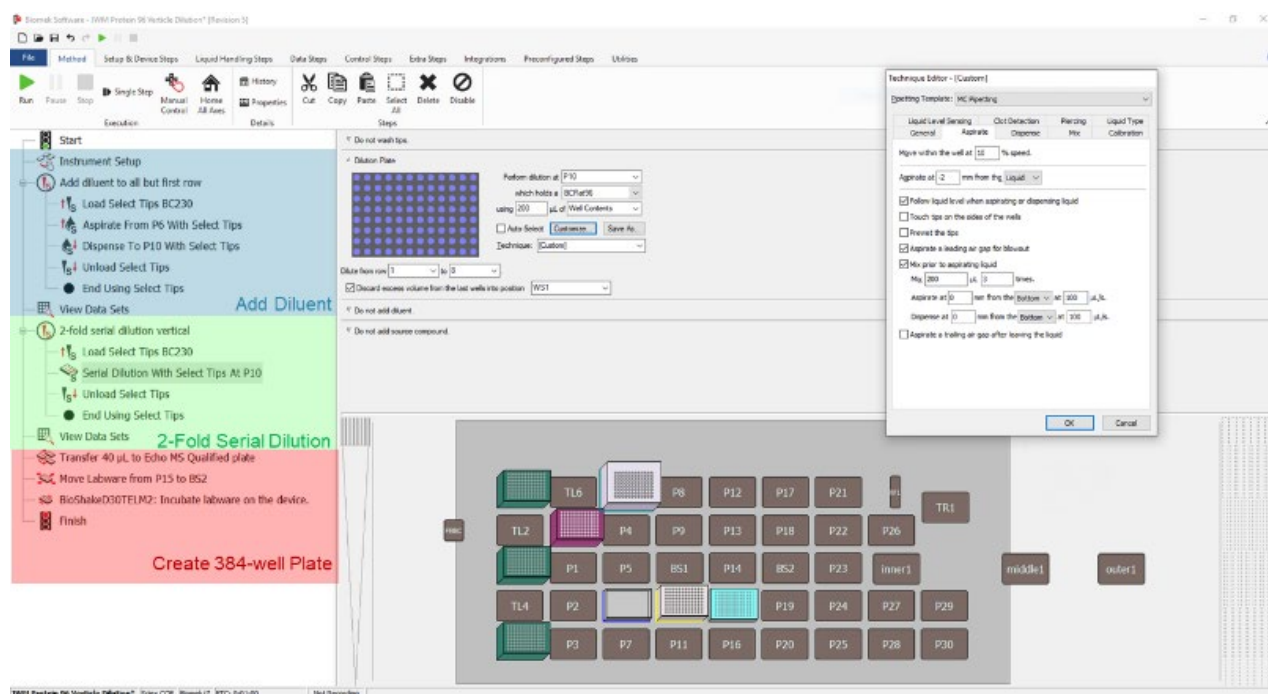
## Introduction

Proteins play a key role in almost every biochemical process. Analyzing intact protein molecules via traditional mass spectrometry enables characterization of these proteins. For example, accurate molecular weight determination is crucial for assessing protein expression and detecting unexpected modifications during development. The need for high-throughput intact protein analysis has increased in the pharmaceutical and biopharmaceutical industries.<sup>3</sup> The most common way to analyze these proteins is by peptide mapping, or a “bottom-up” experiment, in which a protein is digested with an enzyme [such as trypsin] and the resulting peptides are analyzed by liquid chromatography-mass spectrometry [LC-MS] to confirm the sequence and determine site-specific post-translational modifications. Throughput currently limits peptide mapping LC-MS analysis, as minutes or hours are needed to achieve the necessary characterization and extensive sample preparation before analysis by bottom-up applications. An alternative approach is to analyze the intact mass of the protein first and compare the data against a known sequence, allowing

faster screening and shorter analysis time while preserving high sensitivity and mass accuracy.

