# Optimization of Sliding Window Deconvolution Parameters Maximizes Quality of Protein Identification and Relative Quantitation in LC/MS Intact Protein Analysis

**Aaron O. Bailey [1], Paul Gazis [1], Jennifer Sutton [1], Stephane Houel [1], Eugen Damoc [2], Kai Scheffler [3], Jonathan L. Josephs [1], Thermo Fisher Scientific, [1] San Jose, CA, USA; [2] Bremen, Germany; [3] Dreieich, Germany**

## ABSTRACT

Characterization of protein therapeutics is a constantly evolving challenge where biochemical complexity is growing and where regulatory requirements are steadily increasing. Data processing software is the critical link between acquisition of high quality raw data and accurate automatic interpretation of results. The Sliding Window algorithm accommodates separations-based mass spectrometry analysis by "sliding" along a chromatogram to produce a time-integrated result. In this study we focus on the native size exclusion chromatography (SEC) Orbitrap MS analysis of a non-deglycosylated, intact commercial antibody-drug conjugate (ADC). We performed Sliding Window analyses using a range of window widths and window offsets and show that an optimized analysis can be achieved using a window width which is equivalent to the average full width half max (FWHM) of the unique eluting components, and using the smallest possible Window Offset time (equivalent to scan rate). Optimal Sliding Window improved our intact ADC analysis both in terms of qualitative purposes (absolute number of correct mass assignments) as well as in terms of relative quantitation (correct distribution of drug load across all glycoforms).

## INTRODUCTION

The Sliding Window algorithm accommodates separations-based mass spectrometry analysis and proves to be useful and necessary for all types of biotherapeutic proteins. Sliding window acts as an overlay for intact protein analysis by utilizing a deconvolution algorithm multiple times in succession, "sliding" along a chromatogram or electropherogram to produce a time-integrated result. This approach allows a user to analyze mass spectra of protein isoforms which exhibit differential elution profiles, such as separation of mAb subunits or partial separation of intact antibody-drug conjugate (ADC) drug loads. In this study we focus on the native size exclusion chromatography (SEC) Orbitrap MS analysis of an intact, non-deglycosylated, commercial ADC, trastuzumab emtansine. This sample allows us to study a highly complex mixture, while optimizing detection of a broad mixture of specific isoforms.

## MATERIALS AND METHODS

Trastuzumab emtansine (trade name Kadcyla ®) was prepared without pretreatment for trypsin peptide mapping or native intact analysis. For intact mass analysis ADC samples were analyzed using Thermo Scientific™ Vanquish™ Horizon UHPLC system with a Thermo Scientific MAbPac™ SEC-1 size exclusion chromatography (SEC) column coupled directly to a Thermo Scientific Q Exactive™ Plus mass spectrometer with BioPharma option. The mass spectrometer was operated in High Mass Range (HMR) mode to allow improved high mass transmission and scanning up to *m/z* 8000 for native intact analysis (**Fig 1**). MS1 spectra were collected using 10 microscans and an Orbitrap™ resolution setting of 70,000. Intact mass spectra were deconvoluted using the ReSpect™ algorithm in Thermo Scientific BioPharma Finder™ 2.0 software. Deconvolution spectra were annotated was using the amino acid sequence of trastuzumab, with a total of 16 disulfide bonds, alternately fixed modifications of G0/G0F, G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F, +/- alternately fixed modification of MCC linker (avg mass = 221.25) and a variable modification of up to 8 x MCC-DM1 linker-drug (avg mass = 957.53 Da). Average DAR value was automatically calculated based on MCC-DM1 using BioPharma Finder. Annotation was performed using a 20 ppm mass tolerance. All non-annotated entries were manually removed.

**Figure 1. LC-MS Instrumentation for Complete ADC Characterization.** All experiments were performed using a Vanquish UHPLC connected to a Q Exactive Plus with High Mass Range (HMR) mode. Data were analyzed using BioPharma Finder 2.0 software.


## RESULTS

### Sliding Window is a powerful method for deconvolution of intact protein LC-MS data

The Sliding Window algorithm accommodates separations-based intact protein mass spectrometry analyses. The algorithm works by "sliding" along a chromatogram or electropherogram to produce a time-integrated result. Sliding Window is an option in BioPharma Finder software (**Fig 2**), which is paired with a deconvolution algorithm, such as Xtract (for isotopically resolved MS data) or ReSpect (for isotopically unresolved MS data). The unique approach of Sliding Window introduces additional parameters which should be optimized. Namely, window Width ("Target Avg Spectrum Width") and window Offset ("Target Avg Spectrum Offset"), function as x/y-type interdependent parameters which determine how mass spectra are averaged across time in separations-based analyses.

