

## Intact antibody drug conjugate (ADC) analysis

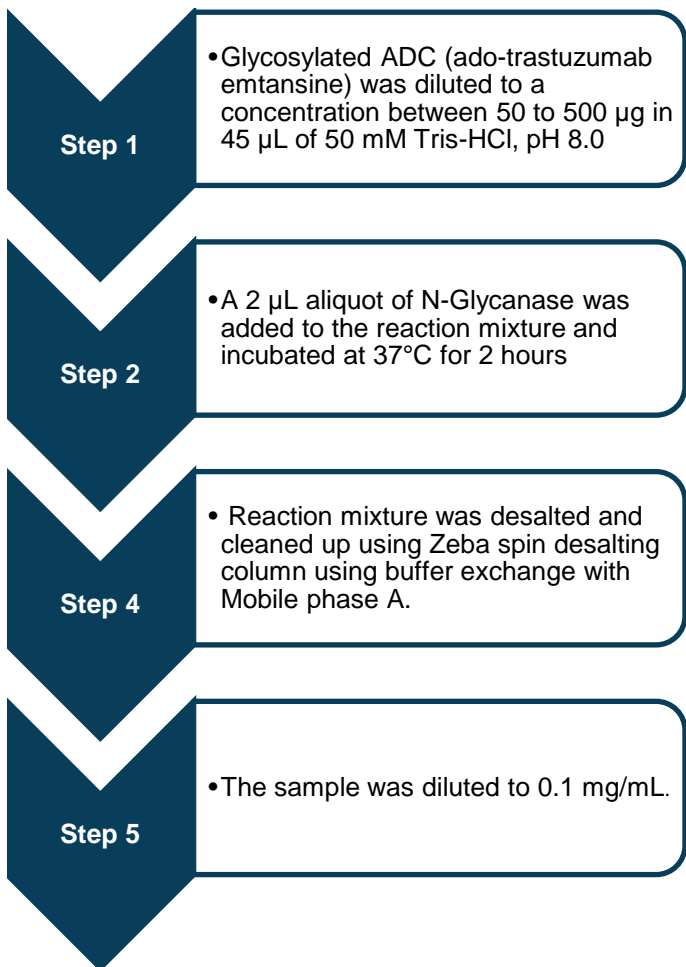
*Routine high-resolution accurate mass analysis of intact ADCs on the X500B QTOF System*

Method details for the routine characterization of an ADC biotherapeutic by high-resolution intact mass analysis using HPLC coupled with the X500B QTOF System, powered by the SCIEX OS Software.



### Sample Prep

A generic sample preparation strategy is shown for deglycosylation and general clean-up of an intact ADC biotherapeutic prior to LC-MS analysis.



### LC Method

|                           |  |            |
|---------------------------|--|------------|
| <i>Column</i>             | Waters Acquity UPLC BEH C4, 2.1mm x 50mm, 300A, 1.7 µm |            |
| <i>Mobile Phase A</i>     | Water, 0.1% Formic acid                                |            |
| <i>Mobile Phase B</i>     | Acetonitrile, 0.1% Formic acid                         |            |
| <i>Flow rate</i>          | 200 µL/min   |            |
| <i>Column temperature</i> | 80° C  |            |
| <i>Injection volume</i>   | 10 µL, 1 µg total protein                              |            |
| <i>Gradient profile</i>   | <b>Time (min)</b>                                      | <b>% B</b> |
|                           | 2.0  | 10         |
|                           | 6.0  | 90         |
|                           | 7.0  | 90         |
|                           | 7.1  | 10         |
|                           | 10   | 10         |

## MS Method

Suggested starting MS method parameters for routine intact ADC analysis as displayed in SCIEX OS. For best sensitivity and resolution, the declustering potential (DP) and collision energy (CE) parameters should be optimized for each individual biotherapeutic.

