REVERSED-PHASE LC PRIMARY STRUCTURE CHARACTERIZATION WORKFLOW

AGILENT LC ADVANCEBIO RP-mAb COLUMNS

In this document Agilent applications chemists share their recommendations for an optimum LC system and its configuration for characterizing biomolecules. They also offer guidance on a generic method to get you started, and how this method can be further optimized to meet your specific separation goals.
Additional application information is available at **www.agilent.com/chem/advancebio**

Agilent 1260 Infinity Bio-Inert LC System

Mobile phases

Eluent A: 0.1% TFA
Eluent B: IPA, ACN, and water with 0.09% TFA

Pump (G5611A)

Typical flow rate for 2.1 mm id columns is 1.0 mL/min

Sample injection (G5667A)

1 to 5 µL injection for samples containing 1 to 5 mg/mL of mAb. Samples can be dissolved in water or eluent A.

Column compartment (G1316C)

60 to 90 °C is a typical temperature for good separation

Detection (G1315D)

UV (210, 280 nm) with a 10 mm bio-inert standard flow cell

Guidelines

Bonded phase: C4 and SB-C8 are routinely used. For alternative selectivity use the Diphenyl column.

Gradient: IPA:ACN:water with 0.1% TFA or 0.1% FA to elute all components of interest.

Sample solubility: Mix with starting mobile phase.

Temperature: Higher column temperature can dramatically improve resolution and recovery of proteins.

Resolution/selectivity: A blend of IPA:ACN provides better resolution. Other organic solvent substitutions can be used for different selectivity. Use of IPA results in increased pressure, which can be managed by column temperature and flow rate.

LC/MS

Desalt protein samples before injection.

Small id columns (e.g. 2.1 mm) are often the best choice with a 0.5 to 1.0 mL/min flow rate.

FA provides better MS signals. Use less TFA in the eluent to enhance MS signals, or use AcOH.

IPA with ACN in the mobile phase provides sharper TIC peaks.

Column selection

Column	C4	SB-C8	Diphenyl
2.1 x 50 mm, 3.5 µm	799775-904	789775-906	799775-944
2.1 x 75 mm, 3.5 µm	797775-904	787775-906	797775-944
2.1 x 100 mm, 3.5 µm	795775-904	785775-906	795775-944
2.1 x 150 mm, 3.5 µm	793775-904	783775-906	793775-944
4.6 x 50 mm, 3.5 µm	799975-904	789975-906	799975-944
4.6 x 100 mm, 3.5 µm	795975-904	785975-906	795975-944
4.6 x 150 mm, 3.5 µm	793975-904	783975-906	793975-944

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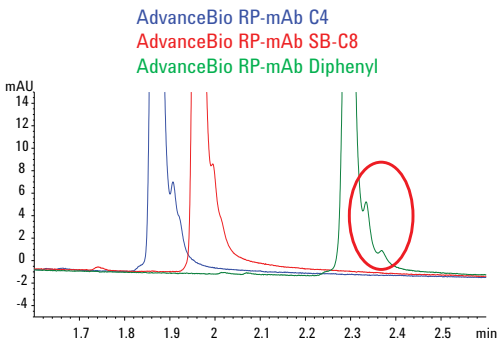
Intact mAb Analysis

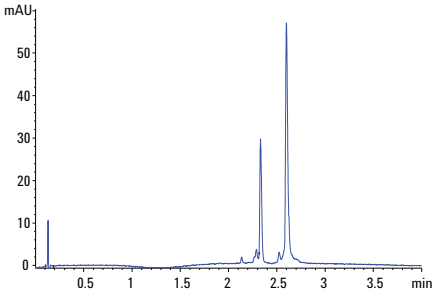
Fast and high-resolution separation



Columns:	2.1 x 100 mm, 3.5 µm
Eluent A:	0.1% TFA in water:IPA (98:2)
Eluent B:	IPA:ACN:Eluent A (70:20:10)
Flow rate:	1.0 mL/min
Gradient:	10-58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B
Injection:	5 µL (1 mg/mL)
Sample:	Herceptin IgG1 variant
Temperature:	80 °C
Detection:	UV, 254 nm

