



In-Depth Protein Sequence Verification by TIMS-enabled MALDI Top-Down Sequencing using OmniScape software

OmniScape software extracts a maximum of sequence information from TIMS-enabled MALDI-TDS data while requiring minimal effort to get from information-rich spectra to high-confidence sequence readout.

Abstract

MALDI Top-Down Sequencing (MALDI-TDS) is a powerful method for rapid sequence characterization of intact proteins. MALDI-TDS is a “fragment all” approach that analyzes N- and C-terminal fragment ions, predominantly singly charged, generated by In-Source Decay in the MALDI ion source (MALDI-ISD) achieving unparalleled depth of sequence readout at high fragment coverage.

Using the timsTOF fleX instrument, MALDI-TDS benefits from accurate mass and high isotopic fidelity, increasing the confidence in sequence readout and proteoform assessment. Trapped Ion Mobility Spectrometry (TIMS) separates MALDI-ISD fragment ions by charge state and terminal origin, allowing for comprehensive sequencing of up to 130 amino acid residues from both termini, including assessment of modifications and verification of protein terminal status via T³-Sequencing.

OmniScape™ software offers an intuitive interface for fast analysis of TIMS-enabled MALDI-TDS data, achieving sequence verification with minimal effort. Key software features include the OmniWave™ algorithm for precise and sensitive peak detection in complex Top-Down MS/MS data and a unique Result Combination workflow for integrating sequencing results from multiple analyses, such as MALDI-ISD and T³-Sequencing.

We applied OmniScape's Confirmation workflow to the sequence analysis of NIST8671 reference mAb, in which 99% of the antibody's amino acid sequence were verified, including all six CDRs, and the modification status of N- and C-terminus was successfully confirmed.

Keywords:
MALDI Top-Down Sequencing, MALDI, MALDI-TDS, In-Source Decay, MALDI-ISD, T³-Sequencing, proteins, biologics, sequence verification, modifications, timsTOF fleX, OmniScape, data analysis, software

Introduction

MALDI Top-Down Sequencing (MALDI-TDS) is an effective method for rapid characterization of intact proteins regarding primary sequence, terminal status and near-terminal modifications. Unlike other top-down MS/MS methods, it is a “fragment all” technique that analyzes N- and C-terminal protein backbone fragments generated by In-Source Decay (MALDI-ISD) during the MALDI ionization process. The predominantly singly charged MALDI-ISD fragments permit a straightforward sequence readout, making MALDI-TDS a simple but highly efficient method for instant sequence verification of protein expression products, such as biologics.

When acquired on the Bruker timsTOF fleX instrument, MALDI-TDS data provide accurate mass and high isotopic fidelity enhancing the confidence in sequence readout and proteoform assessment. TIMS adds ion separation in the gas phase, enabling the dissection of MALDI-ISD fragments by charge state and terminal origin [1,2].

MALDI-TDS analysis on timsTOF fleX typically comprises the following steps:

- 1 MALDI-ISD-TOF analysis (TIMS off):**
The resulting ISD spectrum offers ultimate sequencing depth (up to 130 residues from both termini) based on the analysis of singly charged MALDI-ISD fragment ions.
- 2 TIMS-enabled MALDI-ISD-TOF analysis:**
TIMS separation minimizes isobaric interferences that cannot be resolved by mass spectrometry (MS) alone. This makes TIMS-enabled MALDI-TDS particularly useful for the verification of ambiguous amino acid calls hampered by isobaric signal overlaps in the initial MALDI-ISD-TOF analysis. For certain proteins, TIMS even achieves separation of MALDI-ISD fragments according terminal origin, allowing for dissection of N- and C-terminal MALDI-ISD spectra. This reduces data complexity and simplifies data analysis in de novo sequencing tasks.
Moreover, TIMS separates MALDI-ISD fragment ions according their charge state, allowing for the utilization of 2+ MALDI-ISD fragments for sequence readout. This new capability increases sequence coverage by separating low abundant 2+ fragments from the highly abundant 1+ ions to access sequence regions that are out of reach of 1+ ISD fragments.
- 3 T³-Sequencing (MALDI-ISD-CID-MS/MS):**
By isolating selected N- and C-terminal MALDI-ISD fragment ions for CID-MS/MS analysis, T³-Sequencing [3] allows for verification of the protein's terminal status, extending the sequence readout towards the termini (typically the first 8-10 amino acid residues), which are not directly accessible by MALDI-ISD because of the complex spectral background commonly observed in MALDI-ISD-TOF spectra below m/z 1000.

