ThermoFisher SCIENTIFIC

(U)HPLC columns

SurePac Protein RP MDi columns

Product manual

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Contents

Introduction

Introduction to the column

The Thermo Scientific™ SurePac™ Protein RP MDi™ (Reversed-Phase Monodisperse and Inert) Column is a chromatography column designed for high-resolution separation of proteins including intact monoclonal antibodies (mAb), mAb fragments, bispecifics, Antibody Drug Conjugates (ADCs), and other therapeutic proteins. The stationary phase is compatible with mass spectrometry-friendly mobile phases, including acetonitrile and isopropanol, and acidic modifiers such as trifluoroacetic acid (TFA) or formic acid (FA). The column is packed with supermacroporous, 2.5 µm monodisperse polymer particles (Figure 1), with a highly uniform particle size distribution and broad pore size range. This morphology supports improved mass transfer and efficient separation of both small and large biomolecules, including intact monoclonal antibodies. The polymeric stationary phase is inherently hydrophobic and exhibits broad chemical stability across a wide pH range and thermal stability up to 90 °C, supporting flexible and robust method development. The controlled particle size distribution enables precise column packing and improved lot-to-lot reproducibility. The SurePac Protein RP MDi media is packed in hydrophobic coated stainless-steel hardware to minimize secondary interactions and reduce undesired adsorption of molecules to the metal surfaces.

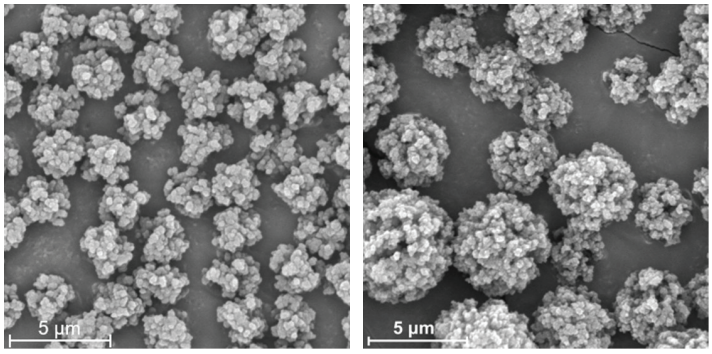


Figure 1. Scanning Electron Microscopy (SEM) image of monodisperse SurePac Protein RP MDi column particles (left) vs. conventional polydisperse particles (right). White scale bars are 5 µm in length.

Operating limits and specification

Table 1. Recommended column operating conditions for optimal performance and extended column lifetime

Column dimension (mm)	Recommended flow rate[1] (mL/min)	Max. column pressure drop[2] psi (bar)	Temperature (°C)	pH
0.3 × 50 mm	0.001 - 0.012	8000 (552)	5 - 90	2 - 10
2.1 × 20 mm	0.2 - 1.0	6500 (448)	5 - 90	2 - 12
2.1 × 50 mm	0.2 - 0.6	6500 (448)	5 - 90	2 - 12
2.1 × 100 mm	0.2 - 0.6	6500 (448)	5 - 90	2 - 12

[1]The maximum usable flow rate is dependent on backpressure. Ensure the column pressure does not exceed the specified pressure limit.
[2]The column pressure drop for a given flow rate is calculated as the pressure of the system with column minus the pressure of system with union in place of column.

Additional requirements for safe column operation

- Always set up the mobile phase flow direction as indicated on the column tag.
- Avoid exposing the column bed to sharp pressure fluctuations that may disrupt the column bed.
- When starting, stopping, or changing the flow rate, a flow ramp rate (mL/min/min) of ~⅓ of the maximum flow rate for the specific column format is recommended.

Getting started

Thermo Fisher Scientific recommends performing an efficiency test on SurePac Protein RP MDi column before use. The purpose of column performance validation is to ensure no damage has occurred during shipping. Steps below outline the necessary process to validate system operation. Test the column using the conditions described on the Certificates of Analysis (CoA) enclosed in the column box. Repeat the test periodically to track the column performance over time. Note that slight variations may be found on two different HPLC systems due to system electronic, hardware, flow path differences, operating environment, reagent quality, column conditioning, and operator technique.

Step 1: Visually inspect the column

Report any visible damage upon receiving the column to Thermo Fisher Scientific immediately.

Step 2: Prepare mobile phases

The SurePac Protein RP MDi column can be used with a variety of mass spectrometry friendly organic solvents such as acetonitrile and isopropanol. For general separations, we recommend adding 0.1% TFA to the mobile phase to achieve optimal chromatographic performance. When coupling the HPLC system to a mass spectrometer, it is recommended to use up to 0.1% FA to improve MS sensitivity and reduce ion suppression.

- **LC/UV experiments**
  - Mobile phase A: Water with TFA (99.9:0.1, v/v).
  - Mobile phase B: Acetonitrile/water with TFA (90:9.9:0.1, v/v/v).
- **LC/MS experiments**
  - Mobile phase A: Water with FA (99.9: 0.1, v/v).
  - Mobile phase B: Acetonitrile/water with FA (90:9.9:0.1, v/v/v).

Step 3: Set up the LC system

Use a biocompatible or inert LC system equipped with a gradient pump, injection valve or autosampler and a detector appropriate for specified application (UV, VWD, DAD, FLD, and/or mass spectrometer). For optimal performance, the system should be configured for low dead volume using small internal diameter with Thermo Scientific™ Viper™ TQ Connectors (e.g., 100 µm ID) and an appropriate detector flow cell (e.g., 2.5 µL semi-micro biocompatible flow cell) that is thoroughly primed prior to use. Operation at elevated column temperatures (70–80 °C) is recommended to improve separation of monoclonal antibodies and antibody fragments. The use of an eluent pre-heater ensures appropriate pre-column mobile phase heating to match column compartment set temperature and minimize on-column thermal induced analyte dispersion for improved peak shape.

