Application Note

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Top-Down Sequence Analysis of Intact Proteins Using an Agilent AdvanceBio 6545XT LC/Q-TOF with ExD

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Abstract

Detailed proteoform characterization is crucial for understanding disease mode of action (MOA) and guiding biotherapeutic development. However, traditional bottom-up proteomics cannot distinguish individual proteoforms because digestion breaks proteins into a mixture of peptides, which scrambles proteoform-level information. Top-down proteomics is a promising alternative that analyzes intact proteins directly, preserving proteoform-level details.

This application note describes the implementation of top-down fragmentation using the Agilent 6545XT AdvanceBio LC/Q-TOF with an electron capture dissociation (ExD) cell. The goal of this publication is to guide new users in setting up top-down mass spectrometry (TDMS) workflows, provide practical insights, and examine how parameters such as collision energy and spectral averaging influence outcomes like sequence coverage and ions detected. This understanding serves as a foundation that will enable future TDMS method development using Agilent Q-TOF mass spectrometers. By integrating accessible instrumentation, reproducible results, and expert ecosystem support, this workflow presents a scalable solution for top-down proteomics, empowering researchers to confidently explore proteoform biology.

Introduction

A proteoform is a specific form of a protein consisting of a unique amino acid sequence and its post-translational modifications (PTMs). A single gene can give rise to hundreds or thousands of distinct proteoforms containing unique patterns of modifications such as phosphorylation, glycosylation, methylation, and acetylation.[1,2] These variations in sequence and PTMs often play critical roles in determining protein function or dysfunction in disease MOAs.[3,4]

While traditional bottom-up proteomics has unrivaled ability to rapidly identify proteins and PTMs, full characterization of proteoforms is not possible via bottom-up due to the reliance on peptide mixtures. For example, the peptides produced during digestion may not be observable in the mass spectrometer, causing gaps in sequence coverage. More importantly, digestion of the protein obscures possible correlations of PTM occurrences or may even make splice variants indistinguishable.

In contrast, top-down proteomics analyzes intact proteins directly, preserving proteoform-level information.[5] Top-down proteomics is becoming increasingly practical and is now being used by researchers to characterize proteoforms from complex samples such as brain and heart tissues.[6–8] Effective sequence analysis of intact proteins requires gas-phase fragmentation techniques outside of commonly implemented collision induced dissociation (CID). Instead of favoring the weakest bonds, electron capture dissociation (ECD) enables radical-driven fragmentation that is more evenly distributed across accessible regions of the protein sequence. Additionally, combining ECD with CID can enhance top-down fragmentation and generate complimentary fragments, leading to more confident sequence assignments.[9,10]

This application note describes the process of performing top-down fragmentation on intact proteins by direct infusion into an Agilent 6545XT AdvanceBio LC/Q-TOF equipped with an ExD cell. The goal of this publication is to provide guidance for how to get started with top-down mass spectrometry using denatured proteins and deliver practical insights including how parameters such as added CID energy and spectral averaging can influence TDMS outcomes like sequence coverage and ion detection. These insights establish a foundation for future TDMS method development on Agilent Q-TOF platforms, enabling researchers to confidently characterize intact proteins and accelerate biopharmaceutical innovation.

Experimental

Chemicals and standards

- ESI-L tuning mix (part number G1969-85000), Agilent Technologies
- Formic Acid, LC/MS Grade, (part number G2453-85060), Agilent Technologies
- InfinityLab Acetonitrile for LC/MS (part number 5191-5101), Agilent Technologies
- InfinityLab Water for LC/MS (part number 5191-5121), Agilent Technologies
- Small protein ECD tuning standard
  - Bovine ubiquitin, 8.6 kDa (part number U6253), Sigma-Aldrich
- Larger protein ECD tuning standard
  - Carbonic anhydrase, 29 kDa (part number C2624), Sigma-Aldrich
- Aldolase, 39 kDa (part number A2714), Sigma-Aldrich
- Enolase, 46 kDa (part number E6126), Sigma-Aldrich

Note: Alternative proteins can be used as tuning standards for top-down analysis. When choosing a tuning standard, it is important to consider sample availability, purity, and storage stability. The presence of disulfide bonds is another important factor to consider, because they often limit fragment detection.

