High Efficiency Protein A Affinity Columns for the LC-MS and 2D LC-MS Analysis of mAb and msAb

A quiver of LC-MS based approaches for the sub-unit and intact and analysis of intact monoclonal antibodies (mAb) and multispecific antibodies (msAb) directly from clarified cell-culture samples employing a newly developed high-efficiency (3.5 µm particles), high-pressure capable (6500 PSI/450 bar) 2.1 X 20 mm Protein A (ProA) affinity column*

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ProA-RP-MS Subunit Analysis

Key Experimental Parameters

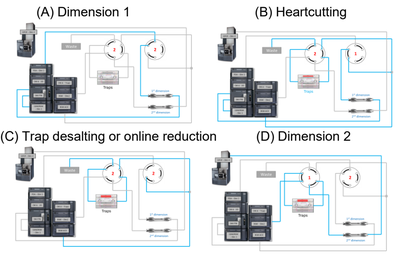
Column: Dim 1 ProA*
Mobile phase A: PBS 2X
Mobile phase B : 100 mM K phosphate, 100 mM KCl, pH3
Step gradient
flow rate: 0.30 mL/min
Temperature 30°C

Trap: BioResolve™ mAb RP Polyphenyl Guard Column, 450Å (2.1 x 30 mm, 2.7 µm)
Dim 2: BioResolve RP mAb Polyphenyl Column, 450Å (2.1 X 50 mm, 2.7 µm)
Mobile phase A: 0.1 % FA in water
Mobile phase B :0.1 % FA in acetonitrile
flow rate: 0.30 mL/min
Temperature 80°C

Sample : Rybrevant™ (amivantamab) diluted in PBS or diluted in cell medium
bsAb load: 0.2 µg

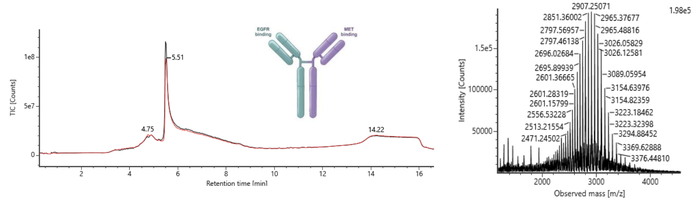
MS: Xevo™ G2-XS™ QTof Mass Spectrometer
Positive mode *m/z* 400-5000
Intact Mass Analysis
Collision energy mode : Off (6V) , Source temp : 100 °C, Desolvation temp : 600 °C, Cone gas : 0 L/h, Desolvation gas : 800 L/h, Capillary voltage: 2.75 kV, Sample cone voltage: 150 V, Intelligent Data Capture: Low
Subunit Mass analysis
Collision energy mode : Off (6V) , Source temp : 100 °C, Desolvation temp : 500 °C, Cone gas : 100 L/h, Desolvation gas : 1000 L/h, Capillary voltage: 2.5 kV, Sample cone voltage: 120 V, Intelligent Data Capture: Low

CDS: waters_connect™ Software

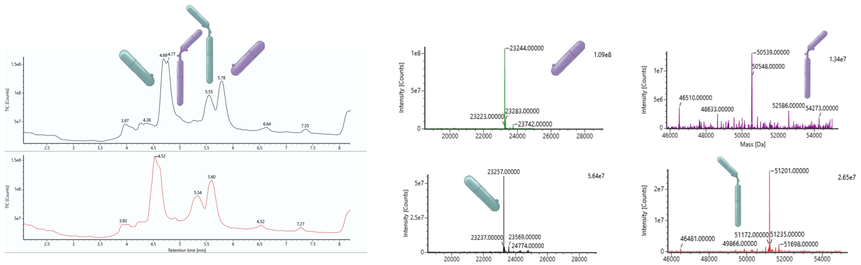


The 2D-LC-MS system consists of 3 pump systems, an autosampler, two CM-A column managers, a PDA detector and a Xevo G2-XS Qtof MS. The system is used in heartcutting mode. (A) in a development phase or for fraction heartcuts, samples are injected on the first dimension followed by UV detection. (B) during the run, on the first dimension, fractions of interest are sent to the trap column by switching positions on the valve of the column manager. (C) At the end of the first dimension run, compounds are trapped on the trap column and can be desalted or reduced online. (D) Finally, the compounds are eluted by sending the flow through the output of the trap column towards the second dimension and detection is performed by ESI-QTOF-MS.

