

MAbPac RP Column

# High Performance RP Chromatography Column for mAb Analysis

## Benefits

- Superior resolution power for monoclonal antibodies and related substances
- High efficiency with low carry-over
- Excellent MS compatibility
- Wide operating pH range: 0–14
- High temperature stability: up to 110 °C
- High throughput

## Keywords

MAbPac, monoclonal antibody, mAb, reverse phase, biopharmaceutical, protein, proteomics, biomolecules, intact protein

The Thermo Scientific™ MAbPac™ RP is a reverse phase (RP) liquid chromatography column designed for mAb characterization, including the separation of mAb and variants, light chain (LC) and heavy chain (HC), Fc and Fab fragments, scFc and F(ab')<sub>2</sub> fragments, using LC/UV or LC/MS. The unique column chemistry provides excellent performance under a broad range of pH, temperature, and mobile phase composition.

## Introduction

The monoclonal antibody (mAb) therapeutics market is growing at a rapid rate owing to increased demand for targeted treatments. Therapeutic mAbs are mostly produced from mammalian cells. These biological products are heterogeneous due to post-translational modifications. Additional modifications such as oxidation can be introduced during the manufacturing process. A comprehensive characterization of mAb purity, aggregates, and variants is required for the final biopharmaceutical product approval and subsequent manufacturing processes. There is a growing trend to obtain intact mass information, as well as the glycan profile, in the QC of mAbs using reverse phase chromatography coupling with high resolution mass spectrometry detection. In addition, LC/MS analysis of mAb fragments such as light chain (LC), heavy chain (HC), Fc, and Fab, scFc and F(ab')<sub>2</sub>,

can quickly reveal the location, as well as nature, of the modification. In most QC environments LC/UV analysis of mAb fragments has been established as high throughput assays.

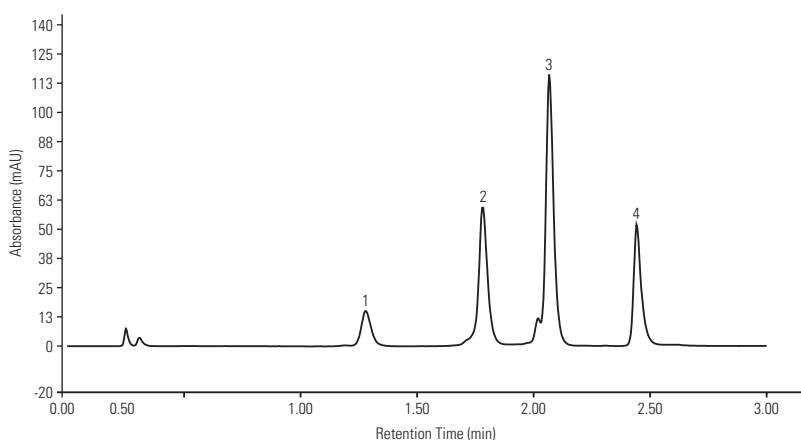
## Column Technology

The MAbPac RP is a reverse phase (RP) column specifically designed for separation of intact monoclonal antibodies (mAbs) and mAb fragments. The stationary phase is fully compatible with mass spectrometry friendly organic solvent such as acetonitrile and isopropanol, as well as low pH eluents containing trifluoroacetic acid or formic acid. The MAbPac RP column is based on wide-pore 4  $\mu\text{m}$  polymer particles that are stable at extreme pH (0–14) and high temperature (up to 110  $^{\circ}\text{C}$ ). The wide-pore size of polymeric particles enables efficient separation of protein molecules with low carry-over.