Instrumentation

- Agilent 6545XT AdvanceBio LC/Q-TOF (part number G6549AA)
- Agilent ExD cell for LC/Q-TOF (part number G1997AA)
- Agilent AJS Source (part number G1959A)

Software

- Agilent ExDControl, v3.7.8
- Agilent MassHunter Data Acquisition for LC/TOF and LC/Q-TOF, v11.0 Update 2
- Agilent ExDViewer, v4.6.28

Sample introduction

Samples were diluted to a concentration of 1–10 µM in 15% acetonitrile and 0.1% formic acid. The solutions were infused with a syringe pump at 10–20 µL/min directly into the Agilent Dual Jet Stream (AJS) source. A New Era syringe pump (model no. 300) was used for direct infusion.

Mass spectrometry methods

Top-down mass spectrometry was accomplished using the 6545XT AdvanceBio LC/Q-TOF equipped with an ExD cell for electron capture dissociation. A targeted MS/MS acquisition method for top-down analysis was created in MassHunter Data Acquisition for LC/TOF and LC/Q-TOF v11.0. Collision energy was set at a fixed value for each acquisition and precursors were added manually to the target list. For ECD only experiments, collision energy was set to 0 V. Further method parameters are listed in Table 1.

Table 1. Q-TOF LC/MS data acquisition parameters.

6545XT Q-TOF MS System	
Ion Source	Dual Agilent Jet Stream Electrospray ionization source
Polarity	Positive
Gas Temperature	325 °C
Drying Gas Flow	7 L/min
Nebulizer	35–50 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	3500 V
Nozzle Voltage	2000 V
Fragmentor	175–300 V
Skimmer	45–125 V
Acquisition Rate	1 spectrum/sec
Acquisition Mode	2 GHz Extended Dynamic Range
Isolation Window	Wide (9 *m/z*)
MS Spectrum Range	200–3200 *m/z*
MS/MS Spectrum Range	200–3200 *m/z*

Note: Large protein transmission is often improved when parameters such as nebulizer pressure and fragmentor voltage are set high (e.g., 50 psi and 300 V). However, setting the fragmentor voltage too high activates the protein and can cause unwanted dissociation, which reduces the availability of intact precursor for top-down fragmentation.

Preparing the instrument for fragmentation of small proteins

The following text provides step-by-step method instructions for tuning the Agilent 6545XT AdvanceBio LC/Q-TOF MS for top-down analysis using ECD. These steps assume the filament current is optimized, and other aspects of the instrument, such as quadrupole isolation efficiencies, are performing within normal range. Reference the ExD cell user guide for more information.

Note: For the purpose of this publication, a small protein is less than 20 kDa.

Tuning the ExD cell for protein transmission and ECD

1. Turn the ExD cell filament on and warm up for 20 minutes. Create or load an ExD cell voltage profile for tunemix transmission with the filament on. Turn on bottle B with tune mix. Check instrument resolution, mass accuracy, and high *m/z* transmission. Ensure the source has been recently cleaned and that the spray is stable before proceeding. This can be evaluated using the total ion current feedback graph in the MassHunter Data Acquisition user interface.
2. Infuse 10 µM ubiquitin in 15% acetonitrile and 0.1% formic acid at a rate of 10 µL/min. Once the signal has stabilized, use ExDControl to perform a coarse autotune to optimize transmission of ubiquitin parent ions. Consider using a profile previously tuned for protein transmission if it provides a better starting point than the profile used for tune mix. Save the autotuned voltage profile with a name that indicates the profile has been tuned for ubiquitin transmission.
3. Load the saved ubiquitin transmission profile into the MS2 slot within ExDControl. In MassHunter, isolate the 11^+ ubiquitin precursor. Then, use ExDControl to run an autotune to optimize the intensity of ubiquitin ECD fragments. ExDControl is installed with a preloaded mass list for ubiquitin precursors and fragments which can be used for autotuning (Table 2). It is recommended to run a coarse tune followed by a fine tune to convert the ubiquitin transmission profile into an effective ECD profile. Save the profile with a name that indicates the profile has been tuned for ubiquitin ECD.

Table 2. Fragment mass list for ubiquitin 11^+ precursor at *m/z* 768.8, which can be used to optimize ECD fragmentation for small proteins via autotune in ExDControl.