Amivantamab present in a cell culture medium (red), injected on the ProA column coupled to a RP column on a 2D LC MS system eluted with the same intensity and resolution as amivantamab diluted in a buffer (black) . MS identification also showed that the same species were identified.



More detailed information on the sample can be obtained working at the subunit level. This can be perfomed by reduction of the disulfide bonds. This step is automated by sending a flow of 10 mM DTT in ammonium bicarbonate on the trap column while the bsAb is trapped. Subsequently, the four chains are separated on the second RP dimension and identified by MS.



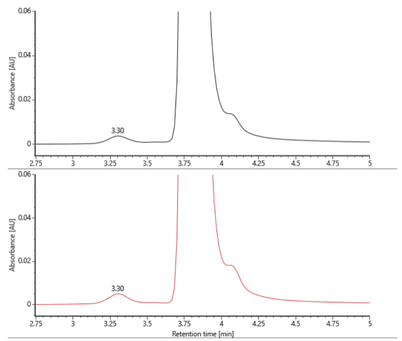
ProA-SEC-MS Intact Analysis

Key Experimental Parameters

Columns: ProA* coupled to an ACQUITY™ Premier Protein SEC Column, 250 Å (4.6 x 150 mm, 1.7 µm)
Mobile phase A: 250 mM amm. acetate
Elution solution : 500 mM acetic acid
flow rate: 0.30 mL/min
mAb load: 20 µg
Sample : Humira™ (adalimumab) diluted in PBS or diluted in cell medium

MS: Xevo™ G2-XS QTof Mass Spectrometer
Positive mode *m/z* 1000-7000
Collision energy mode : Off (6V) , Source temp : 100 °C, Desolvation temp : 500 °C, Cone gas : 100 L/h, Desolvation gas : 600 L/h, Capillary voltage: 2.50 kV, Sample cone voltage: 60 V, Intelligent Data Capture: Low

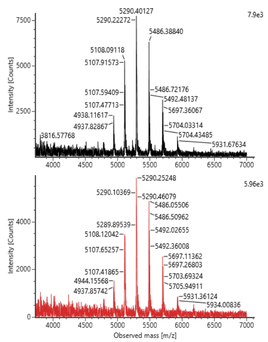
CDS: waters_connect Software



Adalimumab present in a buffer (black) or in a cell culture medium (red) can be purified via the online ProA column coupled to a SEC column prior to MS analysis.

For the two samples, similar chromatographic separation is obtained in terms of intensity and resolution for the separation of HMW and fragments based on the UV280 data.

	% HMW	% monomer	% fragments
mAb (Neat)	0.6	96.0	3.4
mAb in CM	0.6	96.2	3.2



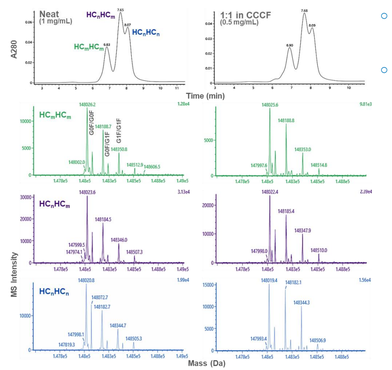
Hyphenating the ProA-SEC column couple to mass spectrometry enables direct mAb identification.

mAb Variant	Avg Mass (Da) and mass error (ppm)	
	Neat	CM
G0F/G0F	148102 (-65)	148101 (-71)
G1F/G0F	148268 (-38)	148266 (-49)
G1F/G1F	148431 (-31)	148429 (-44)

In conclusion, mass variant analysis can be easily performed on in process mAb samples. The high molecular weight fraction is separated from the monomeric mAb peak and from the fragments. In addition, the MS data allow identity confirmation of the mAb of interest. The combination of columns and detectors enable multi determination of size variant and monomer identity.

