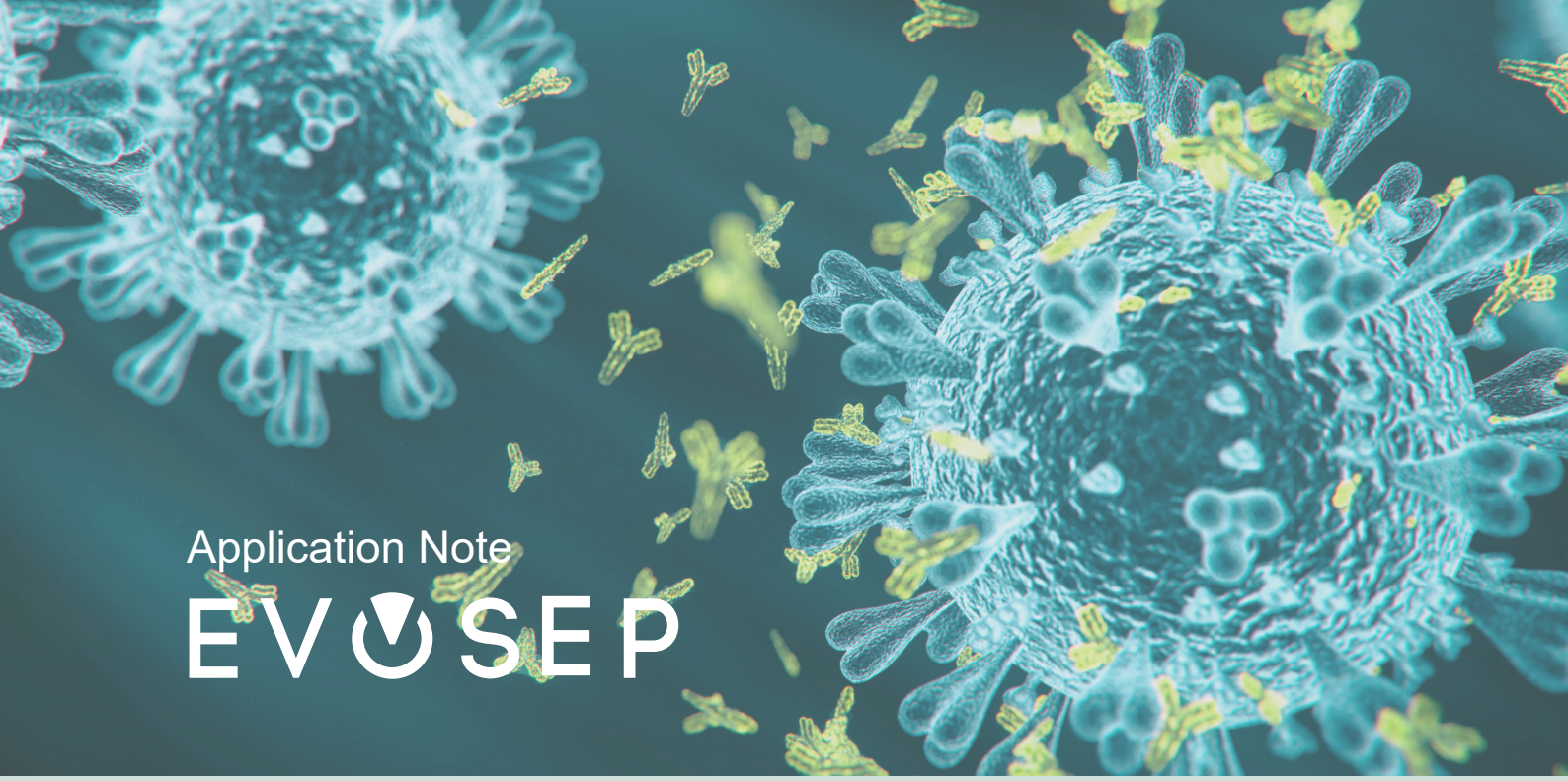

Application Note

EVOSEP

Rapid and robust **PTM peptide mapping for biologics** with the Evosep One and the SCIEX ZenoTOF 7600

1. Introduction

Biologics are a powerful emerging class of therapeutics with superior selectivity and potency, but also with a structural complexity, which makes characterization challenging. However, validation of sequence and quantification of modification states is required during development and production, typically achieved by liquid chromatography and mass spectrometry-based peptide and PTM mapping. With continuous improvement to LC-MS based workflows, we here evaluated the utility of the Evosep One in combination with the ZenoTOF 7600 mass spectrometer for peptide mapping analyses. Amino acid sequences were characterized and modification patterns of a reference monoclonal antibody material (NISTmAb) quantified, exploring sensitivity down to 50 ng tryptic digest loaded on Evotip (Figure 1).