Analytical-format SurePac Protein RP MDi columns (2.1 mm ID) should be operated with a high-pressure mixing UHPLC pump, such as Thermo Scientific™ Vanquish™ Horizon and Thermo Scientific™ Vanquish™ Flex UHPLC Systems, to minimize gradient delay and achieve high-resolution separations. Low-flow SurePac Protein RP MDi columns (0.3 mm ID) are typically operated at flow rates of 1-8 µL/min and are recommended for use with low-flow HPLC systems such as the Thermo Scientific™ Vanquish™ Neo UHPLC System. The 0.3 mm ID SurePac Protein RP MDi column is designed for coupling with high-resolution mass spectrometers, such as the Thermo Scientific™ Q Exactive™ BioPharma Platform, to support accurate intact mass analysis and top-down protein sequencing.

Note: For increased 0.3 × 50 mm format longevity and result consistency, it is recommended to use relatively fresh or completely new out-of-the box Thermo Scientific™ nanoViper™ Fingertight Fittings in combination with a torque wrench (Part No. 6250.2110) for easy and consistent column attachment. Regularly check for nanoViper fingertight fittings thread deterioration. If thread screw-in resistance increases, a change of nanoViper fingertight fittings is recommended. 20 µm and 50 µm internal diameter Viper connections are recommended for the 0.3 × 50 mm column.

Getting started (continued)

Step 4: Condition the column

Set the pressure limit on the pump to ≤ 6500 psi (448 bar) for 2.1 mm and 8000 psi (552 bar) for 0.3 mm ID columns. Slowly ramp up the flow rate: a flow ramp rate (mL/min/min) of ~⅓ of the maximum flow rate for the specific column format is recommended. Wash the column with mobile phase for 30 minutes.

SurePac Protein RP MDi columns are designed to minimize secondary interactions and for low carryover. Depending on the nature of your sample, column conditioning may be required prior to achieving optimal performance. To quickly condition the column, we recommend performing 2-3 sample overload injections of 10× - 25× your standard sample loading and standard gradient method.

Step 5: Verify the performance of the column

Each column is shipped with two Certificates of Analysis (CoA) verifying the resin performance and the column performance. Each CoA provides the test conditions used. These tests can be reproduced to check the performance of your column. The lot qualification tests are performed using a 2.1 × 50 mm column and the gradient and flow rate should be scaled based on column length and diameter, respectively. Note that differences in system configuration may result in differences in retention time and chromatographic performance.

Step 6: Real sample analysis

Once the column performance is satisfactorily confirmed in previous steps, the column is ready for real sample analysis. Equilibrate the column with the desired mobile phase before sample analysis. Note that it is recommended that the column performance test be performed periodically to monitor the condition of the column.

Column care

Column storage and extended care

To maintain the performance of your column between uses, always store the column in mobile phase buffer for short-term storage. For long-term storage (more than 24 hours), it is recommended to store the column in water/acetonitrile (50/50 v/v). Use the plugs the column was shipped with to seal the ends of the column to prevent evaporation of the buffer and drying of the stationary phase.

Operating pH range

The SurePac Protein RP MDi columns operating pH range is 2 to 12 for 2.1 mm ID columns, and pH 2 to 10 for 0.3 mm ID columns.

Operating temperature limit

The SurePac Protein RP MDi columns are stable at temperatures from 5 - 90 °C. The typical operating temperature for protein separation is between 70 °C to 80 °C.

Pressure limit

The column pressure should not exceed 6,500 psi for 2.1 mm ID and 8000 psi for 0.3 mm ID. The back pressure of the column is strongly correlated to the column I.D, length, column temperature, flow rate and type of organic solvent used for mobile phase.

Flow rate

Maximum flow rate will depend on the column I.D, length, column temperature and type of organic solvent used for mobile phase. Please refer to Operating Conditions table (Table 1) for recommended flow rates at 80 °C.

Column cleaning and troubleshooting

Column washing procedure: carry-over may occur if the system is not clean or previous sample may not have completely eluted from the column. Wash the system and the column with acetonitrile/0.1M NaOH (90:10 v/v).

For metal contamination (Fe, Cu, etc.) removal, wash the column in the following sequence:

1. 100 mM NH_4OAc for 10 column volumes.
2. 100 mM Pyrophosphate solution for 100 column volumes.
3. 100 mM NH_4OAc for 10 column volumes.
4. ACN/100 mM NH_4OAc (90:10; v/v) for 20 column volumes.
5. ACN/H_2O (50:50; v/v) for 10 column volumes.

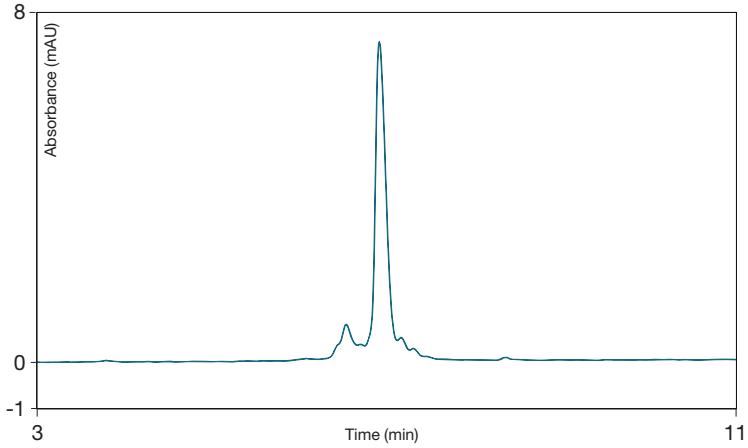
Note: Above procedure may need to be combined with the column washing procedure

Applications

Optimization for protein separations

The SurePac Protein RP MDi column is designed to provide high-resolution and improved separation of proteins. Protein separations are highly sensitive to chromatographic conditions. Key parameters such as gradient slope, flow rate, column temperature, and sample loading must be carefully optimized to achieve consistent retention, good peak shape, and high resolution of closely related species. Figure 2 demonstrates an optimized method for NISTmAb using a 10-minute gradient at a flow rate of 0.4 mL/min, achieving enhanced resolution and accurate quantification between the main peak and associated variants.