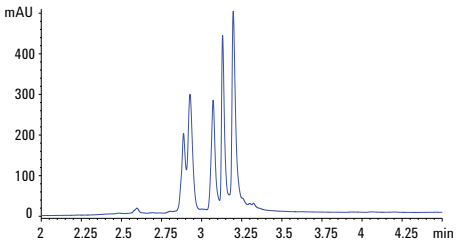
mAb Fragment Analysis

Chemical digestion – heavy chain/light chain



Column:	AdvanceBio RP-mAb Diphenyl, 2.1 x 50 mm, 3.5 µm
Eluent A:	0.1% TFA in water
Eluent B:	IPA:ACN:water (70:20:10) + 0.09 % TFA
Flow rate:	1 mL/min
Gradient:	0 min, 15% B; 0.5 min, 25% B; 1.5 min, 35% B; 1.51 min, 35% B; 3 min, 60% B; 4 min, 60% B
Injection:	1 µL (1 mg/mL) (TECP reduction)
Sample:	Rituximab innovator
Temperature:	80 °C
Detection:	UV, 220 nm

Enzymatic digestion – Fab/Fc regions

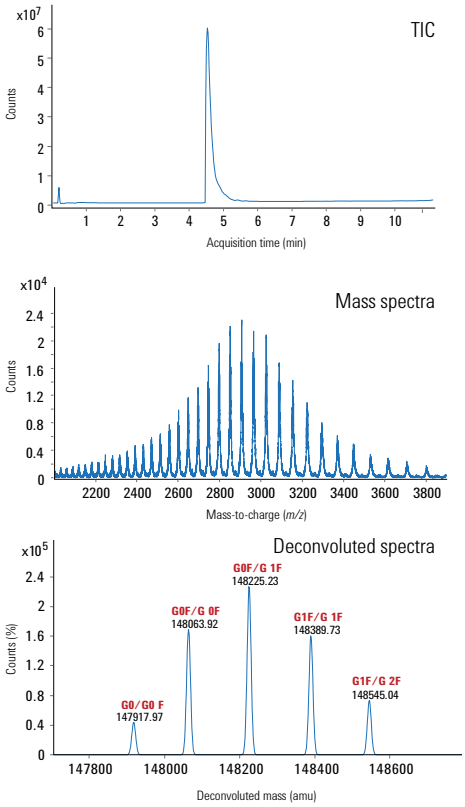


Column:	AdvanceBio RP-mAb C4, 2.1 x 100 mm, 3.5 µm
Eluent A:	0.1% TFA in water
Eluent B:	n-Propanol:ACN:eluent A (80:10:10)
Flow rate:	0.8 mL/min
Gradient:	5-40% B in 5 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B
Injection:	1 µL (2 mg/mL)
Sample:	Herceptin IgG1 variant – papain digested
Temperature:	60 °C
Detection:	UV, 220 nm

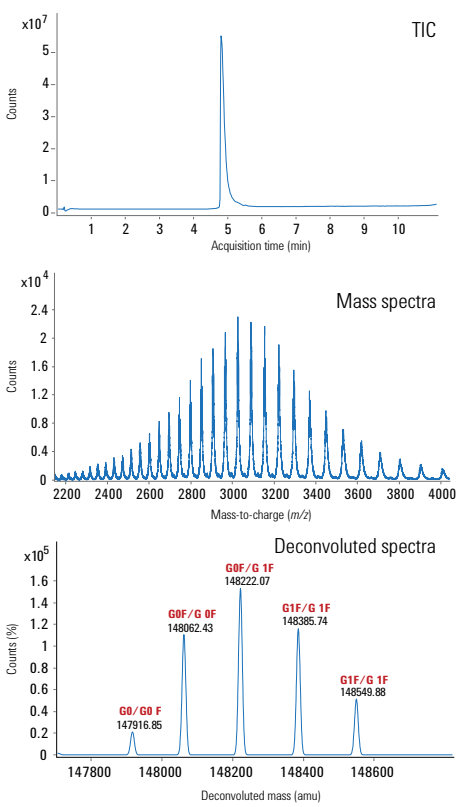
LC/MS Analysis of Intact mAbs

Fast chromatography with excellent peak shape and MS data using formic acid mobile phase

AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm



AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm



Eluent A:	0.1% FA in water
Eluent B:	IPA:ACN:water (80:10:9.9) + 0.1% FA
Gradient:	0 min, 20% B; 4 min, 20% B; 5 min, 40% B; 10 min, 70% B; 11 min, 90% B; 11.1 min, 20% B
Flow rate:	0.6 mL/min
Temperature:	80 °C
Injection:	1 µL (1 µg/µL)
Sample:	Innovator Herceptin
Detection:	Agilent 6530 Accurate-Mass Q-TOF LC/MS

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