Although TIMS-enabled MALDI-TDS datasets are rather small, they contain rich information that requires advanced software tools for efficient extraction of sequence information. Bruker's new OmniScape software addresses this requirement to full extent:

- Minimal effort is needed to convert MALDI-TDS data into scored sequence verification results.
- OmniScape's proprietary OmniWave algorithm excels in detecting peaks in complex top-down MS/MS data, including partially overlapping isotopic clusters.
- The Result Combination workflow seamlessly compiles sequencing results from multiple top-down experiments, such as MALDI-ISD and T³-Sequencing, as well as various ionization modalities, fragmentation techniques, and instrument types.

In this application note, we used OmniScape's Confirmation and Result Combination workflows for the analysis of TIMS-enabled MALDI-TDS data of bovine carbonic anhydrase II, a 29 kDa protein. Additionally, we utilized the new software for sequence verification of the NIST8671 monoclonal antibody (mAb) reference material, achieving an MS/MS coverage of 99%, with all six CDRs covered and confident confirmation of N- and C-terminal status.

Experimental

Sample preparation

Bovine carbonic anhydrase isoenzyme II (CAII; MW 29 kDa; Merck) was dissolved in 0.1 % TFA in water at a concentration of 100 pmol/ μ L. 0.25 μ L aliquots were spotted on the MALDI target plate.

NISTmAb8671 humanized IgG1k monoclonal antibody reference material (Merck) was deglycosylated using IgZERO (Genovis), cleaved into subunits using IdeS (FabRICATOR, Genovis) and dithiothreitol (DTT, Merck) as reducing agent. Resulting subunits (light chain (LC), Fc/2 and Fd) were separated by analytical reversed-phase C4 liquid chromatography with UV detection applying a 10-minutes acetonitrile gradient. Eluting subunits from 3 subsequent LC runs (injected sample amount 30 μ g each) were collected into 1.5 mL tubes. Collected LC fractions were vacuum-dried and reconstituted in 20 μ L 0.1% TFA in water. 0.5 μ L aliquots of the reconstituted subunits were prepared for MALDI analysis.

All samples were prepared on a Bruker MTP AnchorChip 384 BC MALDI target plate. Sample aliquots were spotted first. The dried samples were overlaid with 0.5 μ L of SDHB matrix (Bruker; 25 g/L in 50/50 ACN/H₂O, 0.05% TFA) and dried down under ambient conditions.

Data acquisition

All data were recorded on a Bruker timsTOF fleX instrument equipped with a dual MALDI/ESI ion source. The instrument's TIMS In pressure was reduced to 1.7 mbar to ensure optimum signal intensities in the higher m/z range beyond m/z 8000 [4].

MALDI-TDS data were acquired operating the instrument in positive MALDI-MS, MALDI-TIMS-MS and, in case of T³-Sequencing, MALDI(-TIMS)-MS/MS mode, respectively. Instrument m/z calibration was performed in MALDI operation mode using red phosphorus as a reference substance.

Data processing and analysis

Raw data were preprocessed in DataAnalysis 6.1 software (Bruker). Preprocessing included the following steps:

- Extraction of spectra from ion-mobility regions (in case of TIMS-enabled data)
- Smoothing (Savitzky Golay; smoothing width 0.06 m/z)
- Baseline correction (flatness 0.8)
- Export of preprocessed profile spectra as Simple x-y ASCII files (*.xy).

Data were analysed using Bruker's OmniScape 2025b software. For matching MALDI-MS and T³-Sequencing spectra against target protein sequences using OmniScape's Confirmation workflow, the following parameter settings were applied and saved as OmniScape methods: Mass Error 6 ppm; Min/Max Charges 1/1 (in case of extracted 2+MALDI-MS-TIMS-MS spectra: 2/2); Ion Types (MALDI-MS) a, c, y, z+2; Ion Types (T³-Sequencing) a, b, y, including neutral-loss ions (-NH₃, -H₂O); Noise curve offset (Simple moving average, SMA) was adjusted for individual spectra so that the threshold line matched the upper border of the noise band in the higher m/z region of the spectrum.

The overall MS/MS sequence coverage was compiled across 1+ and 2+MALDI-MS(-TIMS)-TOF and T³-Sequencing analyses utilizing OmniScape's Result Combination workflow.