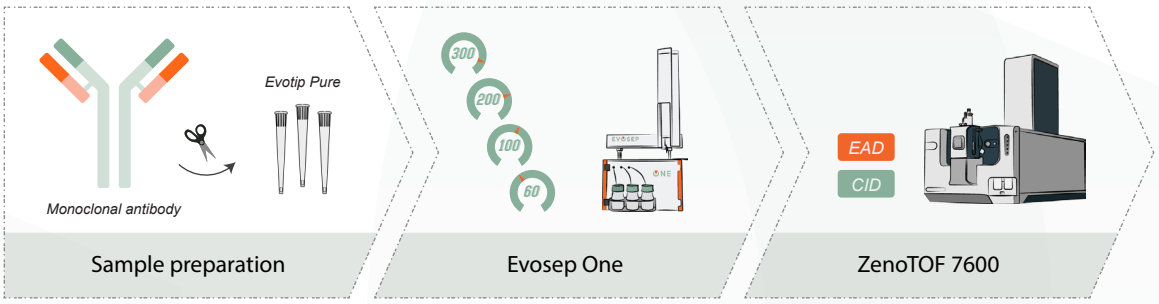

Figure 1: Workflow for monoclonal antibody peptide mapping analysis on the Evosep One connected to the ZenoTOF 7600.

2. Method details

A commercial tryptic digest of NIST reference material (Waters, 186009126) was loaded on Evotip Pure with 50, 100 and 200 ng respectively using the standard loading protocol. Triplicates of each condition were analyzed on the Evosep One with the 60 & 100 samples per day methods using the EV1109 column at 40 degrees and with the 200 & 300 samples per day methods using the EV1107 column at ambient temperature. The ZenoTOF 7600 mass spectrometer was operated in information dependent acquisition (IDA) mode utilizing a Top15 method with either CID or EAD fragmentation by using the OptiFlow Turbo V ion source with the low microflow emitter (SCIEX, 5061574). MS1 scan range was set to 200-1800 mz using 200 ms fill time. MS2 scan settings included charge states 1-6 with accummuation time of 35 ms for 60 & 100 SPD or 30 ms for 200 & 300 SPD with Zeno pulsing enabled and with dynamic collision energy or dynamic ETC for CID and EAD fragmentation, respectively. Peptide mapping analysis was performed using the Sciex Biologics Explorer software (v1.0.2). The Peptide Map workflow considered spectra with mass tolerances of 5 ppm (MS1, except 7 ppm for condition 50ng-200SPD-EAD) & 10 ppm (MS2). Carbamidomethyl (C), Deamidation (N/Q), Gln -> pyro-Glu conversion, Oxidation (M) and Glycosylation with the preconfigured reference database of glycostructures were considered as modifications. The retention time range of considered matches were limited to ≥ 0.02 min for mapped precursors. A spectral library was generated from spectrum-centric searches of CID and EAD runs using MS-GF+ (v2022.04.18) against NISTmAb and background mouse sequence database with semi-tryptic cleavage, allowing oxidation (M) and deamidation (N/Q) as variable modifications and carbamidomethylation (C) as fixed. This was used in Skyline-daily to extract quantitative precursor information and retention times to monitor stability.

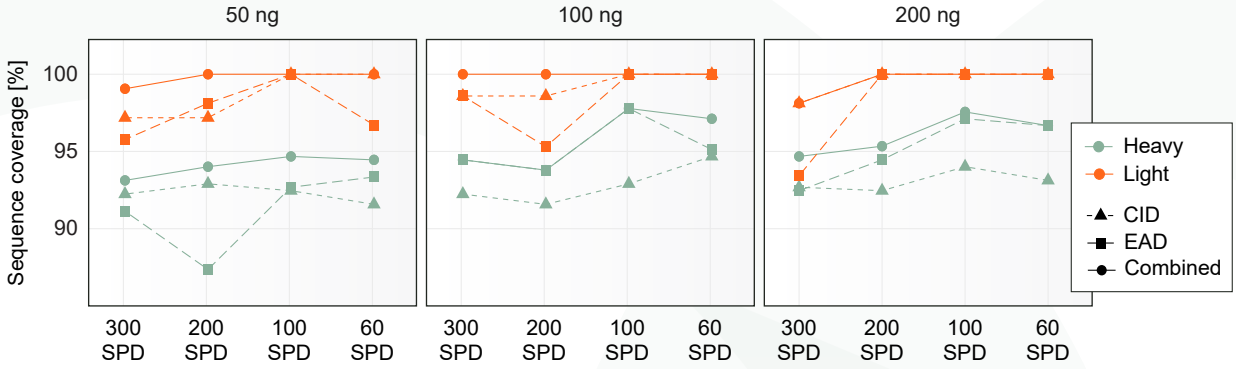

Figure 2: Sequence coverage of reference antibody chains from tryptic peptide map.

3. High sequence coverage

First, we explored the general performance metrics of peptide mapping using the Evosep One combined with the ZenoTOF 7600 as a function of peptide load, sample throughput and employing either CID or EAD fragmentation. In addition, combined data from CID and EAD fragmentation were considered to identify potential synergies of sequential usage of the two orthogonal fragmentation methods. Sequence coverage of mAb chains remained high (essentially, ≥ 90 %) in light of increased throughput and reduced sample loading amount (Figure 2). For the 100 ng load, the light chain sequence coverage was at 100% when calculated by combining CID and EAD fragmentation independent of the sample throughput.