## Applications

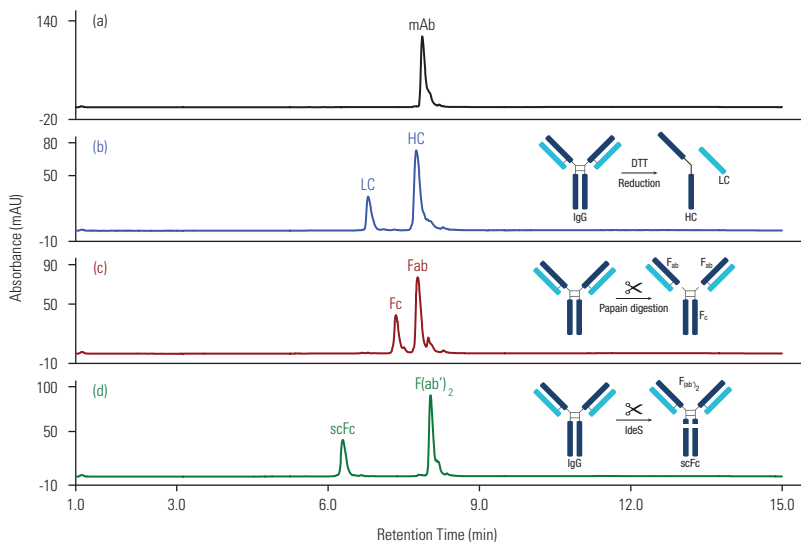
### Fast Separation of Intact Proteins/mAbs

The MAbPac RP column is designed for high resolution and fast separation of proteins. High throughput and high-resolution can be achieved by adjusting both gradient slope and flow rate. The wide-pore nature of polymer particles combined with 4  $\mu\text{m}$  particle size provides excellent resolution for intact proteins/mAbs with high-throughput and low carry-over. Figure 1 shows the baseline separation of four proteins: ribonuclease A, cytochrome C, lysozyme, and mAb, on a 2.1  $\times$  50 mm MAbPac RP column within 3 min.



Column:	MAbPac RP, 4 $\mu\text{m}$		
Format:	2.1 $\times$ 50 mm		
Mobile phase A:	$\text{H}_2\text{O}/\text{TFA}$ (99.9 : 0.1 v/v)		
Mobile phase B:	$\text{MeCN}/\text{H}_2\text{O}/\text{TFA}$ (90: 9.9 : 0.1 v/v/v)		
Gradient:	Time (min)	%A	%B
	-1.0	80	20
	0.0	80	20
	2.5	50	50
	2.7	50	50
	2.8	80	20
	3.0	80	20
Temperature:	80 $^{\circ}\text{C}$		
Flow Rate:	0.6 mL/min		
Inj. Volume:	1 $\mu\text{L}$		
Detection:	UV (280 nm)		
Sample:	1. Ribonuclease A (0.5 mg/mL)		
	2. Cytochrome C (0.5 mg/mL)		
	3. Lysozyme (0.5 mg/mL)		
	4. mAb (1 mg/mL)		

Figure 1. Fast separation of intact proteins/mAb.



Column:	MAbPac RP, 4 $\mu\text{m}$		
Format:	3 $\times$ 50 mm		
Mobile phase A:	$\text{H}_2\text{O}/\text{FA}/\text{TFA}$ (99.88 : 0.1:0.02 v/v/v)		
Mobile phase B:	$\text{MeCN}/\text{H}_2\text{O}/\text{FA}/\text{TFA}$ (90: 9.88 : 0.1:0.02 v/v/v/v)		
Gradient:	Time (min)	%A	%B
	0.0	80	20
	1.0	80	20
	11.0	55	45
	12.0	55	45
	14.0	80	20
	15.0	80	20
Temperature:	80 $^{\circ}\text{C}$		
Flow Rate:	0.5 mL/min		
Inj. Volume:	5 $\mu\text{L}$		
Detection:	UV (280 nm)		
Sample:	(a) Trastuzumab (5 mg/mL)		
	(b) Trastuzumab + DTT (4 mg/mL)		
	(c) Trastuzumab + Papain (2 mg/mL)		
	(d) Trastuzumab + IdeS (2 mg/mL)		