Results

Bovine Carbonic Anhydrase II (MW 29 kDa)

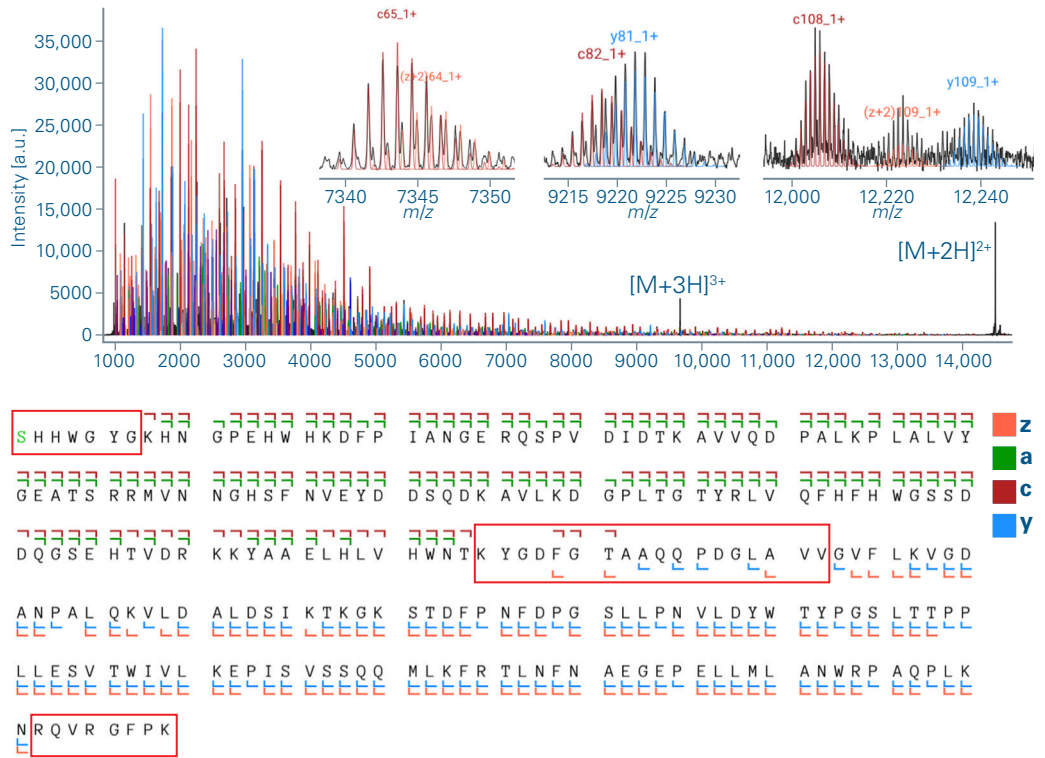


Figure 1. MALDI-MS/MS analysis of bovine CAII.

Top: MALDI-MS/MS spectrum matched against the CAII sequence. The spectrum was acquired in two segments (m/z 1000 – 6900; m/z 6900 – 15,000) which were stitched together after their export as Simple ASCII files. OmniScape's proprietary OmniWave feature finding algorithm ensured robust detection of fragment ion signals even at low signal-to-noise ratio, and reliably dissected partially overlapping isotopic clusters. Resolving power provided by timsTOF fleX enabled isotopic fidelity of fragment ion signals across the entire m/z detection range. Bottom: CAII sequence map obtained from the MALDI-MS/MS spectrum shown in the top panel. Considering 1+ MALDI-MS/MS fragments, the data verified 90.7% of the protein sequence. Colored frames indicate sequence regions that were not completely covered by the spectrum.