## Methods

**Sample preparation:** Protein standards for myoglobin,  $\beta$ -lactoglobulin A and B [BLG A and BLG B, respectively],  $\alpha$  and  $\beta$  casein, chymotrypsin,  $\alpha$ -chymotrypsinogen A and bovine serum albumin were purchased from Sigma Aldrich. Proteins were diluted at 1 mg/mL concentration in 70:30 (v/v), ACN/H<sub>2</sub>O with 0.1% formic acid. Protein stocks were diluted to 20  $\mu$ M before further dilution with the Biomek i7 liquid handler. Assay plates were prepared using the Biomek i7 Automated Workstation from Beckman Coulter® Life Sciences. Using selective tip pipetting, a single row of 12 tips was loaded on the 96-multichannel pipetting head to execute a 2-fold serial dilution vertically down the plate to obtain an 8-point concentration. The samples were then stamped into each quadrant of a 384-well plate [Figure 2B]. Before the analysis on the prototype system, 50  $\mu$ L of the sample was transferred to a well of an Echo® MS+ system-qualified 384-well polypropylene [PP] microtiter plate. The plate was centrifuged at 1,530  $\times$  g for 2 minutes.



**Figure 2:** Sample preparation for replicate analysis is done using the Biomek i7 automated workstation. Briefly, protein samples were 2-fold serially diluted vertically down the plate.

**Acoustic ejection:** Table 1 summarizes the acoustic ejection method conditions.

**Table 1. Acoustic ejection method conditions.**

Parameter	Value
Carrier solvent	70:30 ACN/H <sub>2</sub> O with 0.1% formic acid
Carrier solvent flow rate	400 $\mu$ L/min
Fluid class	AQ
Ejection volume	100 nL
Rep rate	varied
Interval	varied

**Mass spectrometry:** The ZenoTOF 7600+ system was operated using the TOF MS scan with the following parameters as displayed in Table 1.

**Table 2. ZenoTOF 7600+ system conditions.**

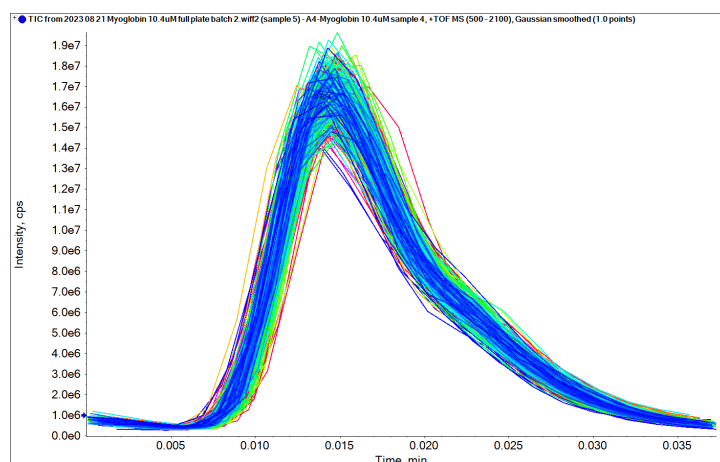
Parameter	Value
Polarity	Positive
Ion source gas 1	90 psi
Ion source gas 2	40 psi
Curtain gas	35 psi
Source temperature	300°C
Ion spray voltage	5000 V
CAD gas	10
MS method	TOF MS
Time bins to sum	40

**Data processing:** The SCIEX OS software was used to process the data qualitatively and quantitatively. The mass reconstruction workflow within the SCIEX OS software was used for peak area determination of proteins.

## Peak area reproducibility

The speed of the Echo<sup>®</sup> MS+ system with ZenoTOF 7600+ system allows for 384 samples completed in as little as 8 minutes. Data is collected in a single data file and automatically split during post-processing, with the well position indicated in the split data file. Due to the low volume of the acoustic ejections in the nanoliter range, a significant concern is the reproducibility of the ejections across an entire 384-well plate.

An overlay of 384 split ejections of 10.4 $\mu$ M intact myoglobin is shown in Figure 3 with a calculated %CV of peak area under 10%.



**Figure 3: Overlay of the TIC for 384 ejections of intact myoglobin.** Overall, the calculated %CV of the peak area was <10%.

## Data processing using the SCIEX OS software

The SCIEX OS software enables targeted analysis and deconvolution of large intact molecules on a per-well basis, providing TIC, extracted MS for a single well, and reconstructed mass. The ejection status, time, and volume can be observed on a per-well basis to check the data quality. The split data file can be processed for the target protein mass at a reconstruction resolution of 5000. The reconstruction mass output range was set between 15 kDa and 20 kDa. The reconstructed masses were found at 16,951 Da, matching literature values (Figure 4).<sup>4</sup> Once a specific processing method is determined, the results file can be generated through batch submission after acquisition. The reviewed data in SCIEX OS can be directly integrated with a laboratory information management system (LIMS) for key information storage.