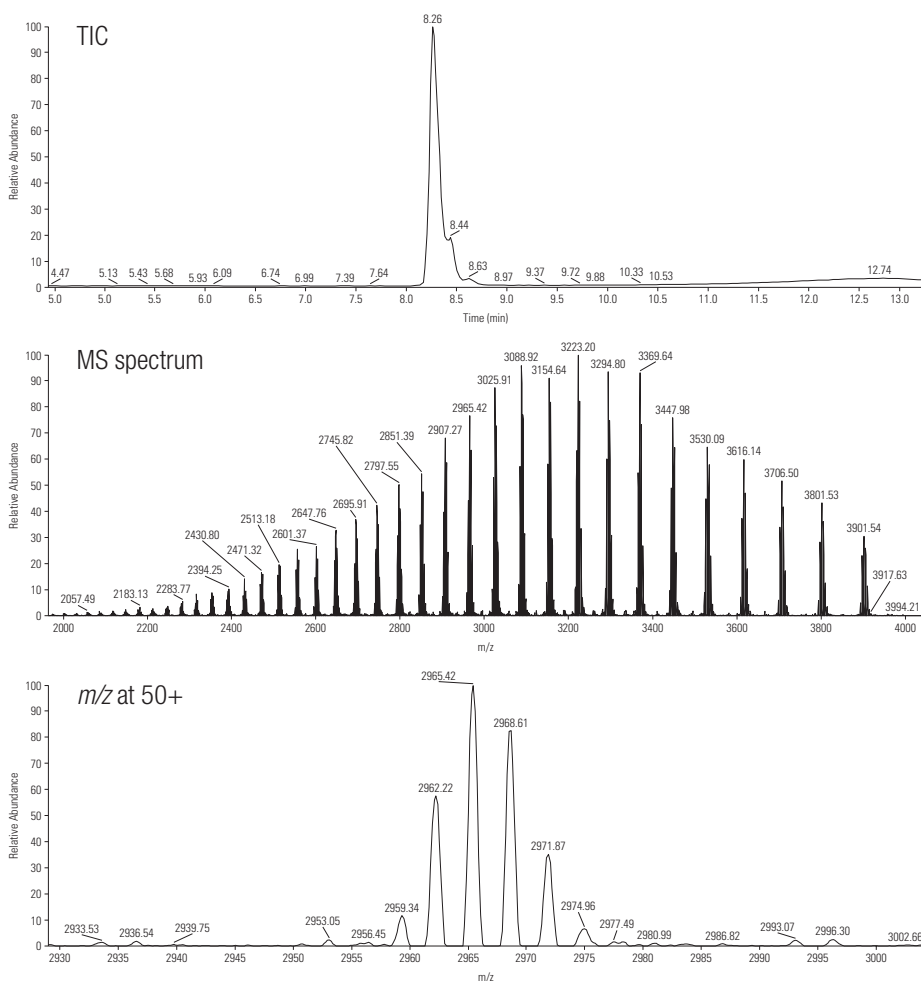
Figure 2. Separation of mAb and mAb fragments.

### Separation of 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 mAbs and sequencing of all the peptides. However, “peptide mapping” is time consuming. A simpler and more direct way to analyze mAb variants and locate the modifications is to measure mAb fragments, which can either be generated by chemical reduction or by enzymatic digestion. Figure 2 shows the analysis of trastuzumab intact molecule and fragments. LC and HC (Figure 2b), Fc and Fab (Figure 2c), scFc and F(ab')<sub>2</sub> (Figure 2d) are baseline separated on a 3 × 50 mm MAbPac RP column using a 10 min gradient. Light chain (LC) and heavy chain (HC) are generated by the reduction of mAb, Fc and Fab fragments are generated by papain digestion, and single-chain Fc (scFc) and F(ab')<sub>2</sub> fragments are generated by IdeS digestion.

### LC/MS analysis of Intact mAb and mAb fragments

High resolution mass spectrometers such as the Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ Mass Spectrometer provide accurate mass information of large biologic molecules such as mAbs. The MAbPac RP column can be directly coupled to the mass spectrometer for MS detection of mAb and mAb fragments. While trifluoroacetic acid (TFA) as ion-pairing reagent provides excellent separation results, TFA can suppress ionization in the LC/MS interface, causing a drop in signal. This can be mitigated by reducing the TFA concentration to 0.02% and additional use 0.1% Formic acid (FA). Figure 3 shows the intact mass detection of trastuzumab. The top trace shows the total ion-current chromatogram. The middle trace shows the MS spectrum of trastuzumab in the mass range of 2,000 to 4,000 *m/z*. The bottom trace shows a zoom-in spectrum of trastuzumab with 50+ charges. The cluster shows the glycosylation profile of trastuzumab.