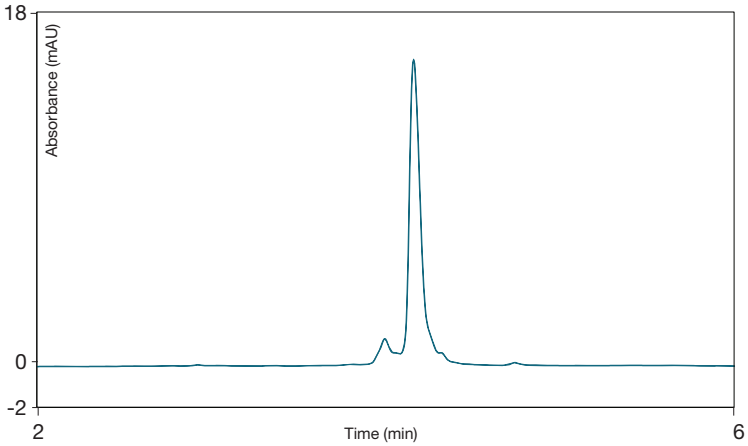
Figure 3 illustrates a fast 4-minute gradient to support high-throughput analytical workflows. Although resolution of closely eluting variants is reduced compared to the 10-minute gradient method, the fast gradient still provides acceptable separation between the main peak and its associated variants. The high resolving power of the SurePac Protein RP MDi column allows sensitive detection of these closely eluting species.



Column	SurePac Protein RP MDi column, 2.1 × 50 mm		
Mobile phases	A: H_2O/TFA (99.9:0.1 v/v); ACN/H_2O/TFA (90:9.9:0.1 v/v)		
Gradient	Time (min)	%A	%B
	0.0	63.0	37.0
	1.0	63.0	37.0
	11.0	58.0	42.0
	13.0	58.0	42.0
	13.0	63.0	37.0
	18.0	63.0	37.0
Temperature	80 °C PH; 80 °C CC; 45 °C PCC		
Flow rate	0.4 mL/min		
Inj. volume	0.5 µL		
Detection	UV (280 nm)		
Sample	NISTmAb (1 mg/mL)		

PH - Pre-heater; CC - Column compartment; PCC - Post column cooler

Figure 2. NISTmAb analysis using optimized method with 10-minute gradient at 0.4 mL/min.



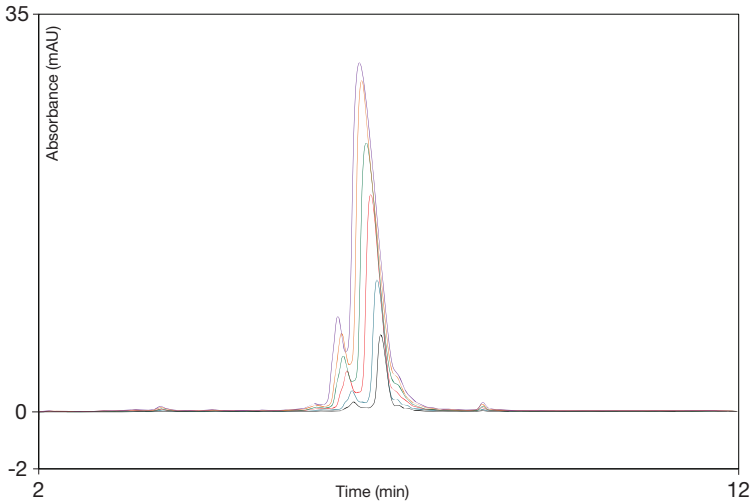
Column	SurePac Protein RP MDi column, 2.1 × 50 mm		
Mobile phases	A: H_2O/TFA (99.9:0.1 v/v); ACN/H_2O/TFA (90:9.9:0.1 v/v)		
Gradient	Time (min)	%A	%B
	0.0	63.0	37.0
	1.0	63.0	37.0
	5.0	58.0	42.0
	7.0	58.0	42.0
	7.0	63.0	37.0
	12.0	63.0	37.0
Temperature	80 °C PH; 80 °C CC; 45 °C PCC		
Flow rate	0.4 mL/min		
Inj. volume	0.5 µL		
Detection	UV (280 nm)		
Sample	NISTmAb (1 mg/mL)		

Figure 3. NISTmAb analysis using optimized method with 4-minute gradient at 0.4 mL/min.

Applications (continued)

Loading capacity

Total mass of protein loaded into the column can have significant effect on peak width and resolution. To evaluate the loading capacity of the phase, dynamic loading analysis was performed on a 2.1 × 50 mm column as shown in Figure 4. The chromatograms show loading masses of 0.5–5.0 µg of NISTmAb. At lower sample loads (0.5–1 µg), the main NISTmAb peak and minor variants remain sharp and symmetrical, indicating operation within the optimal dynamic loading range of the column. At higher sample loads (≥2 µg), peak width increases more significantly, consistent with the onset of column overloading effects, which may reduce resolution of minor variants surrounding the main mAb peak. Dynamic loading capacity is influenced by protein molecular weight and structural characteristics. Larger proteins, such as intact monoclonal antibodies, are generally more sensitive to overloading due to mass transfer limitations and pore accessibility constraints. Therefore, loading behavior should be evaluated individually for each protein. The example provided may be used as a general reference for other protein analytes.