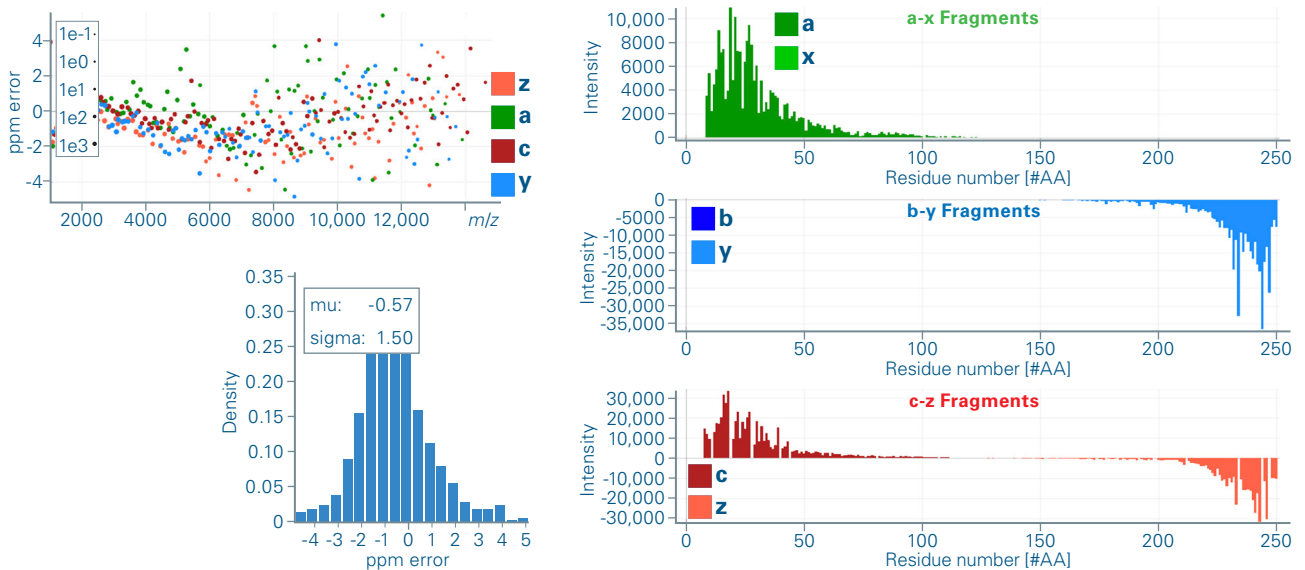


Figure 2. Result statistics provided by OmniScape for sequence verification of bovine CAII based on the MALDI-MS/MS spectrum depicted in Figure 1.

The fragment ion statistics (right) indicates a well-shaped intensity distribution for the dominating, MALDI-MS/MS specific ion types a, c, y and z+2. The mass error plots (left) illustrate the outstanding MALDI-MS/MS data quality delivered by timsTOF fleX. Accurate-mass detection throughout a wide m/z range enabled high-confidence sequence readout at unparalleled coverage.

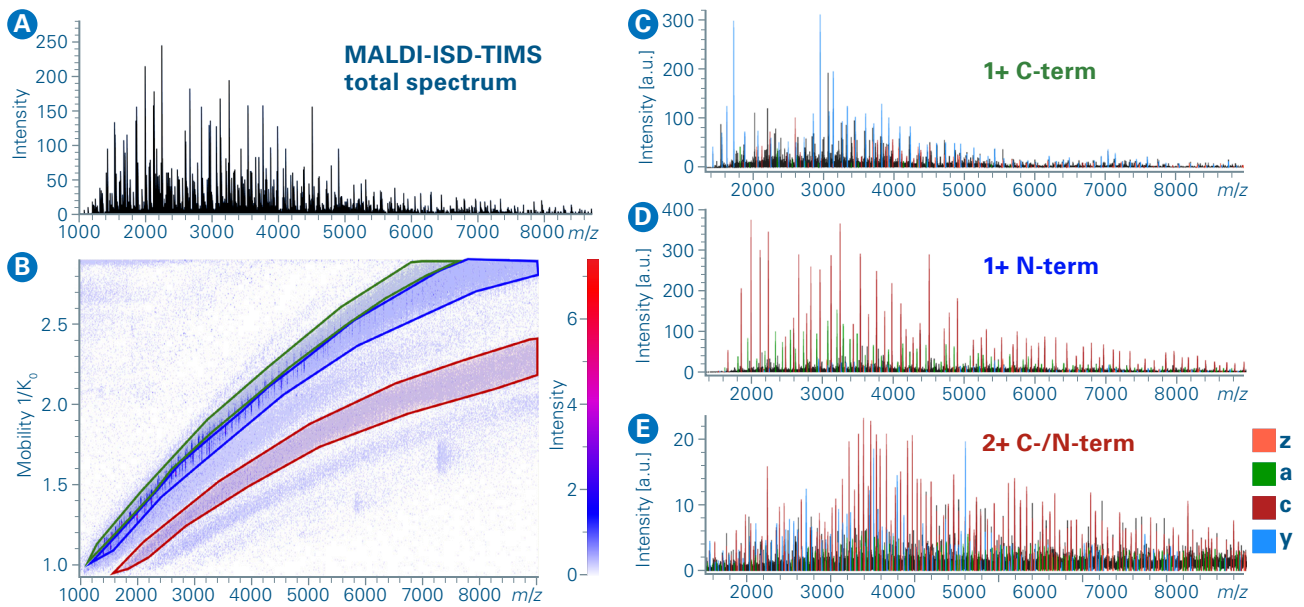


Figure 3. TIMS-enabled MALDI-ISD-TOF analysis of bovine CAII.