ProA-MS Intact Analysis

Extended Gradient ProA-MS Native Intact Analysis of msAb HC Pairing



- A280 ProA elution gradient chromatograms and deconvoluted HRMS data for the mock msAb analyzed neat and diluted 1:1 in non-transfected cell culture (CCCF)
- HC_n is the native HC and HC_m is the mutated HC with reduced ProA affinity (Δ mass = +3.05)

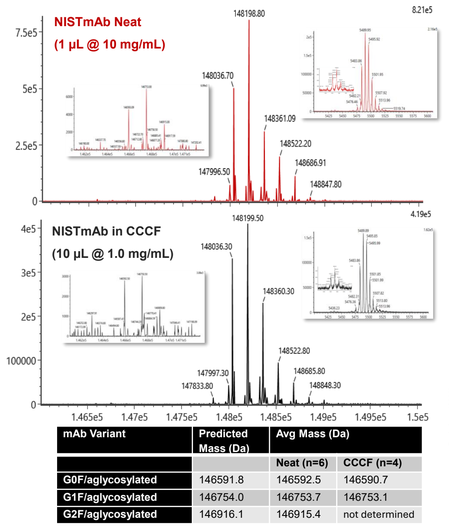
	HC_mHC_m (range, n=2)	HC_nHC_m (range, n=2)	HC_nHC_n (range, n=2)
Mock msAb (Neat)	148026.0 (± 0.5)	148023.4 (± 0.4)	148020.4 (± 0.9)
Mock msAb in CCCF	148025.7 (± 0.2)	148022.7 (± 0.5)	148019.2 (± 0.5)
Predicted MW (G0F/G0F)=	148026.0	148022.9	148019.9

Key Experimental Parameters

mobile phase A: 100 mM amm. acetate
mobile phase B: 200 mM formic acid
flow rate: 0.20 mL/min
gradient: 0% to 10% B (1 min.)
10% to 65% B (10 min.)
msAb load: 10 µg
column: see below*
LC: ACQUITY™ Premier UPLC™ Chromatography System

MS: Xevo G3 QTof Mass Spectrometer
Collision energy mode : Off (6V) , Source temp : 120 °C, Desolvation temp : 500 °C, Cone gas : 50 L/h, Desolvation gas : 600 L/h, Capillary voltage: 2.00 kV, Sample cone voltage: 150 V, Intelligent Data Capture: Custom (1)
CDS: waters_connect Software

High-Throughput ProA-MS Native Intact Analysis of NISTmAb

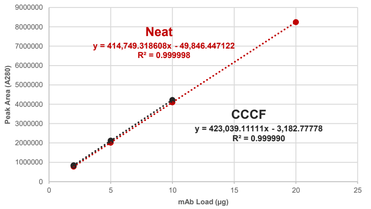


- Deconvoluted high-throughput HRMS data for NISTmAb analyzed neat and diluted to 1.0 mg/mL in non-transfected cell culture (CCCF)
- Left insets show zoom of trace level singly glycosylated variants (masses provided in table) and right insets shows raw data for z=27 and zoom of singly glycosylated variants
- MS signal lower for CCCF sample, likely due higher levels of adducts as A280 peak areas are comparable as shown in response curves
- Response curves indicate linear response for the determination of mAb titer with Pro-MS method

Key Experimental Parameters (different from above)

flow rate: 0.10 mL/min
gradient: 0% to 10% B (2 min.) then 10% to 100% B (1 min.)
NISTmAb load: 10 µg and varying for response curve

mAb Variant	Predicted Mass (Da)	Avg Mass (Da)	
		Neat (n=6)	CCCF (n=4)
G0F/aglycosylated	146591.8	146592.5	146590.7
G1F/aglycosylated	146754.0	146753.7	146753.1
G2F/aglycosylated	146916.1	146915.4	not determined



* BioResolve Protein A Affinity Column, MaxPeak™ Premier Technology, 3.5 µm, 2.1 x 20 mm

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