The screenshot shows the 'MS Method' configuration window in SCIEX OS. The window title is 'MS Method' and it is currently in 'Running' status. The main configuration area is titled 'Intact ADC method' and includes the following sections:

- Method Overview:**
  - Device: X500 QTOF
  - Ion Source: TurboSpray
  - Method duration: 10 min
  - Estimated cycles: 1137
  - Total scan time: 0.527524 sec
  - Intact protein mode: ON
  - Options:  Large proteins (>70 kDa),  Decrease detector voltage
- Source and Gas Parameters:**
  - Ion source gas 1: 40 psi
  - Ion source gas 2: 40 psi
  - Curtain gas: 30 psi
  - CAD gas: 7 psi
  - Temperature: 400 °C
- Experiment (TOF MS):**
  - Polarity: Positive
  - Spray voltage: 5000 V
  - TOF start mass: 900 Da
  - Declustering potential: 250 V
  - TOF stop mass: 4500 Da
  - DP spread: 0 V
  - Collision energy: 20 V
  - Accumulation time: 0.5 s
  - CE spread: 0 V
- Advanced Experiment Settings:**
  - Time bins to sum: 120
  - Channel 1:
  - Channel 2:
  - Channel 3:
  - Channel 4:

The bottom status bar shows 'Data Acquisition' with 'MS' selected and buttons for 'Start', 'Stop', and 'Save...'.

## Batch

In Batch setup, open the 'Automated Calibration Editor' window in order to select the use of the autocalibration function. Designate use of the 'X500 ESI Positive Calibration Solution', and then determine how often you would like the system to perform a fast, automated calibration. These short calibrations will be added automatically to your queue once you have submitted a sample batch.

The screenshot shows the 'Batch' software interface. At the top, there's a navigation bar with 'Batch' and 'Running' status. Below it is a toolbar with buttons like 'Auto-Calibrate...', 'Plate Layout...', 'New', 'Open', 'Save', 'Print...', 'Manage', and 'Submit'. The main area contains a table with columns: Sample Name, MS Method, LC Method, Rack code, Vial position, and Data File. The first row is 'Intact protein' with 'intact protein analysis MS' as the MS Method and 'Intact\_10min' as the LC Method. Overlaid on this is the 'Batch - Automatic Calibration Editor' dialog box. The dialog has a title bar and a close button. The main text says 'Provide ion reference and calibrant delivery settings to be applied automatically, at the correct frequency during acquisition'. There are three main settings: 'Ion reference table' (a dropdown menu currently open showing a list of solutions with 'X500 ESI Positive Calibration Solution' selected), 'Calibrate every' (a dropdown menu set to '3' samples), and 'Calibrant delivery' (a dropdown menu set to 'CDS'). There are also 'Edit...', 'OK', and 'Cancel' buttons.

This is a close-up of the 'Batch - Automatic Calibration Editor' dialog box. The 'Ion reference table' dropdown is now closed and shows 'X500 ESI Positive Calibration Solu...'. The 'Calibrate every' dropdown is set to '3' samples. The 'Calibrant delivery' dropdown is set to 'CDS'. The 'CDS channel' dropdown is set to '1'. The 'Edit...' button is visible next to the 'Ion reference table' dropdown. The 'OK' and 'Cancel' buttons are at the bottom right.

# Pharma and Biopharma



## Data Processing

### Process intact biotherapeutic data in BioPharmaView™ Software 2.0.

Input the protein sequence, and assign potential modifications in the 'Assay Information' window. Click on the 'Add Modifications' tab to insert the drug conjugate mass information, as well as expected location. In this case the DM1/Emtansine drug conjugate and linker is conjugated to lysine residues within the protein. The DM1/Emtansine has an average mass of 957.37 Da.