(A) Total TIMS frame MALDI-ISD-TOF spectrum. **(B)** TIMS heatmap (mobilogram) obtained from MALDI-ISD-TIMS-TOF analysis. TIMS separates MALDI-ISD fragments by charge state. Without their separation from the highly abundant 1+ signals, the majority of low abundant 2+ fragments would remain undetected. As a further benefit, TIMS allows to partially dissect 1+ MALDI-ISD fragment ions by their origin from either N- or C-terminus, thus reducing the level of data complexity significantly. **(C), (D)** Annotated MALDI-ISD-TOF spectra extracted from ion mobility regions accommodating singly charged N- or C-terminal fragment ions. Extracted spectra show a clear dominance of either C-terminal y- and z+2-ion series (panel C) or N-terminal a- and c-ion series (panel D). Terminally dissected MALDI-ISD spectra are particularly useful for verifying ambiguous amino acid calls, e.g., caused by isobaric overlaps in the initial MALDI-ISD-TOF analysis, and simplify data interpretation in *de novo* sequencing tasks. **(E)** Annotated MALDI-ISD-TOF spectrum extracted from the mobility region of doubly charged fragment ions. Although being of lower abundance, charge-separated 2+ fragment ions provided additional sequence information from sequence regions not covered in the 1+MALDI-ISD spectrum (see Figure 4).

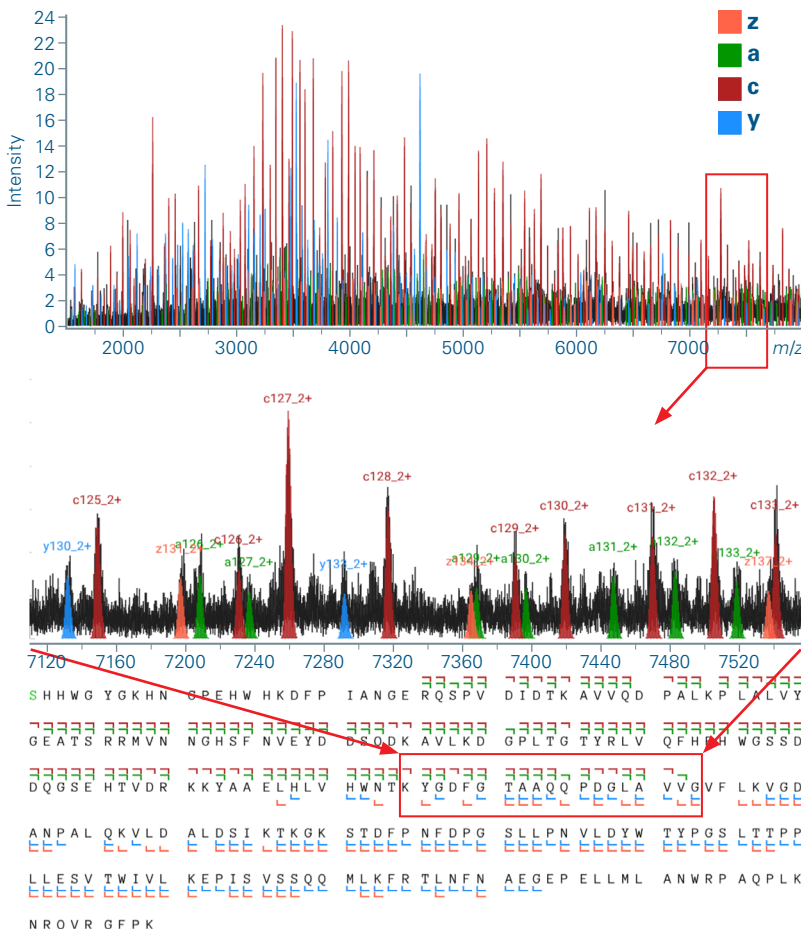


Figure 4. MALDI-ISD-TOF spectrum extracted from the mobility region accommodating 2+MALDI-ISD fragment ions of bovine CAII. Doubly charged MALDI-ISD fragments provided for extended depth of sequence readout. In particular, the 2+ c-type ion series increased the coverage for the central part of the CAII sequence.