Column:	MAbPac RP, 4 μm		
Format:	3 × 50 mm		
Mobile phase A:	H <sub>2</sub> O/FA/TFA (99.88 : 0.1:0.02 v/v/v)		
Mobile phase B:	MeCN/H <sub>2</sub> O/FA/TFA (90: 9.88 : 0.1:0.02 v/v/v/v)		
Gradient:	Time (min)	%A	%B
	0.0	80	20
	1.0	80	20
	11.0	55	45
	12.0	55	45
	14.0	80	20
	15.0	80	20
Temperature:	80 °C		
Flow Rate:	0.5 mL/min		
Inj. Volume:	1 μL		
MS Detection:	positive-ion mode		
Mass Spec:	Q Exactive Plus		

Sample: Trastuzumab (5 mg/mL)

Figure 3. Intact mAb and glycosylation profile by LC/MS.

During characterization, mAb is often reduced to LC and HC. Mass spectrometry analysis of these fragments can quickly review and localize the modifications. Figure 4 shows the separation of trastuzumab LC and HC on a 3 × 50 mm MAbPac RP column. Total ion chromatogram (TIC) and UV spectrum show identical retention time of mAb fragments. The mass spectrum of LC shows multiple charge states of a single polypeptide chain while the mass spectrum of the HC shows multiple glycosylation forms of the heavy chain.

Column: MAbPac RP, 4 μm  
 Format: 3 × 50 mm  
 Mobile phase A: H<sub>2</sub>O/FA/TFA (99.88 : 0.1:0.02 w/v/v)  
 Mobile phase B: MeCN/H<sub>2</sub>O/FA/TFA (90: 9.88 : 0.1:0.02 v/v/v/v)  
 Gradient:

Time (min)	%A	%B
0.0	80	20
1.0	80	20
11.0	55	45
12.0	55	45
14.0	80	20
15.0	80	20

Temperature: 80 °C  
 Flow Rate: 0.5 mL/min  
 Inj. Volume: 1 μL  
 UV Detection: 280 nm  
 MS Detection: positive-ion mode  
 Mass Spec: Q Exactive Plus

Sample: Reduced trastuzumab (4 mg/mL)