Precursor	ECD fragment *m/z* values
Ubiquitin 11^+ (*m/z* 768.8)	552.9, 596.4, 724.9, 789.0, 800.3, 803.4, 950.5, 957.5, 960.7, 1347.2

Note: Some vendors supply bovine ubiquitin, which has two additional glycine residues at the C-terminus and a mass of 8560 Da. The 11^+ precursor ion will consequently appear at *m/z* 779.1. A suitable ECD fragment list for this species is 609.9, 636.4, 717.9, 735.0, 942.8, 946.1, 957.5, 959.5, 965.1, 1347.2.

4. Using the targeted acquisition method detailed in the experimental section, acquire ubiquitin ECD spectra for 1 minute. Load the resulting .d file into ExDViewer to evaluate the data quality. ECD fragments should be greater than 200 counts and the overall sequence coverage should be > 95% using ECD alone. Figure 1 shows an example of a good-quality ubiquitin ECD spectrum. If ECD fragment abundances are low, increase the filament heating current by +.05 A followed by an additional fine autotune to optimize ubiquitin fragments. See the ExD cell user guide for further troubleshooting.

   Note: The automatically generated ion intensity graph in ExDViewer is a useful tool for evaluating the quality of isotopically resolved fragmentation spectra. A high-quality fragmentation spectrum often minimizes unassigned ions while exhibiting abundant fragment ion signals, such as is seen in Figure 1B.

5. The MS1 and MS2 profiles optimized using ubiquitin may now be used to collect ECD spectra on proteins of interest with an auto or targeted acquisition method. If the protein of interest is larger than approximately 20 kDa, continue to steps 6–9 to optimize the ExD cell for fragmentation of larger protein.

Tuning the ExD cell for fragmentation of larger proteins

6. In ExDControl, load the MS1 profile tuned for ubiquitin transmission and the MS2 profile tuned for ubiquitin ECD. While in the MassHunter tune context, begin infusing 10 µM carbonic anhydrase in 15% acetonitrile and 0.1% formic acid. After signal has stabilized, perform an ExDControl fine tune on carbonic anhydrase precursors to improve transmission.
7. Isolate the denatured carbonic anhydrase precursor at 1117.3 *m/z*. The most abundant precursor should be above 30K counts before isolation and ECD. Perform a fine autotune on carbonic anhydrase ECD fragments. ExDControl is installed with mass lists for carbonic anhydrase (Table 3); however, custom mass lists can be created for any analyte of interest.

Table 3. Fragment mass list for carbonic anhydrase 26^{+} precursor at *m/z* 1117.3

Precursor	ECD fragment *m/z* values
Carbonic Anhydrase 26^{+} (*m/z* 1117.3)	375.2, 421.2, 506.7, 575.3, 588.3, 626.4, 664.3, 726.4, 755.9, 776.5, 784.6, 816.6, 887.7, 904.2, 939.0, 944.7, 945.7, 960.2, 983.5, 1025.2, 1149.9, 1181.9, 1239.3, 1292.4, 1296.7, 1309.2