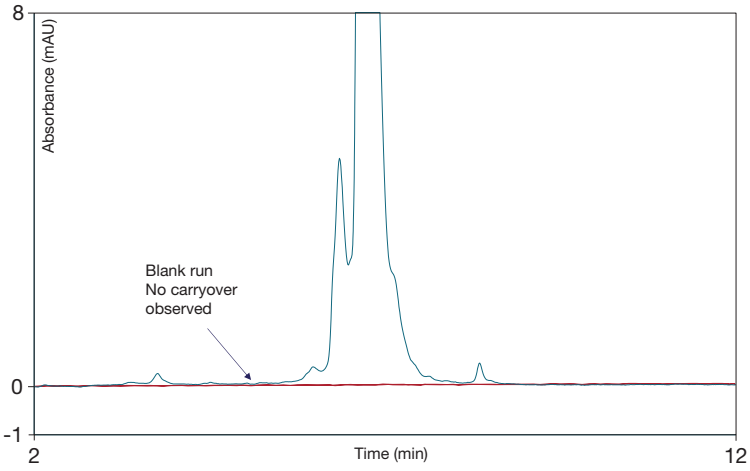


Column	SurePac Protein RP MDi column, 2.1 × 50 mm		
Mobile phases	A: H_2O/TFA (99.9:0.1 v/v); ACN/H_2O/TFA (90:9.9:0.1 v/v)		
Gradient	Time (min)	%A	%B
	0.0	63.0	37.0
	1.0	63.0	37.0
	11.0	58.0	42.0
	13.0	58.0	42.0
	13.0	63.0	37.0
	18.0	63.0	37.0
Temperature	80 °C PH; 80 °C CC; 45 °C PCC		
Flow rate	0.4 mL/min		
Inj. volume	0.5 µL - 5 µL		
Detection	UV (280 nm)		
Sample	NISTmAb (1 mg/mL)		

Figure 4: Overlaid chromatogram showing the dynamic loading analysis of NISTmAb.

Applications (continued)

The SurePac Protein RP MDi 2.5 µm stationary phase is designed for very low carryover. Figure 5 shows the overlaid chromatograms for a 3.0 µL injection volume (3 µg sample load) run using 1 mg/mL NISTmAb and the following blank run with no injection. No carryover was detected in the blank run, demonstrating the low carryover properties of the stationary phase even at high mass loading levels, which enables consecutive protein injection runs without interference due to carryover from previous injections.

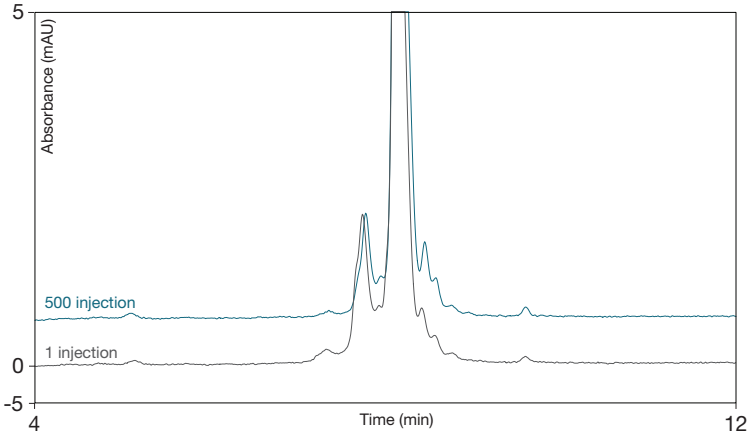


Column	SurePac Protein RP MDi column, 2.1 × 50 mm		
Mobile phases	A: H_2O/TFA (99.9:0.1 v/v); ACN/H_2O/TFA (90:9.9:0.1 v/v)		
Gradient	Time (min)	%A	%B
	0.0	63.0	37.0
	1.0	63.0	37.0
	11.0	58.0	42.0
	13.0	58.0	42.0
	13.0	63.0	37.0
	18.0	63.0	37.0
Temperature	80 °C PH; 80 °C CC; 45 °C PCC		
Flow rate	0.4 mL/min		
Inj. volume	3.0 µL		
Detection	UV (280 nm)		
Sample	NISTmAb (1 mg/mL)		

Figure 5: Overlaid chromatogram showing a 3.0 µg injection and elution of NISTmAb using 10 minute gradient and the following blank run to measure carryover.

Ruggedness

Column ruggedness is a critical characteristic for ensuring accurate, reproducible results and extended column lifetime. The ruggedness of the SurePac Protein RP MDi 2.1 × 50 mm column was evaluated under extended use conditions using repeated injections of NISTmAb. Over 500 injections, the column showed highly consistent chromatographic performance, with minimal variation in retention time and peak width (PWHH), demonstrating its robustness and suitability for routine analytical applications.