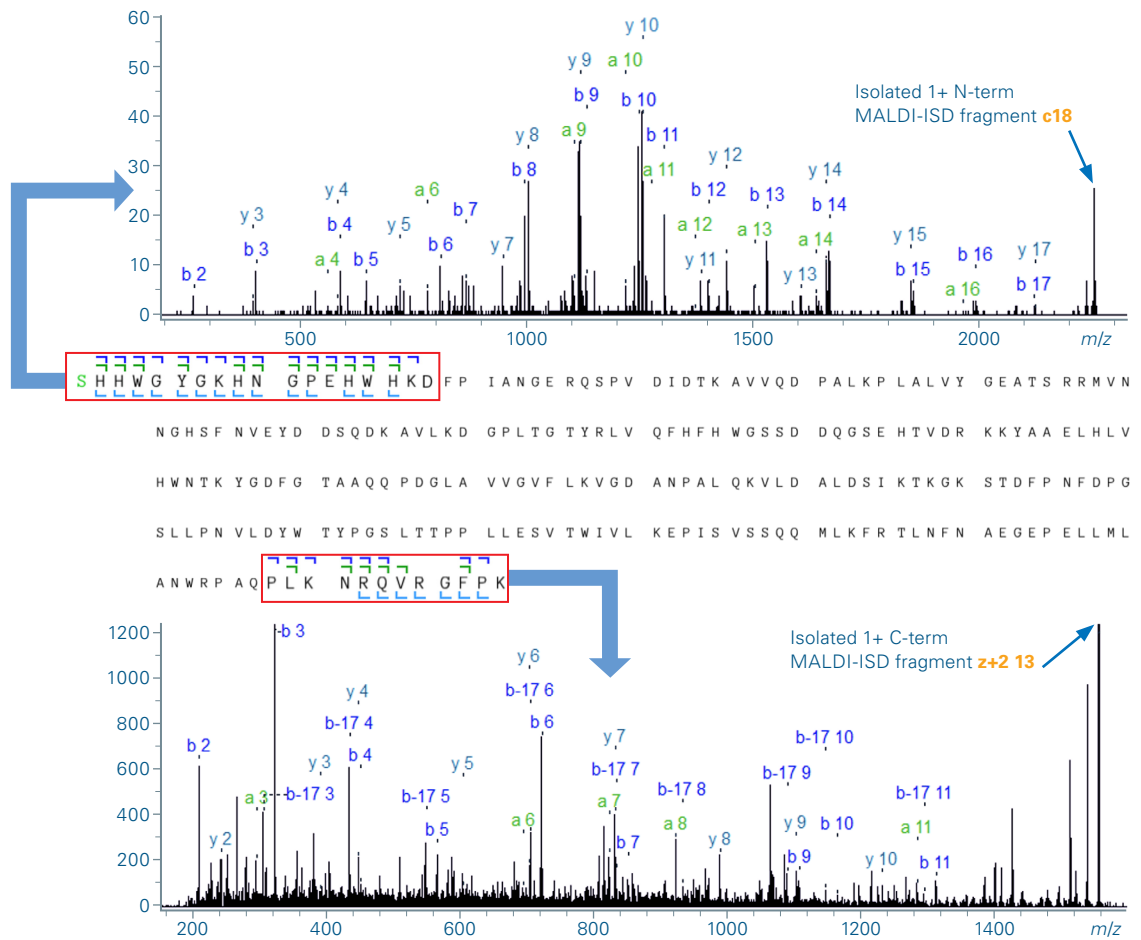


Figure 5. Verification of terminal status in bovine CAII by T³-Sequencing of selected N- and C-terminal 1+MALDI-ISD fragments.

Top: CID-MS/MS spectrum of MALDI-ISD fragment c18 confirmed the presence of the acetylated protein N-terminus and extended the N-terminal sequence readout down to amino acid position H2. Bottom: CID-MS/MS spectrum of MALDI-ISD fragment (z+2)13 extended the C-terminal sequence readout to the second C-terminal amino acid residue.

Name	SC [%]
▼ CAII_bovine	100.00
• CAII MALDI-ISD TTFleX	90.70
• CAII MALDI-ISD TIMS 1+ C-term TTFleX	47.29
• CAII MALDI-ISD TIMS 1+ N-term TTFleX	45.35
• CAII MALDI-ISD TIMS 2+ TTFleX	79.84
• CAII T3 c18 TTFleX	6.59
• CAII T3 z13 TTFleX	4.26



Figure 6. Combined sequence map from MALDI-ISD and T³-Sequencing analysis of bovine CAII.