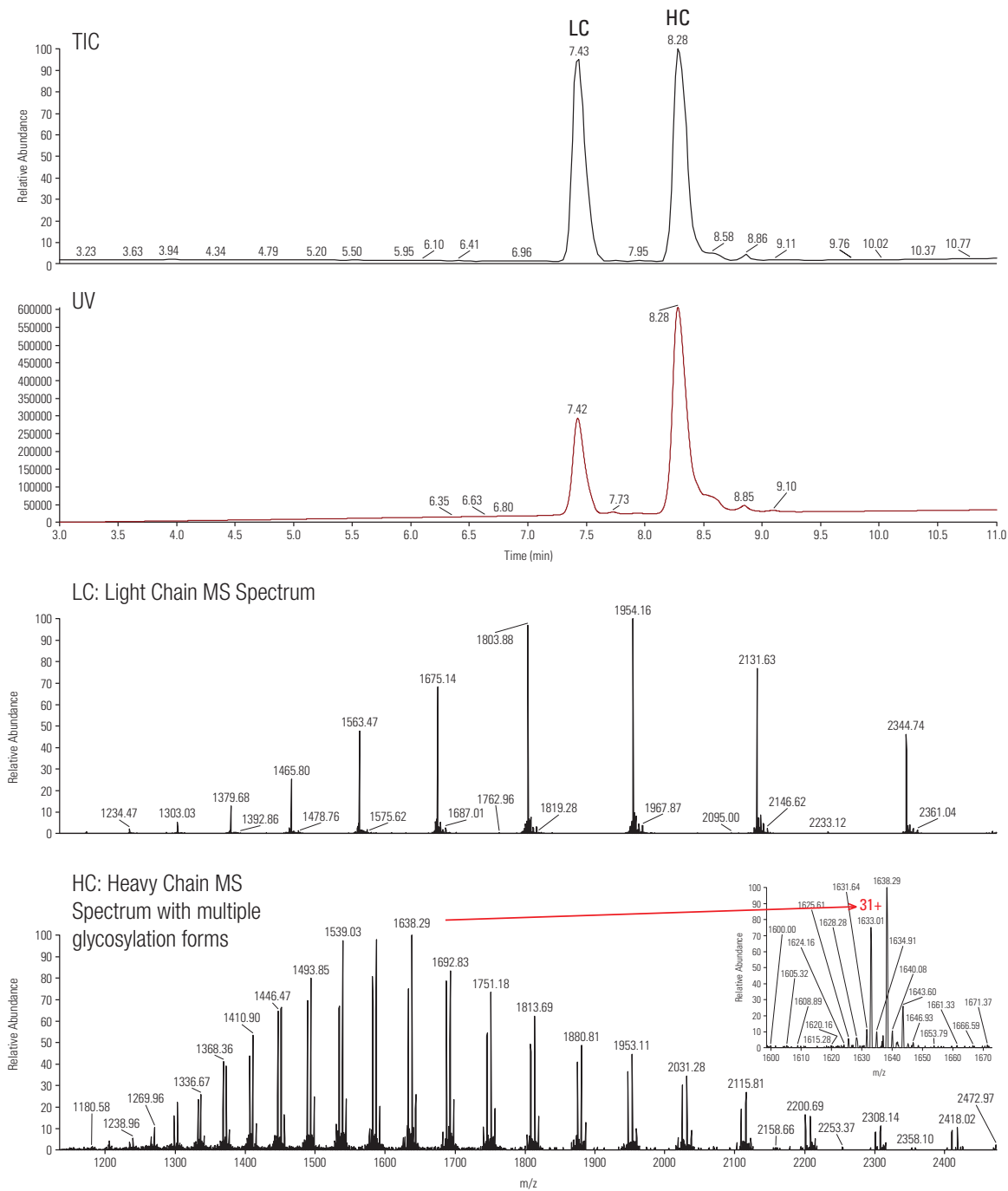


Figure 4. LC/MS analysis of mAb fragments.

## LC/MS analysis of mAb fragments containing oxidation variants

Development of high-throughput release and characterization assays is critical for the effective support of the rapidly growing biologics pipeline for biotherapeutics. Oxidation of mAb is commonly monitored during process optimization, formulation development, and stability studies. The mAb can be broken down into scFc, LC, and Fd' fragments using DTT reduction followed by IdeS enzyme. Figure 5a shows the baseline separation of the scFc, LC, and Fd' fragments of trastuzumab. This sample was previously treated with H<sub>2</sub>O<sub>2</sub>, resulting in oxidation of a methionine in the Fc region (confirmed by peptide mapping, data not shown). The oxidized scFc eluted before the

unmodified scFc. Figure 5b shows the +10 charge state of scFc at *m/z* 2525.60 and Figure 5c shows the +10 charge state of unmodified scFc at *m/z* 2524.08. The delta mass between oxidized peak and unmodified peak corresponds to one oxygen mass. This example demonstrates that oxidation occurring in the scFc, LC, and Fd' regions can be simultaneously monitored by this assay, without going through complete digestion of mAb and peptide mapping. This simple reduction/digestion assay coupling with fast separation of the mAb fragments using the MAbPac RP column and high resolution accurate mass detection using the Q Exactive HF Hybrid system makes it an ideal method for high throughput screening of mAb oxidation.