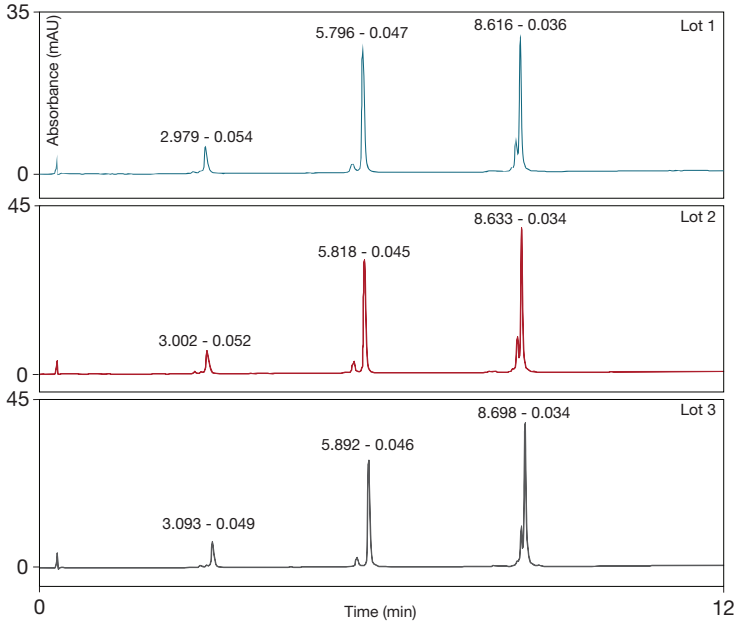
Column	SurePac Protein RP MDi column, 2.1 × 50 mm		
Mobile phases	A: H_2O/TFA (99.9:0.1 v/v); ACN/H_2O/TFA (90:9.9:0.1 v/v)		
Gradient	Time (min)	%A	%B
	0.0	63.0	37.0
	1.0	63.0	37.0
	11.0	58.0	42.0
	13.0	58.0	42.0
	13.0	63.0	37.0
	18.0	63.0	37.0
Temperature	80 °C PH; 80 °C CC; 45 °C PCC		
Flow rate	0.4 mL/min		
Inj. volume	1.0 µL		
Detection	UV (280 nm)		
Sample	NISTmAb (1 mg/mL)		

Figure 6: Overlaid chromatograms of NISTmAb analysis over 500 injections using the SurePac Protein RP MDi column (2.1 × 50 mm). Retention times are normalized to aid comparison of variant separation of the detailed view of NISTmAb variants.

Applications (continued)

Lot-to-lot reproducibility

Using a 10-minute gradient SurePac Protein RP MDi columns from three different media lots were analyzed with a 3-protein mixture to evaluate column-to-column and lot-to-lot reproducibility. The chromatograms shown in Figure 7 demonstrate highly consistent retention times and peak widths across three different lots. The monodisperse particle technology in the SurePac Protein RP MDi platform ensures uniform packing, delivering excellent batch-to-batch reproducibility and consistent chromatographic performance.



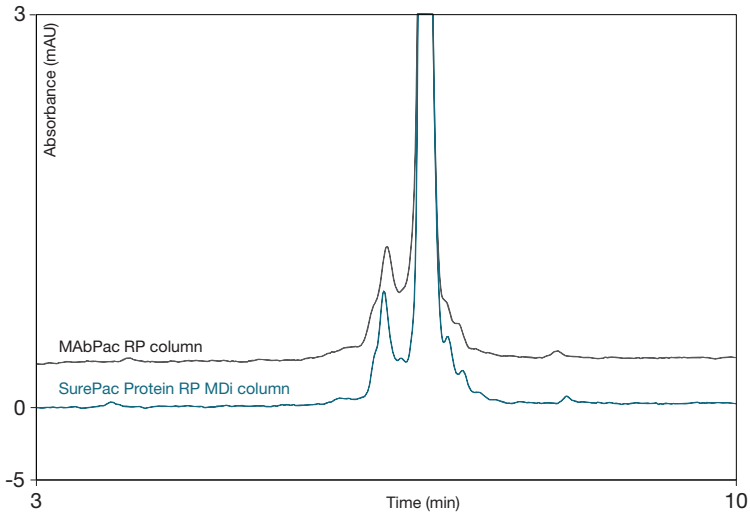
Column	SurePac Protein RP MDi column, 2.1 × 50 mm		
Mobile phases	A: H_2O/TFA (99.9:0.1 v/v); ACN/H_2O/TFA (90:9.9:0.1 v/v)		
Gradient	Time (min)	%A	%B
	-5.0	80.0	20.0
	0.0	80.0	20.0
	1.0	80.0	20.0
	11.0	50.0	50.0
	13.0	50.0	50.0
	13.1	80.0	20.0
	17.2	80.0	20.0
	18.0	80.0	20.0
Temperature	80 °C PH; 80 °C CC; 45 °C PCC		
Flow rate	0.1 mL/min		
Inj. volume	3.0 µL		
Detection	UV (280 nm)		
Sample	Peak 1: Ribonuclease A (1.0 mg/mL) Peak 2: Lysozyme (0.5 mg/mL) Peak 3: NISTmAb (1.0 mg/mL)		

Figure 7 : Chromatograms of three different lots using a 10 minute gradient at 0.4 mL/min flow rate.
Legend: Peak number - RT (min) – PWHH (peak width half height).

Applications (continued)

Column comparison

Figure 8 compares the performance of the SurePac Protein RP MDi column against our current Thermo Scientific™ MAbPac™ RP Column. The SurePac Protein RP MDi column provides superior resolution and peak definition, enabling more effective separation of closely eluting monoclonal antibody variants. Overall, SurePac Protein RP MDi columns provide a clear advantage in variant separation, supporting more accurate analysis and quantification.