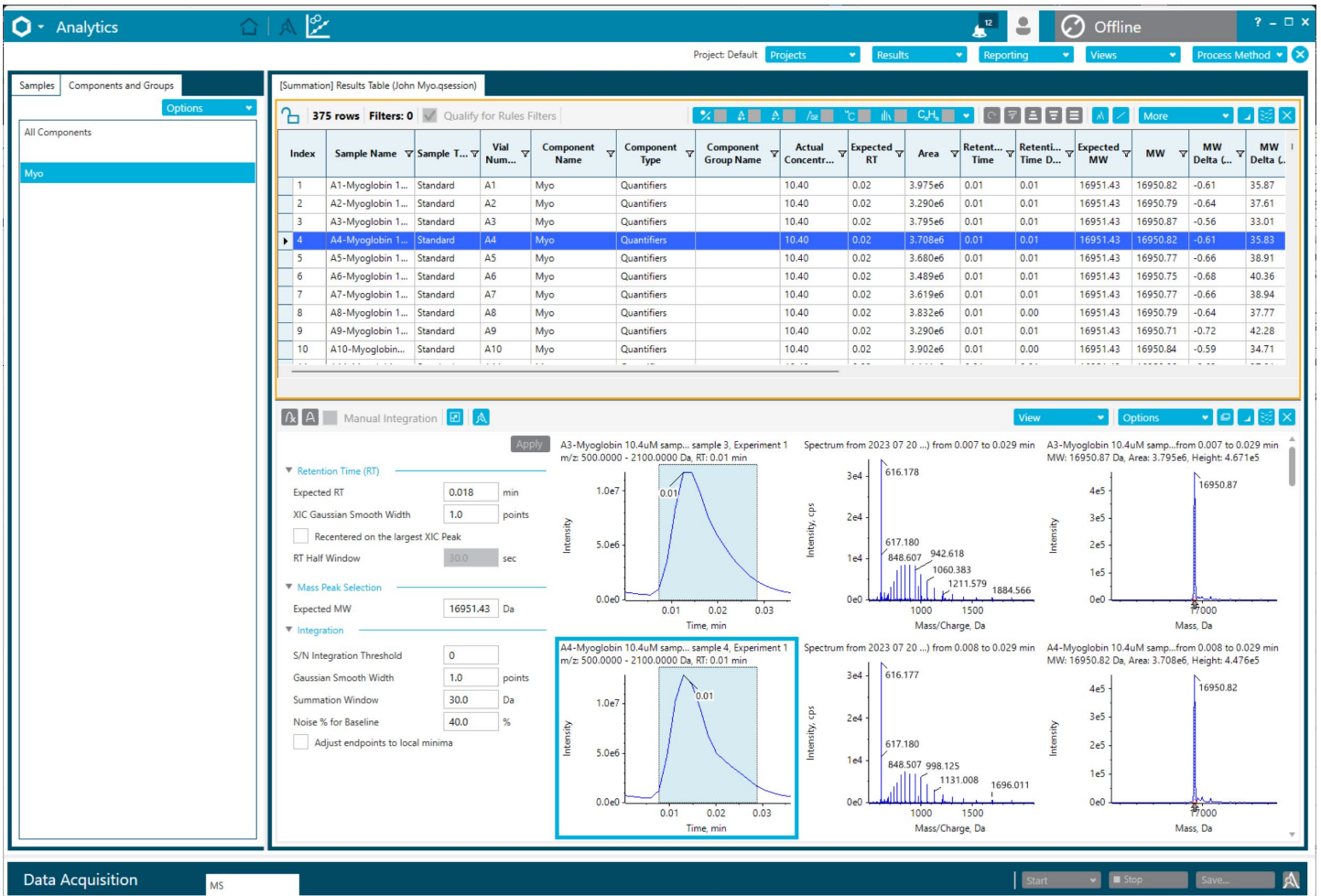
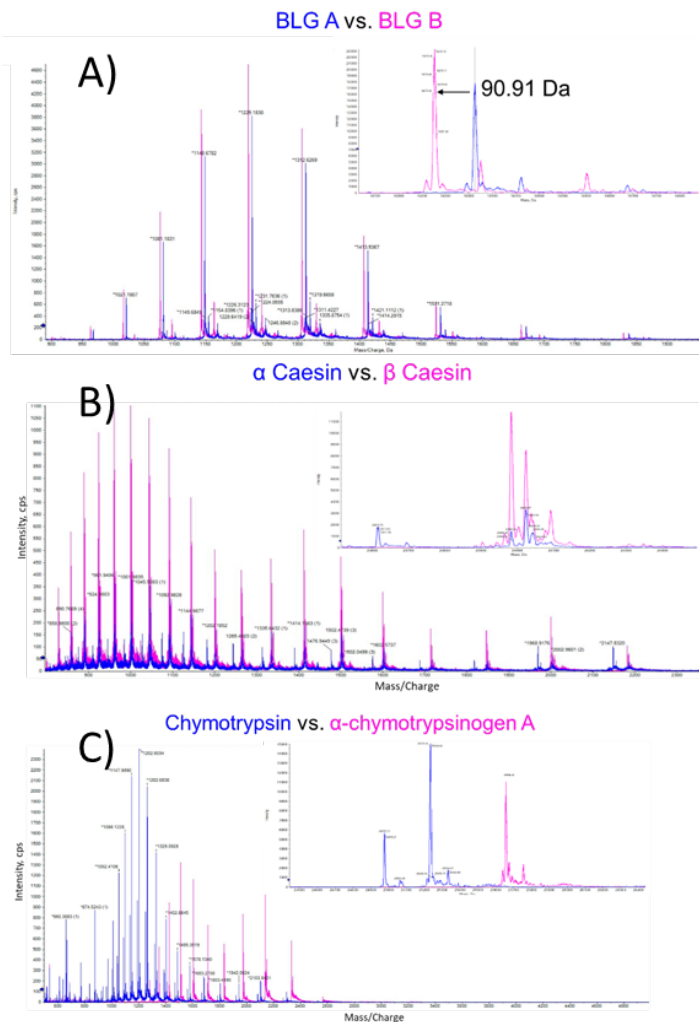