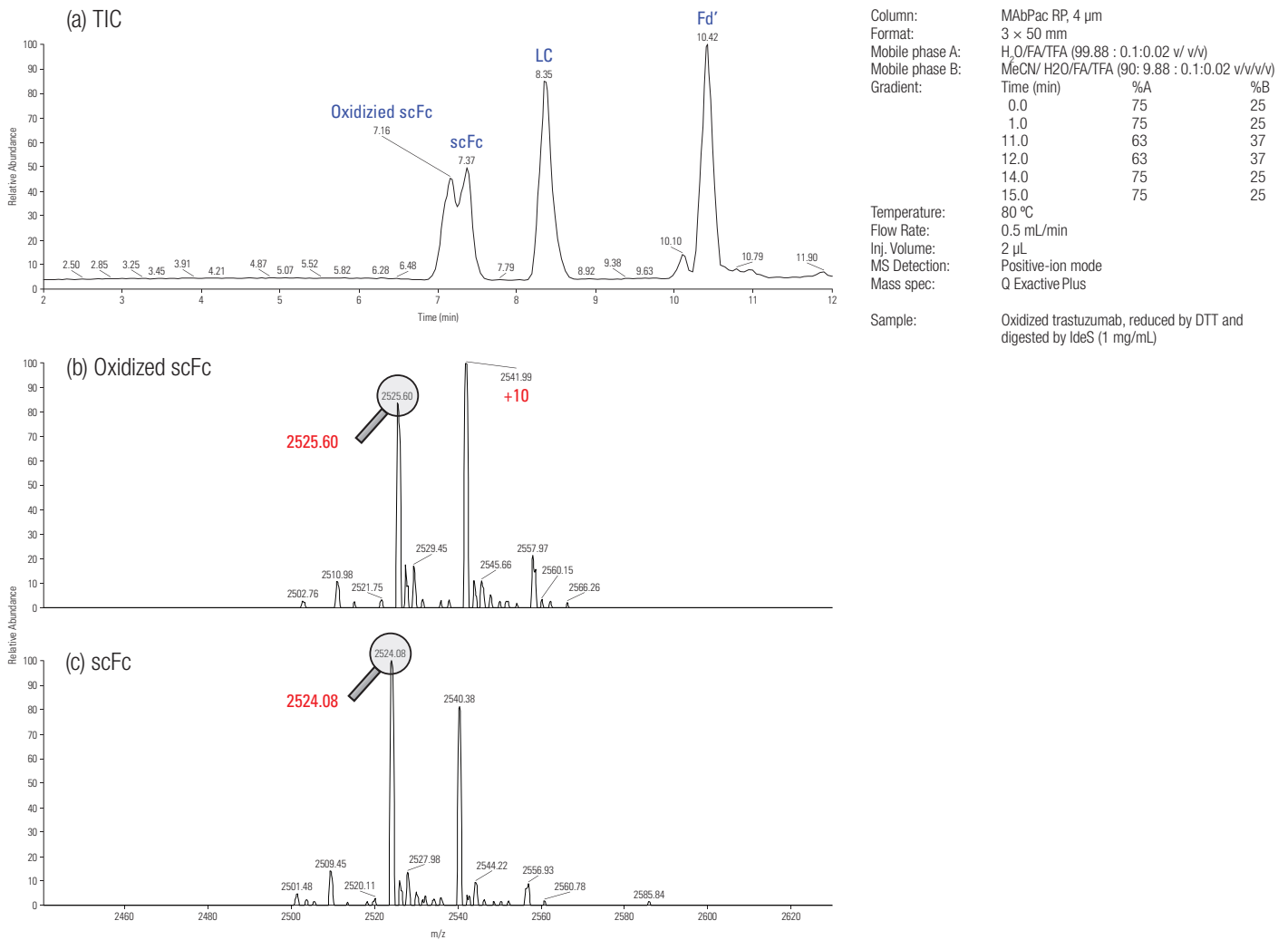


Figure 5. LC/MS analysis of oxidized scFc.