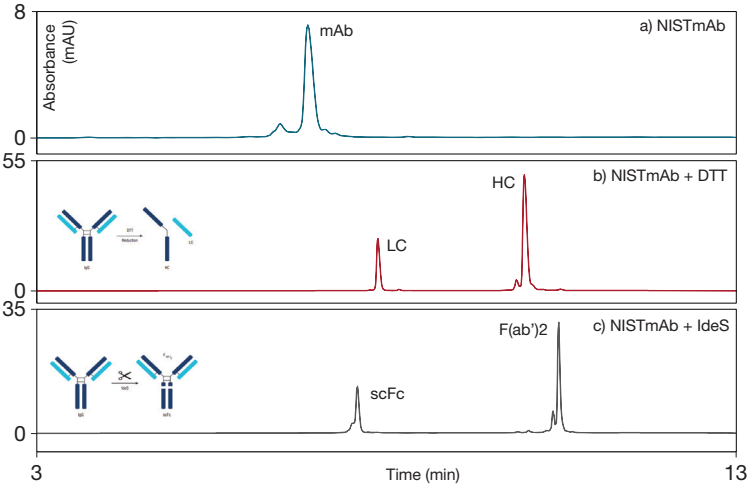


Columns	SurePac Protein RP MDi column, 2.1 × 50 mm MAbPac RP column, 2.1 x 50 mm		
Mobile phases	A: H_2O/TFA (99.9:0.1 v/v); ACN/H_2O/TFA (90:9.9:0.1 v/v)		
Gradient	Time (min)	%A	%B
	0.0	63.0	37.0
	1.0	63.0	37.0
	11.0	58.0	42.0
	13.0	58.0	42.0
	13.0	63.0	37.0
	18.0	63.0	37.0
Temperature	80 °C PH; 80 °C CC; 45 °C PCC		
Flow rate	0.4 mL/min		
Inj. volume	0.5 µL		
Detection	UV (280 nm)		
Sample	NISTmAb (1 mg/mL)		

Figure 8: Comparison of NISTmAb separation using SurePac Protein RP MDi column (2.5 µm, 2.1 × 50 mm) and MAbPac RP column (4 µm, 2.1 x 50 mm) using an optimized 10-minute gradient method. Retention times are normalized for comparison of variant separation.

Separation of mAb and mAb fragments

Monoclonal antibodies are heterogeneous. Comprehensive analysis of mAb post-translational modifications, such as deamidation, C-terminal lysine truncation, N-terminal pyroglutamation, methionine (Met) oxidation, and glycosylation, requires complete digestion of the mAbs and sequencing of all the peptides. However, “peptide mapping” is complex, time consuming and labor intensive. A direct and simplified approach for characterizing mab variants and locating modifications is to analyze mAb fragments. Light chain (LC) and heavy chain (HC) are generated by the reduction of mAb, scFc and F(ab’)2 fragments are generated by IdeS digestion. Figure 5 shows the analysis of Intact NISTmAb and its fragments using SurePac Protein RP MDi column with a 10-min gradient.



Column	SurePac Protein RP MDi column, 2.1 × 50 mm		
Mobile phases	A: H_2O/TFA (99.9:0.1 v/v); ACN/H_2O/TFA (90:9.9:0.1 v/v)		
Gradient	Time (min)	%A	%B
	0.0	85.0	15.0
	1.0	85.0	15.0
	11.0	55.0	45.0
	13.0	55.0	45.0
	13.0	85.0	15.0
	18.0	85.0	15.0
Temperature	80 °C PH; 80 °C CC; 45 °C PCC		
Flow rate	0.4 mL/min		
Inj. volume	0.5 µL		
Detection	UV (280 nm)		
Sample	a) NISTmAb (1 mg/mL) b) NISTmAb + DTT (4 mg/mL) c) NISTmAb + IdeS (2 mg/mL)		

Figure 9: Analysis of NISTmAB and fragments using 10-minute gradient at 0.4 mL/min: a) NISTmAb; b) NISTmAb LC and HC; c) NISTmAb scFc and F(ab’)2 fragments

Applications (continued)

LC–MS analysis of intact NISTmAb and its subunits

The LC–MS experiments were performed to demonstrate that the SurePac Protein RP MDi column (2.5 µm, 2.1 × 50 mm) is compatible with MS-friendly mobile phases and supports accurate mass identification of intact NISTmAb and its subunits. While the use of formic acid as a mobile phase additive, instead of trifluoroacetic acid, results in reduced chromatographic resolution due to weaker ion-pairing effects, it improves ionization efficiency and enables acquisition of high-quality mass spectra.

The Thermo Scientific™ Orbitrap Exploris™ 480 Mass Spectrometer was coupled to the Vanquish Horizon System. The mass spectrometer was operated with Thermo Scientific™ Xcalibur™ 4.7 Software. Instrument calibration was performed using Thermo Scientific™ Pierce™ FlexMix™ Calibration Solution. The protein mode with positive ion polarity and low-pressure settings was used for this study. Data was analyzed with Thermo Scientific™ Freestyle™ 1.8 and BioPharma Finder™ 5.4. Softwares and MS conditions are summarized in Tables 2 and 3, while LC conditions are provided alongside the corresponding chromatograms.

Table 2. Summary of tune parameters

MS ion source parameters	Settings for intact mAb analysis	Settings for DTT-reduced mAb analysis	Settings for IdeS digested mAb analysis
Sheath gas, a.u.	40	25	25
Auxiliary gas, a.u.	10	10	10
Vaporizer temperature, °C	250	150	150
Source voltage, kV	3.5	3.8	3.8
Ion transfer tube temperature, °C	325	320	320

Table 3. Summary of MS parameters

MS method parameters	Settings for intact mAb analysis	Settings for DTT-reduced mAb analysis	Settings for IdeS digested mAb analysis
Scan type	Full MS	Full MS (two segments)	Full MS (two segments)
Total run time, min	18	0 to 8	0 to 8
		8 to 18	8 to 18
Full MS mass range, m/z	2200-5500	800-2600	800-2600
			1400-3500
Resolution setting	30 000	240 000	15 000
AGC target value, %	100	300	300
Max injection time, ms	200	200	200
In-source CID, V	80	N/A	N/A
Microscans	10	10	5
			10

Applications (continued)

Intact mAb analysis

LC–MS analysis of intact NISTmAb revealed a dominant chromatographic peak with minor contributions from additional proteoforms (Figure 10). Despite this, deconvolution of the mass spectra enabled the identification of multiple proteoforms corresponding to different glycosylation patterns (Table 4). Experimentally determined average masses were in good agreement with theoretical values, with deviations ranging from 3.6 to 4.4 ppm. All detected intact species exhibited C-terminal lysine clipping and N-terminal pyro-glutamate formation on the heavy chain. Disulfide bond formation was confirmed by hydrogen loss on cysteine residues. The observed glycoforms differed in the degree of galactosylation (0, 1, or 2 galactose residues across both chains), reflecting the expected structural heterogeneity of NISTmAb.