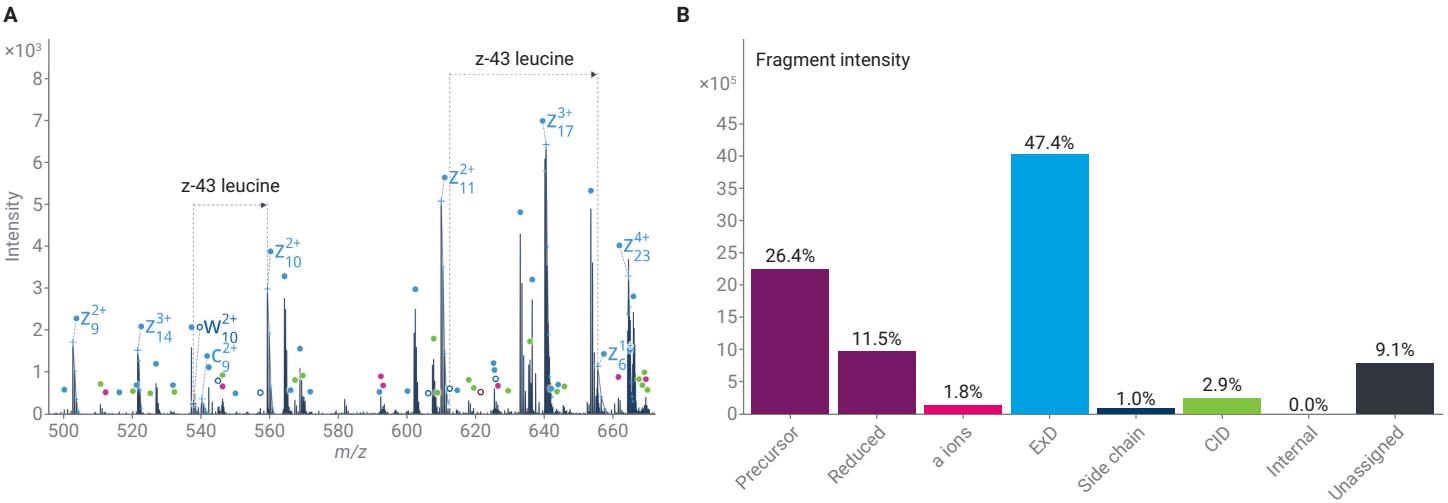

Figure 1. Example ubiquitin ECD spectrum and corresponding intensity graph. (A) In this example ubiquitin ECD spectrum, blue annotations indicate ECD-type ions, while green annotations indicate CID-type ions. Secondary side chain fragments, represented by w ions, are automatically labeled with an arrow pointing to the corresponding z ion. (B) The intensity graph displayed in ExDViewer. Purple bars correspond to the precursor and reduced charge precursors. Unassigned ion signal represents isotope clusters with protein-like isotope ratios but that did not match the target sequence. Internal ions were not considered.

8. Using the targeted acquisition method detailed in the experimental section, acquire carbonic anhydrase ECD spectra for 1 minute. Load the resulting .d file into ExDViewer to evaluate the data quality. Using default ExDViewer settings, the overall sequence coverage for 26^{+} carbonic anhydrase should be greater than 50% and ECD ion intensities should be greater than CID ion intensities.

   If sequence coverage is poor, run an additional ExDControl fine tune on carbonic ECD fragments. If coverage is still poor, increase the filament heating current by +.05 A and re-tune on carbonic anhydrase fragments. Keep in mind that MS1 and MS2 profiles should be retuned if the filament current is adjusted. Refer to the ExD cell user guide for further information on troubleshooting.

9. The MS1 and MS2 profiles optimized using carbonic anhydrase may now be used to collect ECD spectra on proteins of interest. Proceed with setting up MassHunter acquisition method and data collection. If direct infusion cannot be used, samples can be introduced using alternative techniques such as liquid chromatography.

ExDViewer Deconvolution workflow for top-down data analysis

ExDViewer is a tool for analyzing protein fragmentation data, including ECD, CID, and other fragment types, to confirm expected sequences. It also aids in identifying unknowns related to an expected sequence. The ExDViewer deconvolution algorithm excels at identifying protein fragment ions in complex MS/MS spectra, making it well suited for top-down analysis. The first step in using ExDViewer for top-down data analysis is to define a target sequence in the Sequence Editor. Sequences can be imported from a JSON file, ExDViewer .target file, or pasted in manually. Both fixed and variable modifications can be defined.

To begin top-down data analysis, open the Deconvolution workflow interface and load the .d file to the "Add Spectra" field. Select your target from the targets list under "Add Target". Ensure that the "Average Spectra" option is selected (Figure 2). Default values for all other parameters work well for both peptides and proteins. After selecting a target, proceed to the spectrum selection tab. Select the desired spectra for averaging. If needed, the averaging window can be manually adjusted by adjusting the width of the gray bar in the TIC plot. After selecting spectra, the "Run Now" button becomes active, which initiates the deconvolution workflow. The other workflow tabs (peak picking, deconvolution, matching) are used to set custom workflow parameters; however, default settings work well for most experiments.