Compilation of results using OmniScape's Result Combination workflow required only few mouse clicks to report the overall sequence coverage. Complementary sequence information extracted from 1+ and 2+ MALDI-ISD data and T³-Sequencing of selected N- and C-terminal fragments yielded a total MS/MS sequence coverage of 100% for CAII, a 29 kDa protein.

Sequence verification of NISTmAb8671RM subunits

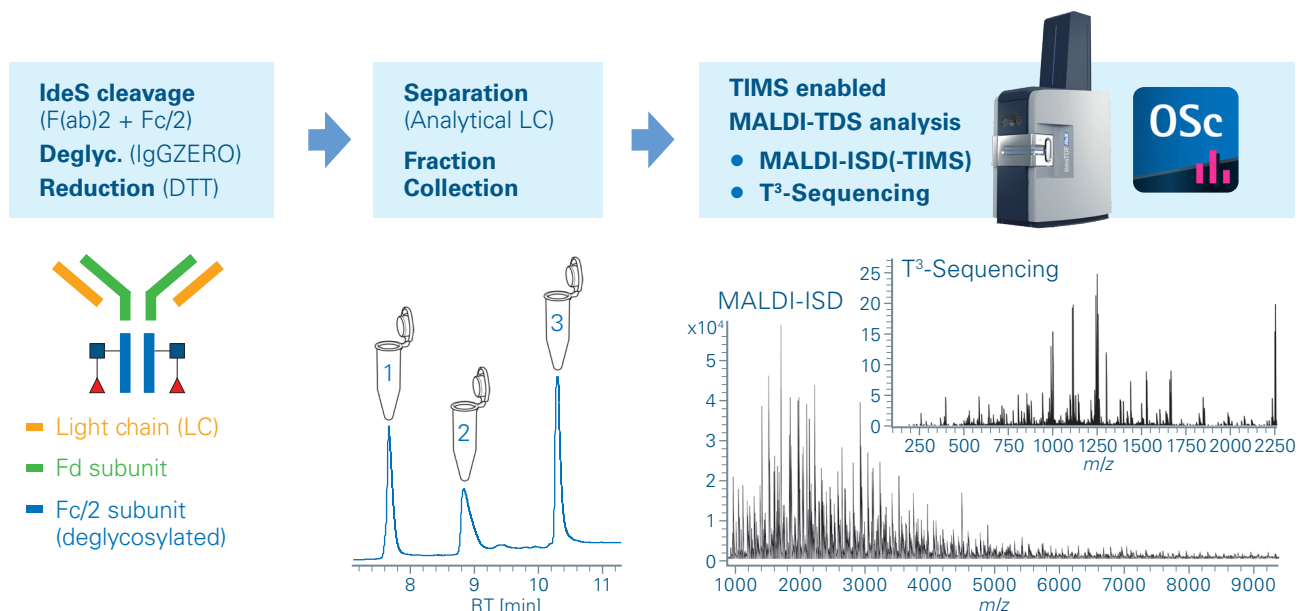


Figure 7. Scheme of the analysis workflow for sequence verification of NISTmAb8671RM.

IdeS cleavage and reduction yielded 23-25 kDa subunits ideally suited for TIMS-enabled MALDI-TDS analysis, enabling virtually complete sequence verification.