To achieve 100% sequence coverage with CID or EAD performed independently, the maximum throughput at 100 ng load is 100 samples per day. The best sequence coverage of the heavy chain was achieved at 97.5 % when using the 100 samples per day method with 100 ng sample amount (Figure 2). Next, we explored the depth of the generated peptide maps, as a function of throughput, sample load and fragmentation method, using the number of mapped precursors as metric. In order to explore potential modifications, state-specific biased precursors were grouped by modification class.

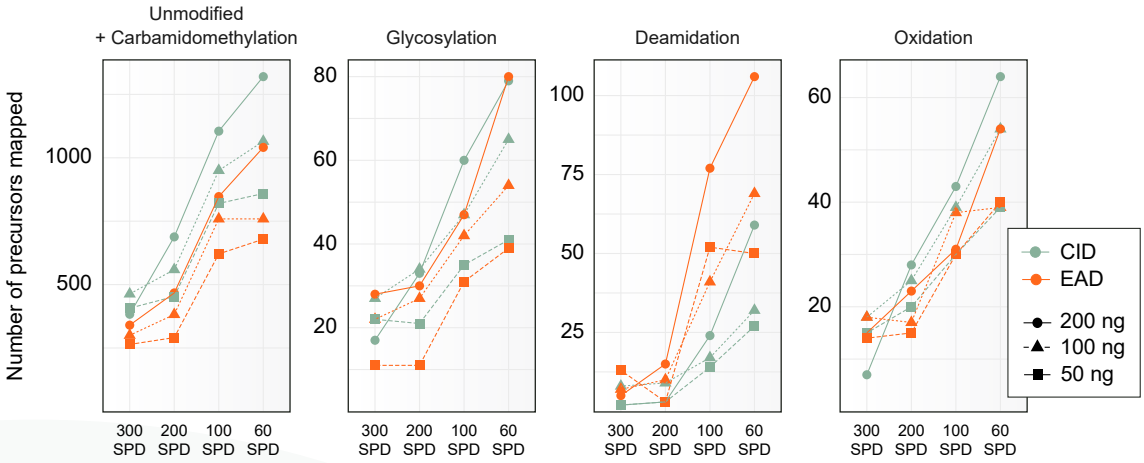

Figure 3: Number of precursors mapped as function of Evosep One method, sample load, fragmentation method and modification class.

4. Excellent reproducibility

We performed 50 simultaneus injections with the 60 samples per day method and evaluated the retention time stability to have low standard deviations and the MS1 XIC area reproducibility to have low coefficients of variation (CV. A representative set of 33 peptides is summarized in the box plot below (Figure 4A).The MS1 area under the curve observed across the 50 injections was robust, as shown for a subset of peptides (Figure 4B) and summarized with all manually reviewed peptides in the boxplots below with a median CV of 10% with 9.8% for raw intensities and 7.8% following a median normalization of raw intensities (Figure 4C).

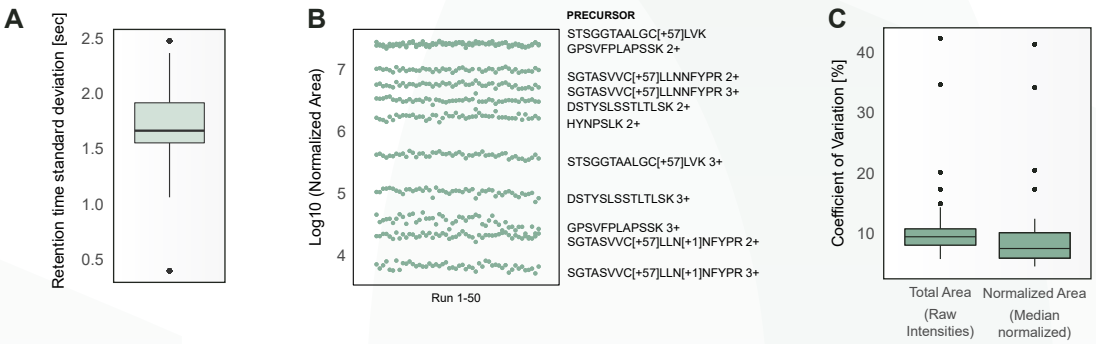

Figure 3: (A) Retention time stability from 50 injections. (B) Area reproducibility from selected peptides and (C) boxplot showing area reproducibility for all peptides with and without normalization.

5. Conclusion

The combination of short and standardized methods on the Evosep One and high precision facilitated on the ZenoTOF 7600 mass spectrometer represents an excellent setup for high throughput peptide mapping analyses. It enables rapid peptide mapping, which for example can be used for quality control with great sensitivity, which is needed for low yield applications. EAD fragmentation provides coverage close to CID fragmentation while in many cases maintaining the capacity to localize the site of interest and identify more labile modifications such as glycosylation. Retention time stability and quantitative precision appear suited also for quantitative studies and development of targeted assays, as validated here for the 60 samples per day method.

To summarize, the Evosep one system provides a high performing platform for generating biologics peptide maps at high throughput and with high sensitivity, highlighting the benefits of disposable trap column design coupled to chromatographic refocusing in the space of biopharmaceutical development.