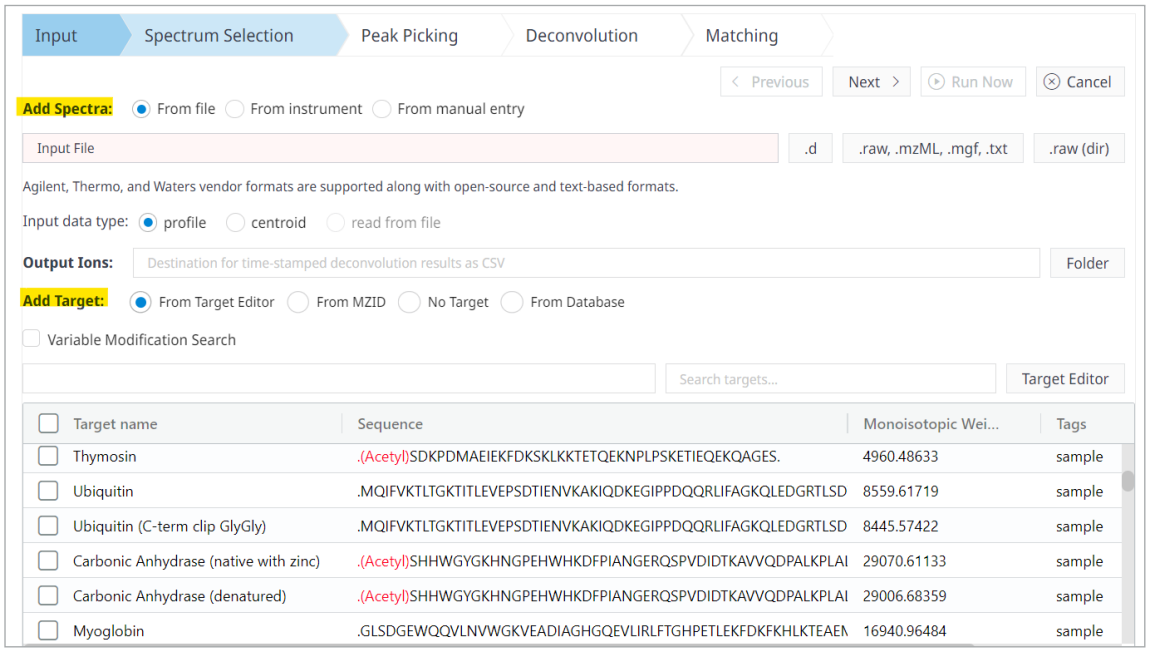

Figure 2. The ExDViewer deconvolution input page. The Add Spectra and Add Target fields are highlighted in yellow.

Results and discussion

Top-down mass spectrometry is emerging as a powerful approach for characterizing intact proteoforms, offering insights that are often inaccessible through traditional bottom-up approaches. Here, we demonstrate how electron capture dissociation expands the capabilities of the Agilent 6545XT AdvanceBio LC/Q-TOF, enabling sequence analysis of intact proteins.

To illustrate the advantages of ECD fragmentation, we compare fragmentation results for ubiquitin (8 kDa) and carbonic anhydrase (29 kDa) using CID or ECD. For ubiquitin, CID required optimization of collision energy to achieve 91% sequence coverage with 126 unique fragment ions detected.

In contrast, ECD alone yielded complete (100%) sequence coverage and 166 unique fragment ions detected. This resulted in the assignment of multiple complementary ions, leading to more confident sequence confirmation (Figure 3A).

Carbonic anhydrase presents a more challenging case for top-down fragmentation. For example, using CID only, the maximum sequence coverage for carbonic anhydrase was limited to 41% with 40 V CE. Using higher CE values resulted in fewer detectable unique fragment ions and reduced sequence coverage. However, using ECD, the sequence coverage of carbonic anhydrase was 62% with 156 unique fragment ions detected (Figure 3B and 3C), underscoring the value of ECD for fragmentation of larger proteins.