These results demonstrate the suitability of the SurePac Protein RP MDi column for rapid and reliable LC–MS characterization of intact mAbs.

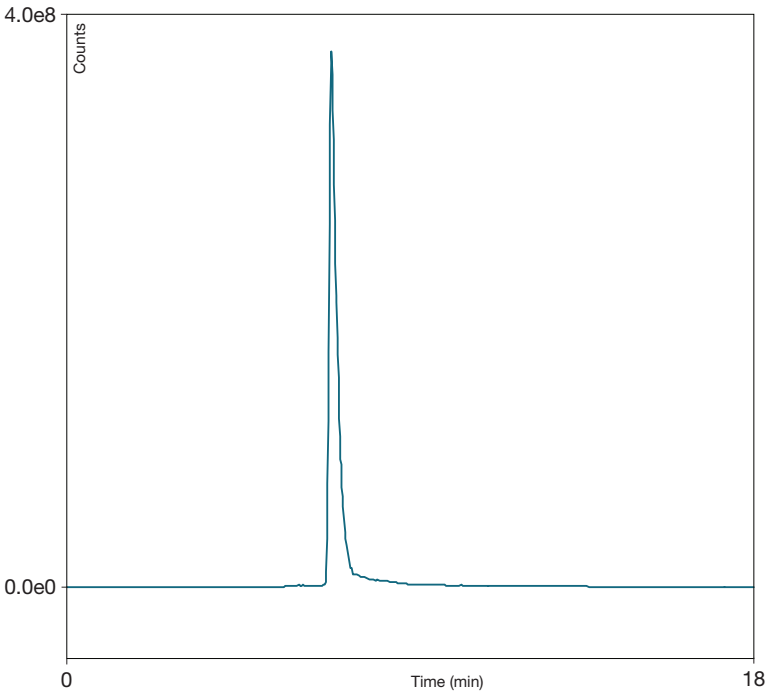


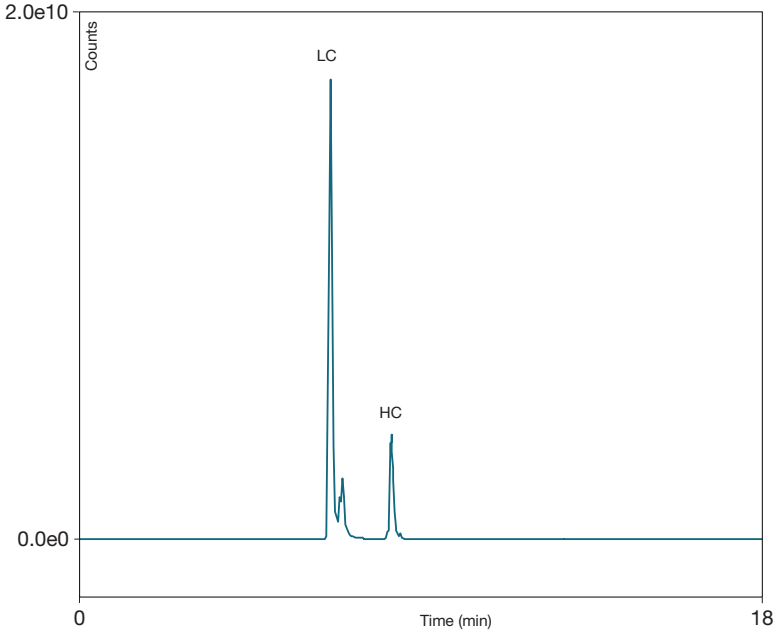
Figure 10. Total ion chromatogram of intact NISTmAb.

Column	SurePac Protein RP MDi column, 2.1 × 50 mm		
Mobile phases	A: H_2O/TFA (99.9:0.1 v/v); ACN/H_2O/TFA (90:9.9:0.1 v/v)		
Gradient	Time (min)	%A	%B
	0.0	70.0	30.0
	1.0	70.0	30.0
	11.0	60.0	40.0
	13.0	60.0	40.0
	13.0	70.0	30.0
	18.0	70.0	30.0
Temperature	80 °C PH; 80 °C CC; 45 °C PCC		
Flow rate	0.4 mL/min		
Inj. volume	0.5 µL		
Detection	UV (280 nm)		
Sample	NISTmAb (1 mg/mL)		

Applications (continued)

Reduced mAb analysis

Thorough analysis of post-translational modifications requires peptide mapping, which is time consuming. A simpler and direct option to analyse the mAb variants and locate the modifications is to measure the subunits of it. Following reduction with DTT, the heavy chain and light chain subunits were analyzed to provide further structural confirmation. The LC–MS data show clear separation and detection of the individual chains (Figure 11).



Column	SurePac Protein RP MDi column, 2.1 × 50 mm		
Mobile phases	A: H_2O/FA (99.9:0.1 v/v); BACN/H_2O/FA (90:9.9:0.1 v/v/v)		
Gradient	Time (min)	%A	%B
	0.0	85.0	15.0
	1.0	85.0	15.0
	11.0	55.0	45.0
	13.0	55.0	45.0
	13.0	85.0	15.0
	18.0	85.0	15.0
Temperature	80 °C PH; 80 °C CC; 45 °C PCC		
Flow rate	0.4 mL/min		
Inj. volume	0.25 µL		
Detection	UV (280 nm)		
Sample	DTT-reduced NISTmAb (4 mg/mL)		

Figure 11. Total ion chromatogram of DTT-reduced NISTmAb showing separation of LC and HC.