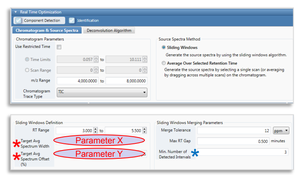



**Figure 2. Sliding Window.** Width and Offset are critical "X/Y"-type parameters for Sliding Window (red asterisks). Window Offset is expressed as a % of window Width. Another parameter "Min Number of Detected Intervals" allows redundancy to be utilized as a means of confidently reporting true positive data (blue asterisk).

Using the minimal possible value of % Offset allows for greatest number of redundant detections of component while the window moves along the LC time axis. This is critical for confidently detecting minor components which may be eluting for only short time periods (**Fig 3**).

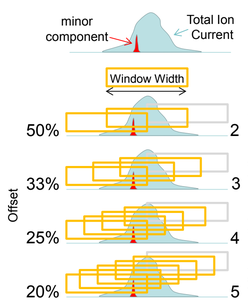
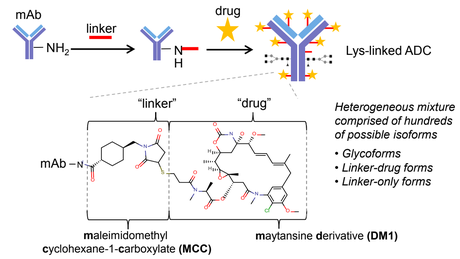



**Figure 3. Window Width and Offset Work Together.** Different isoforms in chromatographic separations can be detected multiple times using Sliding Window. Redundant detections allow the components to confidently reported. Using a minimum % Offset will achieve a maximum number of deconvolution events and thus maximize the redundancy of possible component detections.

### Lysine-linked ADC offers challenge of heterogeneous mixture for Sliding Window

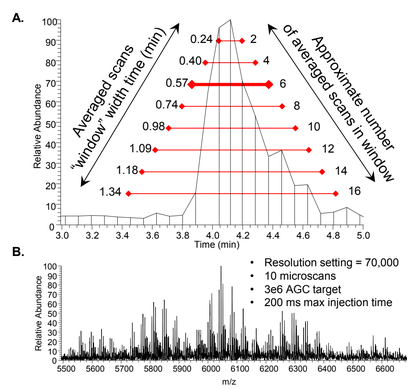
Antibody drug conjugates (ADCs) are complex mixtures, involving diverse chemical modifications which arise from the manufacture of the core antibody as well as the drug conjugation chemistry. Major forms of lysine-linked ADCs differ in N-glycan composition, the number of linker-drugs attached, as well as potential linker-only attachments (**Fig 4**).

**Figure 4. Multi-stage assembly of a lysine-linked ADC results in a highly complex mixture of isoforms with similar masses.**




The lysine-linked ADC, trastuzumab emtansine, is observed in intact analysis as a complex mixture. Native MS improves separation of sequential charge state envelopes for resolving heterogeneity (**Ref 1**). Using a very high resolution Orbitrap setting of 70,000, we show that direct sample analysis without deglycosylation results in highly resolved mass peaks corresponding to individual glycoforms, linker-drug forms, and linker-only forms.

**Figure 5. Native SEC-MS analysis of an intact ADC.** (A) Native SEC-MS chromatogram of trastuzumab emtansine acquired at Orbitrap R=70,000 setting with 10 microscans. The scan rate in the LC-MS analysis can be observed using a "stick" display to show each spectrum acquired. Red lines depict a range of window Widths for averaging mass spectra, with units in minutes (left) and approximate number of scans (right). (B) Mass spectrum averaging data from 6 scans across the LC peak. Details for MS method are shown.