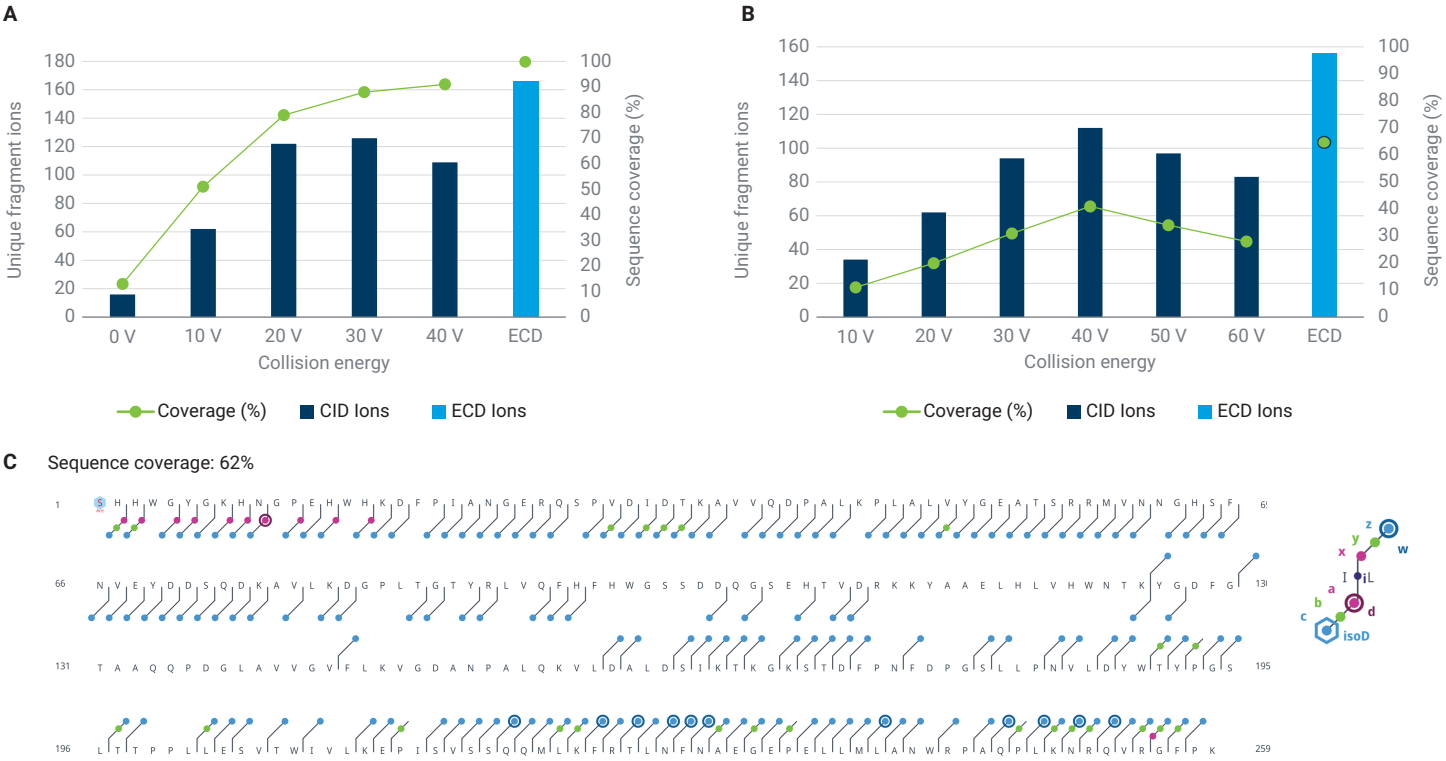

Figure 3. Fragmentation results for (A) 11^+ ubiquitin and (B) 26^+ carbonic anhydrase, showing the number of unique fragment ions detected as a function of collision energy or using ECD alone. Sequence coverage is illustrated as a green line on the secondary axis. (C) The sequence coverage map of 26^+ carbonic anhydrase using ECD only. Distinct fragment types are color-coded. A dot at a given position indicates at least one fragment corresponding to that position was matched. Multiple detected charge states are collapsed into a single dot but can be viewed in a tooltip in ExDViewer.

Combining ECD with supplemental collision energy can enhance TDMS results, particularly for large proteins

Electron capture without dissociation of product ions (ECnoD) is a common phenomenon in top-down analysis that limits the detection of sequence informative ions. Combining ECD with low-level collisional activation can disrupt non-covalent interactions and facilitate the dissociation of c and z-type fragment ions. Collisional activation can also produce CID-type fragments that are complementary to ECD ions and can improve sequence coverage, particularly for proline rich sequences. Table 4 summarizes the optimized ECD and ECD + CID sequence coverage results presented in this publication and details about how the results were achieved.

Table 4. Top-down method and results summary for proteins 8–46 kDa.

Protein	Mass	Sequence Coverage	Precursor Charge	Method
Ubiquitin	8 kDa	100%	11^+	ECD only, 47 spectra
Myoglobin	17 kDa	95%	16^+	ECD only, 47 spectra
Carbonic Anhydrase	29 kDa	67%	26^+	ECD + 10 V CE, 47 spectra
Aldolase	39 kDa	38%	29^+	ECD + 40 V CE, 47 spectra
Enolase	46 kDa	23%	40^+	ECD + 40 V CE, 47 spectra

Unlike peptides, intact proteins require empirically optimized collision energies (CE) for effective fragmentation, as traditional formulas for peptide CE estimation often fail to account for the complexity of protein structure. Factors such as molecular weight, charge state, sequence composition, and presence of disulfide bonds all influence a protein's sensitivity to collisional activation.