As shown in Table 4, the experimentally measured mass of the light chain (23,109.310 Da) is in excellent agreement with the theoretical value (Δ = 1.6 ppm), confirming its integrity.

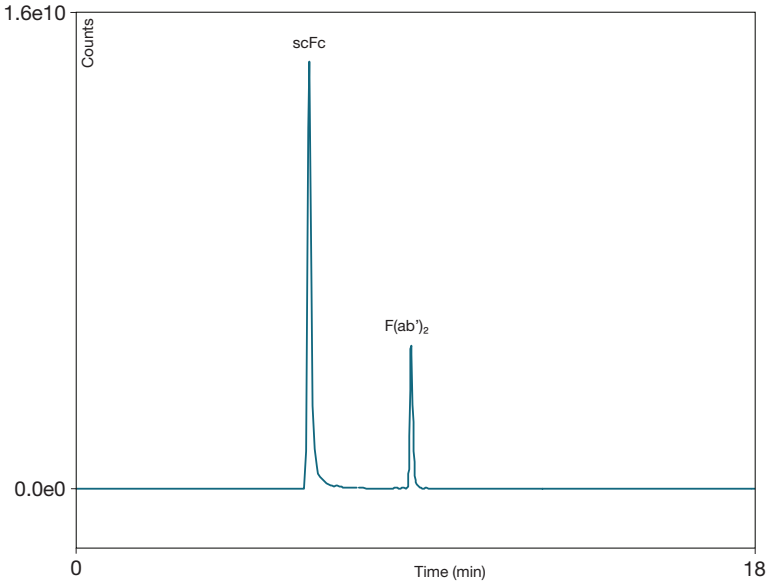
The heavy chain species exhibit mass deviations in the range of 0.7 to 2.5 ppm, indicating high mass accuracy despite structural heterogeneity. The recorded modifications of intact mAb were only observed on the heavy chain, e.g. lysine clipping, N-terminal pyro-glutamate formation and different level of glycoforms. Additionally, as the sample was reduced with DTT, cysteine groups forming inter-chain disulfide bridges were reduced.

These results further confirm the presence of expected modifications and demonstrate reliable subunit-level characterization.

Applications (continued)

IdeS-digested mAb analysis

To further localize modifications and provide a complementary view of mAb structural complexity, NISTmAb was digested with IdeS, generating F(ab')$_2$ and scFc fragments. Figure 12 shows the separation of these subunits.



Column	SurePac Protein RP MDi column, 2.1 × 50 mm		
Mobile phases	A: H_2O/FA (99.9:0.1 v/v); BACN/H_2O/FA (90:9.9:0.1 v/v/v)		
Gradient	Time (min)	%A	%B
	0.0	85.0	15.0
	1.0	85.0	15.0
	11.0	55.0	45.0
	13.0	55.0	45.0
	13.0	85.0	15.0
	18.0	85.0	15.0
Temperature	80 °C PH; 80 °C CC; 45 °C PCC		
Flow rate	0.4 mL/min		
Inj. volume	0.5 µL		
Detection	UV (280 nm)		
Sample	IdeS digested NISTmAb (2 mg/mL)		

Figure 12. Total ion chromatogram of IdeS-digested NISTmAb showing separation of scFc and F(ab')$_2$.

Table 4. Theoretical and experimental masses of intact NISTmAb and its subunits

	Chain	Proposed modifications	Average mass (Da)	Theoretical mass (Da)	Δ ppm
Intact mAb	Intact	1xG0F_G0F	148 036.69	148 037.15	3.2
	Intact	1xG0F_G1F	148 198.64	148 199.30	4.4
	Intact	1xG1F_G1F	148 360.78	148 361.44	4.4
	Intact	1xG1F_G2F	148 523.04	148 523.58	3.6
Reduced mAb	HC	1xA2G0F	50 898.98	50 899.02	0.7
	HC	1xA2G1F	51 061.28	51 061.16	-2.5
	HC	1xA2G2F	51 223.32	51 223.30	-0.5
IdeS-digested mAb	F(ab')2	N/A	97 608.67	97 609.04	3.8
	Chain	**Proposed modifications**	**Monoisotopic mass (Da)**	**Theoretical mass (Da)**	**Δ ppm**
	scFc	1xA2G0F	25 216.460	25 216.432	-1.1
	scFc	1xA2G1F	25 378.516	25 378.485	-1.2
	scFc	1xA2G2F	25 540.566	25 540.538	-1.1
Reduced mAb	LC	N/A	23 109.310	23 109.273	1.6

As summarized in Table 4, the $F(ab')_2$ fragment shows mass deviation of 3.8 ppm and includes modifications such as N-terminal pyro-glutamate formation or H loss due to disulfide bridges formed within cysteine residues.

The scFc fragments exhibit excellent agreement with theoretical masses (Δ ≈ 1.1–1.2 ppm) and correspond to different glycoforms that differ by the degree of galactosylation (0, 1, or 2 galactose residues). These variants are consistent with commonly observed modifications in the scFc region.

Overall, the analysis of IdeS-generated fragments enables more detailed localization of modifications and confirms the structural assignments observed at the intact and reduced levels.

The assigned species are consistent with known NISTmAb glycoforms and post-translational modifications, including scFc glycosylation, N-terminal pyroglutamate formation, C-terminal lysine clipping. Exact combinations and stoichiometries of co-occurring modifications were assigned based on mass agreement and should be interpreted as putative compositions unless confirmed by orthogonal analyses (e.g. LC-MS/MS).

Ordering information

Description	Particle size	Dimension	Quantity	Part No.
SurePac Protein RP MDi columns	2.5 µm	0.3 × 50 mm	Each	43722-050332
		2.1 × 20 mm	Each	43722-022132
		2.1 × 50 mm	Each	43722-052132
		2.1 × 100 mm	Each	43722-102132

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