### Sliding Window optimization for combination of minimum Offset value and FWHM window Width

Trastuzumab emtansine is comprised of a mixture of isoforms owing to combinations of distinct N-glycans, MCC-DM1 linker-drug additions, and MCC linker additions (**Ref 2**). In BioPharma Finder, 10 isoforms were generated which correspond to the trastuzumab sequence with 5 known glycoform combinations, +/- MCC linker. A variable modification of 0-8 MCC-DM1 linker-drugs expanded our search list to 90 total isoforms. Our native SEC-MS ADC data were analyzed using ReSpect deconvolution with Sliding Window (**Fig 2** and **Fig 6**). We varied Width and Offset to determine the optimal combination of settings which provided the greatest number of isoforms within a mass accuracy of 20 ppm (**Fig 7**). We found that a Width of 0.5 min and minimum possible Offset value (17%, equivalent to scan rate) provided the greatest number of annotated isoforms. We further determined that the Width of 0.5 min was nearly equivalent to the Full Width Half Max (FWHM) measured for an extracted ion chromatogram (XIC) of an individual isoform (**Fig 8**).

**Figure 6. Detailed parameter settings for ReSpect deconvolution and isoform annotation, and automatic DAR calculation.**

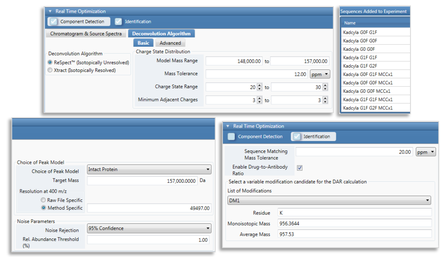


**Figure 7. Optimization Exercise for Sliding Window.** Sliding Window deconvolution was performed iteratively while varying Width and Offset. A list of 90 expected isoforms were searched with a mass accuracy of 20 ppm. X axis shows Offset, Y axis show Width, and a heat map of cells show the total number of isoforms found (automatically annotated) in each Sliding Window experiment. A width of 0.5 min and lowest possible offset value of 17% (equivalent to scan rate) provided the greatest number of annotated isoforms.

| Sliding Window Width (min) \ Offset | 50% | 33% | Minimum % |
|---|---|---|---|
| 0.3 | 33 | 53 | 54 = 29% |
| 0.5 | 30 | 54 | **67** = 17% |
| 0.8 | 26 | 48 | 65 = 11% |
| 1.2 | 26 | 44 | 60 = 8% |
| 1.5 | 0 | 44 | 58 = 6% |

Annotated Isoform IDs

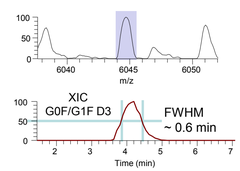
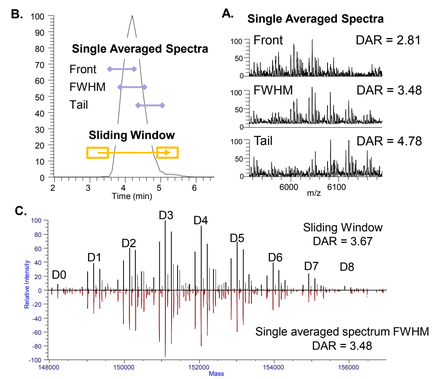



**Figure 8. Optimal Window Width reflects FWHM of individual isoform**. ReSpect deconvolution showed a well-resolved mass at m/z 6044.5 corresponds to the isoform 'G0F/G1F D3'. An XIC plot shows the FWHM (0.6 min) of 'G0F/G1F D3' is nearly equal to the optimal value determined for Sliding Window Width (0.5 min).

### Sliding Window provides accurate DAR measurement

The MCC-DM1 modification was used to calculate an weighted average drug-to-antibody ratio (DAR), which is a critical attribute for intact ADC analysis and reflects ADC potency. Sliding Window moves along LC-MS spectra to determine isoform distribution and quantity changes over chromatographic time. When isoforms do not perfectly co-elute, the conventional method of single averaged spectrum deconvolution may introduce error in DAR measurement.

**Figure 9. Optimized Sliding Window deconvolution provides accurate DAR value.** (A) Averaging spectra across the front, FWHM, or tail of an LC peak can influence the DAR value when isoforms to not perfectly co-elute. (B) Higher drug-load isoforms elute later in native SEC-MS, so DAR value increases over time, which may introduce error in DAR measurement.




## CONCLUSIONS

- Sliding Window deconvolution allows quantitative measurements for LC-MS or other separations-based intact protein analysis
- Optimal window Offset for ADC is equivalent to scan rate
- Optimal window Width is FWHM of XIC of individual isoforms

## REFERENCES

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## TRADEMARKS/LICENSING



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