In this study, combining ECD with low-level collisional activation consistently increased the number of unique fragment ions detected across all proteins analyzed (Figure 4). While ECD alone was sufficient for providing complete sequence coverage (100%) of ubiquitin, supplemental collisional activation proved benficial for the analysis of larger proteins. For example, adding 10 V of collisional activation to carbonic anhydrase improved sequence coverage by 5%. However, exceeding 10 V decreased the overall sequence coverage despite producing abundant CID fragments (Figure 4B). This suggests that excessive collisional activation can lead to over fragmentation and the loss of sequence informative ions.

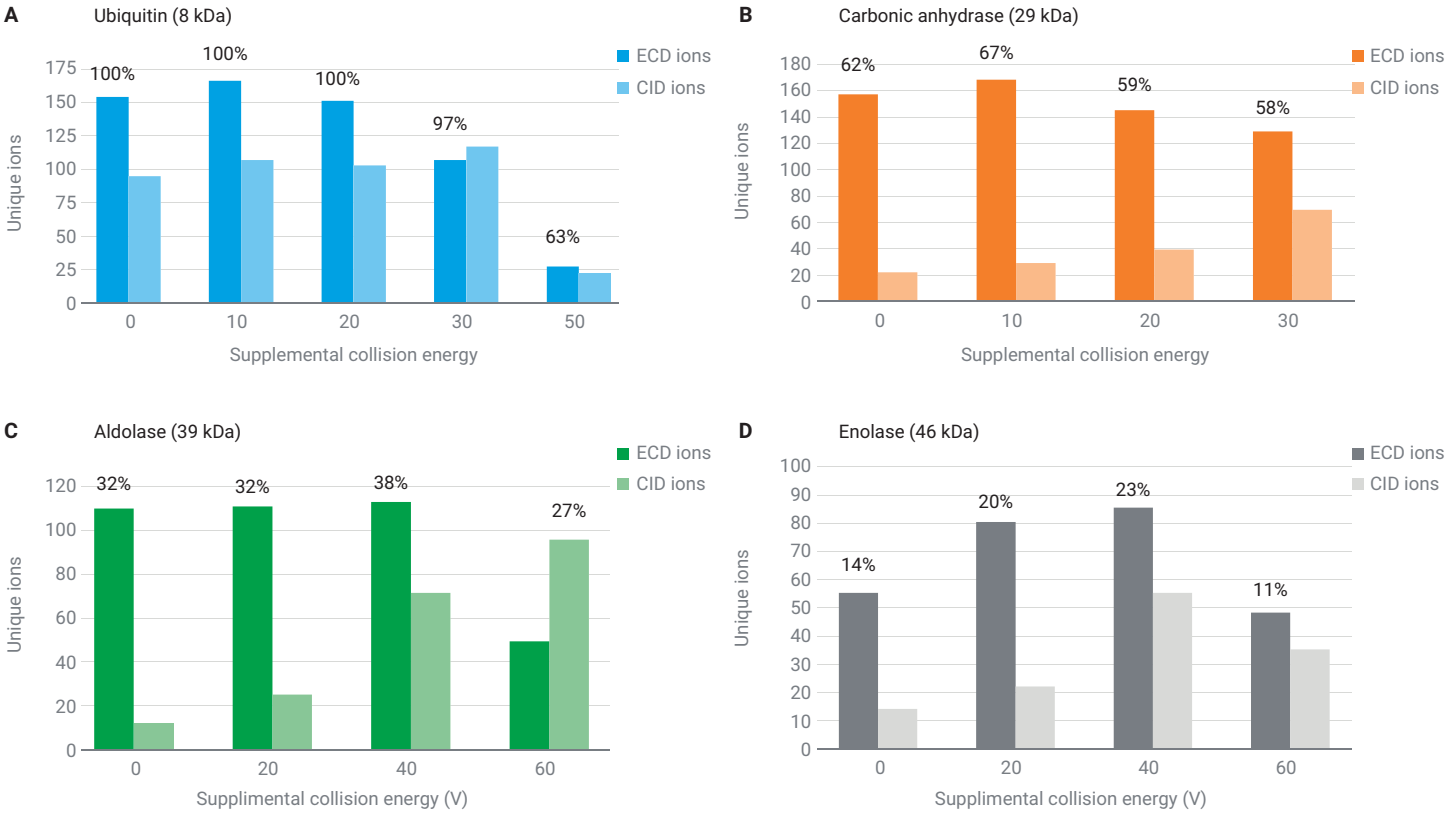

Figure 4. Unique fragment ions detected from combined ECD + CID experiments targeting (A) ubiquitin, (B) carbonic anhydrase, (C) aldolase, and (D) enolase. The numbers on top of each column correspond to the sequence coverage obtained under each condition.