The screenshot displays the BioPharmaView software interface. The 'Assay Information' window is active, showing the 'Sequence Features' tab. The protein is identified as 'Trastuzumab emtansine (ADC)'. The protein sequence is displayed in four chains (LC1, HC1, HC2, LC2). The 'Modifications' section shows a table of modifications, including Emtansine, GOF, GIF, and G2F, with their respective positions and mass shifts. A 'Disulfide Bonds' table is also visible, showing 16 disulfide bonds between chains.

| Chains | Type | Name     | Position  | Maximum Mods per Chain | Modified AA | Applies To | Workflow Usage | Mass Shift |
|--------|------|----------|-----------|------------------------|-------------|------------|----------------|------------|
| 1      | 1-4  | Internal | Emtansine | 2                      | n/a         | K          | Both           | 957.3723   |
| 2      | 2-3  | Internal | GOF       | -                      | N           | N          | Both           | 1444.5339  |
| 3      | 2-3  | Internal | GIF       | -                      | N           | N          | Both           | 1606.5867  |
| 4      | 2-3  | Internal | G2F       | -                      | N           | N          | Both           | 1768.6395  |

|    | From Chain | To Chain | From Cysteine | To Cysteine |
|----|------------|----------|---------------|-------------|
| 1  | 1          | 1        | 23            | 88          |
| 2  | 1          | 1        | 134           | 194         |
| 3  | 4          | 4        | 23            | 88          |
| 4  | 4          | 4        | 134           | 194         |
| 5  | 1          | 2        | 214           | 223         |
| 6  | 4          | 3        | 214           | 223         |
| 7  | 2          | 2        | 22            | 96          |
| 8  | 2          | 2        | 147           | 203         |
| 9  | 3          | 3        | 22            | 96          |
| 10 | 3          | 3        | 147           | 203         |
| 11 | 3          | 2        | 229           | 229         |
| 12 | 3          | 2        | 232           | 232         |
| 13 | 3          | 3        | 264           | 324         |
| 14 | 3          | 3        | 370           | 428         |
| 15 | 2          | 2        | 264           | 324         |
| 16 | 2          | 2        | 370           | 428         |

# Pharma and Biopharma



Navigate to the 'Intact Protein' tab complete processing parameters and to generate the protein forms for matching.

**Processing Parameters**

Matching Tolerance: ± 15.00 Da  
 Start m/z: 2000.00  
 Stop m/z: 4500.00  
 Start Mass: 140167.12 Da  
 Stop Mass: 161374.75 Da

RT Range Processing: Time Selection  
 Perform LC Peak Detection  
 Start RT: 4.11 min  
 Stop RT: 5.24 min

Maximum Number of Combined Modifications per Protein: 20

**Batch Processing Parameters**

Retention Time Tolerance: ± 1.00 min

**Batch Processing Pass / Fail Criteria**

Reconstruction Area Limits: ± 10.0 %  
 Required Form Minimum: ≥ 80 %  
 Restricted Form Maximum: ≤ 120 %

**Characterized Proteins**  Reduced Protein Form

| Batch Usage | Protein Name   | Modifications | User Defined | Mono. Mass  | Avg. Mass | Match... | Reconstruction Area | Retention Time |
|-------------|----------------|---------------|--------------|-------------|-----------|----------|---------------------|----------------|
| 1 Optional  | Deglycosylated |               |              | 145075.6702 | 145167.12 |          |                     |                |
| 2 Optional  | Deglycosylated | Emtansine - 1 |              | 146033.0425 | 146125.67 |          |                     |                |
| 3 Optional  | Deglycosylated | G0F - 1       |              | 146520.2041 | 146612.47 |          |                     |                |
| 4 Optional  | Deglycosylated | G1F - 1       |              | 146682.2569 | 146774.61 |          |                     |                |
| 5 Optional  | Deglycosylated | G2F - 1       |              | 146844.3097 | 146936.75 |          |                     |                |
| 6 Optional  | Deglycosylated | Emtansine - 2 |              | 146990.4147 | 147084.21 |          |                     |                |
| 7 Optional  | Deglycosylated | Emtansine - 1 |              | 147477.5763 | 147571.01 |          |                     |                |
| 8 Optional  | Deglycosylated | G0F - 1       |              | 147639.6292 | 147733.16 |          |                     |                |
| 9 Optional  | Deglycosylated | Emtansine - 1 |              | 147801.6820 | 147895.30 |          |                     |                |
| Optional    | Deglycosylated | G2F - 1       |              | 147947.7870 | 148042.76 |          |                     |                |