Figure 4: The data from Figure 6 can be processed in an automated, high-throughput fashion. A defined target of multiply charged ions can be deconvoluted, and a results table can be used to review data before report generation.

## Proteoform analysis

Six protein standards in sequential wells were analyzed using the Echo® MS+ system with ZenoTOF 7600+ system. This is an example of protein modification analysis in a high-throughput screening environment. Bovine  $\beta$ -lactoglobulin [BLG] has 2 genetic variants, BLG-A and BLG-B, which differ in 2 out of 162 residues.<sup>5</sup> In the case of sequence mutations, differences in the intact mass can rapidly reveal variances in the primary sequences by comparing the 2 data files [Figure 5A]. The  $\alpha$  and  $\beta$ -casein proteins from bovine milk were analyzed in a subsequent well to evaluate phosphorylation differences [Figure 5B]. Phosphorylation is essential for precisely controlling cellular processes, allowing cells to respond and adapt to various signals and environmental conditions. It is a versatile and dynamic mechanism for regulating protein function, signal transduction, enzyme activity, cell cycle progression and membrane transport.<sup>6,7</sup> Chymotrypsinogen is an inactive precursor of chymotrypsin, an enzyme that breaks down proteins.<sup>8</sup> Chymotrypsinogen remains inactive until it reaches the digestive tract to prevent undesired cleavage. Activation occurs when trypsin cleaves a specific peptide bond on  $\alpha$ -chymotrypsinogen [pink trace of Figure 5C] to form active chymotrypsin [blue trace of Figure 5C]. This leads to the formation of  $\nu$ -chymotrypsin, which further reacts with other molecules to produce  $\alpha$ -chymotrypsin. The yield of  $\alpha$ -chymotrypsin can be influenced by factors such as inhibitors, pH, temperature and calcium chloride.



**Figure 5: Intact analysis of 3 sets of 10 $\mu$ M proteins.** A) BLG A [blue] vs. BLG B [pink], B)  $\alpha$  and  $\beta$ -casein [blue vs. pink, respectively] and C) chymotrypsin and  $\alpha$ -chymotrypsinogen A [blue vs. pink, respectively].

## Conclusions

- Rapid proteoform differentiation can be achieved effectively via the Echo® MS+ system with ZenoTOF 7600+ system
- Increased versatility of the acoustic method enables additional analytical time per sample when needed
- Low consumption of 100 nL per ejection enables sample analysis with a limited amount
- A platform MS method can be applied for a wide range of intact protein analysis
- SCIEX OS software enables fast mass reconstruction and quantitation

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