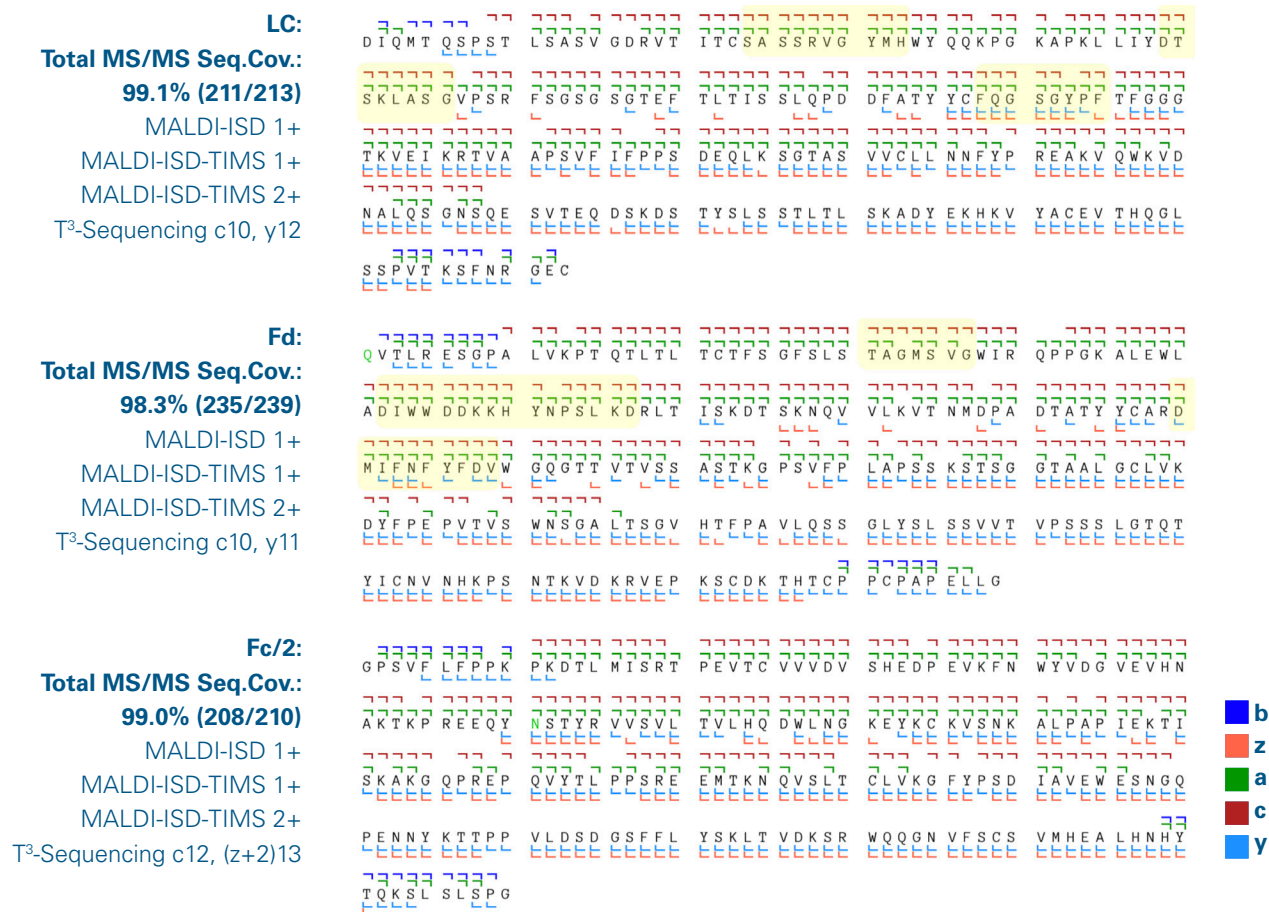


Figure 8. Overview over the total sequence verification result obtained from TIMS-enabled MALDI-TDS analysis of NISTmAb8671RM.

Sequence coverage values were calculated by OmniScape's Result Combination workflow based on integration of results obtained from 1+ and 2+MALDI-ISD data, and N- and C-terminal T³-Sequencing. Overall, 99% of the antibody sequence were confidently verified, including all six CDRs (highlighted in yellow background color). For the heavy chain, a pyroGlu modified N-terminal glutamine and deletion of the C-terminal lysine residue were confirmed.

Conclusions

- OmniScape maximizes the sequence information extractable from TIMS-enabled MALDI-TDS data acquired on timsTOF fleX, requiring minimum effort to get from preprocessed spectra to scored sequence verification results.
- OmniScape's proprietary OmniWave algorithm enables robust and sensitive peak detection in complex top-down MS/MS data leveraging the high mass accuracy and isotopic fidelity of timsTOF fleX data.
- The new OmniScape workflow benefits from TIMS separation of MALDI-ISD fragment ions. For the first time, mobility separation of MALDI-ISD fragments by charge state allowed for the analytical utilization of low abundant 2+ fragment ions, resulting in extended sequencing depth.
- TIMS based dissection of N- and C-terminal MALDI-ISD spectra clarifies ambiguous amino acid calls caused by isobaric signal overlap and simplifies data interpretation in *de novo* analysis tasks.
- The Result Combination workflow featured by OmniScape reports the total sequence coverage obtained from MALDI-ISD and T³-Sequencing analyses as well as from multiple ionization modalities, fragmentation types and instrument platforms. This enhances the sequencing quality and the accuracy of proteoform annotations.

References

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