Navigate to the 'Settings' icon and review your global 'Intact Protein Settings'

**Intact Protein Settings**

**Chromatographic Data Processing**

Peak Threshold: ≥ 5.00 %  
 Gaussian Smoothing: 0.90 points  
 Number of TOFMS Spectra to Combine: 3 ± scans

**Reconstruction Processing**

Iterations: 20  
 Signal To Noise Threshold: ≥ 20.00  
 Resolution: 1000  
 Gaussian Smoothing: 0.00

**Protein Results**

Relative Result Threshold: ≥ 5.00 %

**Chromatogram Peaks Labeling**

Label Matching Tolerance: 0.10 Minutes  
 Display Labels For: All Peaks

Reset to Default

OK Cancel

# Pharma and Biopharma

Intact protein deconvolution can be performed in seconds, on either a single datafile, or on multiple samples using the batch processing function. Review your intact ADC protein deconvolution results in the BioPharmaView Software window. Annotated reconstruction mass graph (bottom right) hyperlinks to the raw spectra (middle right) to confirm peak identity and show fidelity between raw and deconvoluted data. Detailed information on drug load can be found on the bottom left, with automated calculation of the drug to antibody ratio (DAR) presented clearly in the window.

**Characterize Standard for Intact Protein**

Sample # 1 Experiment # 1

**Processing Parameters**

Matching Tolerance: ± 15.00 Da

m/z Range: 2000.00 to 4500.00

Mass Range: 140167.12 to 157835.49 Da

**RT Range Processing**

Automatic

Time Selection  4.11 to 5.24 min

Perform LC Peak Detection

*Processing settings have changed since characterization was performed. Please reprocess the data to get current results.*

**Results** Matched Unmatched Modifications Summary

| View                                | Protein | Modification | Mean Ratio To Protein |
|-------------------------------------|---------|--------------|-----------------------|
| <input checked="" type="checkbox"/> | Deglyco | Emtansine    | 3.66                  |

**% of Total Area by Multiplicity**

| Protein | Modificati... | Multiplicity | % of Total Area | Summed Area |
|---------|---------------|--------------|-----------------|-------------|
| Deglyco | Emtansine     | 0            | 1.61            | 1.26e4      |
| Deglyco | Emtansine     | 1            | 7.70            | 6.02e4      |
| Deglyco | Emtansine     | 2            | 16.41           | 1.28e5      |
| Deglyco | Emtansine     | 3            | 21.74           | 1.70e5      |
| Deglyco | Emtansine     | 4            | 21.47           | 1.68e5      |
| Deglyco | Emtansine     | 5            | 16.62           | 1.30e5      |
| Deglyco | Emtansine     | 6            | 10.75           | 8.40e4      |
| Deglyco | Emtansine     | 7            | 2.82            | 2.20e4      |
| Deglyco | Emtansine     | 8            | 0.88            | 6.91e3      |

**BPC/TIC Graph**

Intensity, cps vs Time, min. Peak at 4.55 min.

**TOF MS Graph**

Intensity, cps vs m/z, Da. Peaks labeled: 2903.4918, 2760.1578, 2644.4982, 2648.3767, 2526.3056, 2468.2540, 2388.6486, 2359.0567, 2884.7642, 2522.4662, 3085.1567, 2980.8987, 3105.0199, 3269.5101, 3503.0495.

**Reconstruction Graph**

Reconstructed Intensity vs Mass, Da. Peaks labeled: 146336.04, 145378.27, 147291.29, 148036.01, 151852.82, 152066.73.

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Document number: RUO-MKT-02-4522-A



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