Similar trends were observed for aldolase (39 kDa) and enolase (46 kDa). For both proteins, 40V of supplemental collisional activation was found to be optimal, increasing sequence coverage by 6% and 9%, respectively. Beyond 40V, sequence coverage and ion identifications were negatively impacted (Figures 4C and 4D), reinforcing the importance of optimizing collisional activation to maximize top-down sequence coverage.

Impact of spectral averaging and method reproducibility

Spectral averaging increases signal to noise ratios and often results in improved top-down sequence coverage. However, observed coverage gains diminish as more spectra are averaged (Figure 5). Therefore, the best results will be obtained with sample introduction strategies such as direct infusion that enable extended acquisition times needed for averaging. Despite the benefit of extended averaging, meaningful top-down data can still be obtained on LC/MS timescales.

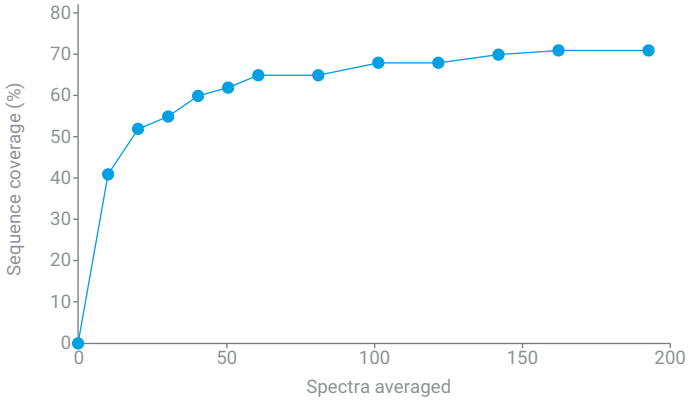

Figure 5. The sequence coverage percent of the 26^+ carbonic anhydrase precursor increases with more averaged spectra.

To assess top-down sequence coverage reproducibility, the tuning procedure described above was applied to the replicate analysis of carbonic anhydrase 26^+ precursor. Table 5 summarizes sequence coverage across replicates collected days or even weeks apart. The standard deviation of day 1–7 replicates is ±2.15%, while same day measurements on days 1 and 4 show a tighter deviation of ±1.51%. Additionally, two different Agilent users applied the method on the same instrument, demonstrating robustness and transferability between users.

Table 5. Reproducibility of sequence coverage obtained from the 26^+ carbonic anhydrase using ECD only. Seven days over a span of 52 days were selected as sampling points to evaluate sequence coverage reproducibility.

Replicate	Day	Operator	Carbonic Anhydrase Sample	Carbonic Seq. Coverage (26^+)
1,2,3	Day 1	User 1	Sample 1	60%, 61%, 60%
4	Day 2	User 1	Sample 1	60%
5	Day 9	User 1	Sample 1	62%
6	Day 36	User 2	Sample 2	67%
7,8,9	Day 44	User 1	Sample 2	61%, 62%, 64%
10	Day 49	User 1	Sample 2	62%
11	Day 52	User 2	Sample 2	60%

Conclusion

This application note presents a practical framework for implementing top-down fragmentation of denatured proteins using the Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer with an ExD cell. These results provide a baseline for expected results across a range of protein sizes and charge states. By optimizing key parameters such as collision energy and acquisition time, researchers can enhance fragment ion generation and improve sequence coverage of intact proteins. The combination of ECD with low-level collisional activation consistently increases the number of unique fragment ions detected, supporting confident proteoform-level analysis. This approach is particularly relevant for characterizing mid-sized, industrially relevant proteins such as erythropoietin, interleukin-6, and protein hormones like insulin and human growth hormone. Proteoform-level analysis of these proteins can reveal structural variants and modifications which are important biotherapeutic quality attributes. The integrated solution for top-down analysis presented here enables researchers to gain deeper insights from proteoform biology, accelerating biopharmaceutical innovation.

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