### Analysis of Antibody-Drug-Conjugate (ADC)

Antibody-drug-conjugate has been proved to be a very effective cancer therapy. Due to the heterogeneous nature of the ADC, it is critical to characterize its multiple drug-to-antibody ratio (DAR) forms. The MAbPac RP column can be utilized in the separation of mAb and its conjugates. In Figure 6, ADCs were prepared using The SiteClick™ Antibody Labeling System from Thermo Fisher Scientific. Most of the antibody glycan branches terminate with galactose-N-acetylglucosamine (Gal-GlcNAc-) or with N-acetylglucosamine (GlcNAc-). Removal of the terminal Gal residue with  $\beta$ -galactosidase unmask the majority of terminal GlcNAc labeling

sites for the subsequent enzymatic  $\beta$ -galactosyl transferase (GalT) reaction. After cleavage of terminal Gal residues by  $\beta$ -galactosidase, each N-linked glycan will contain, on average, 2 terminal GlcNAc residues per heavy chain (4 terminal GlcNAc per antibody). The azide-activated antibodies were then conjugated with dibenzocyclooctyne (DIBO)-activated Val-Cit-PAB-Monomethyl Auristatin E (MMAE) toxin in a copperless click reaction, resulting in a mixture of drug-loaded antibody species with 0 to 4 MMAE molecules. The unmodified mAb and ADCs with DAR values ranging from 0 to 4 are well resolved on the MAbPac RP column (Figure 7).

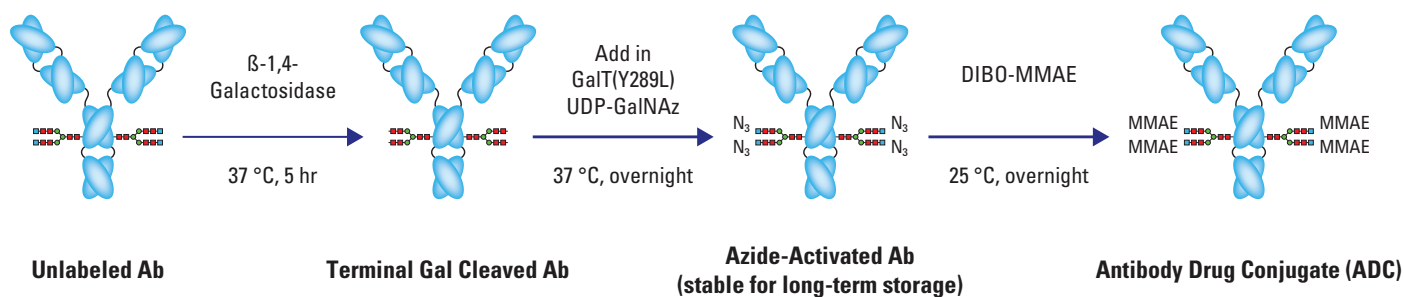
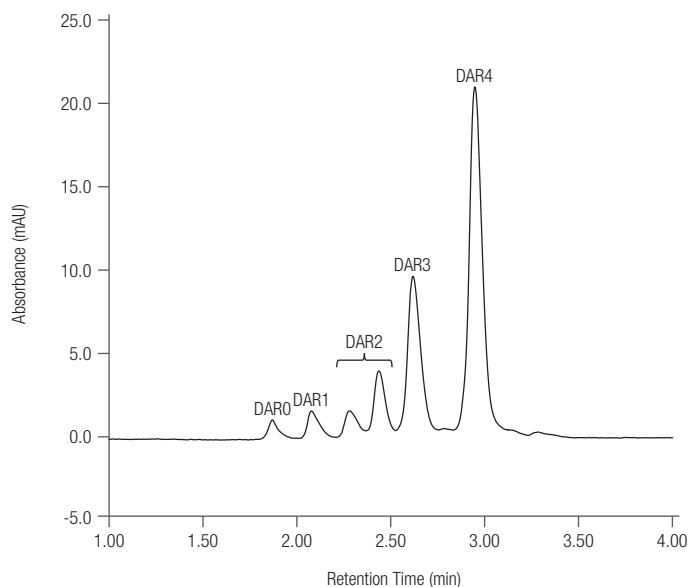


Figure 6. Site-selective antibody-drug conjugates (ADCs).

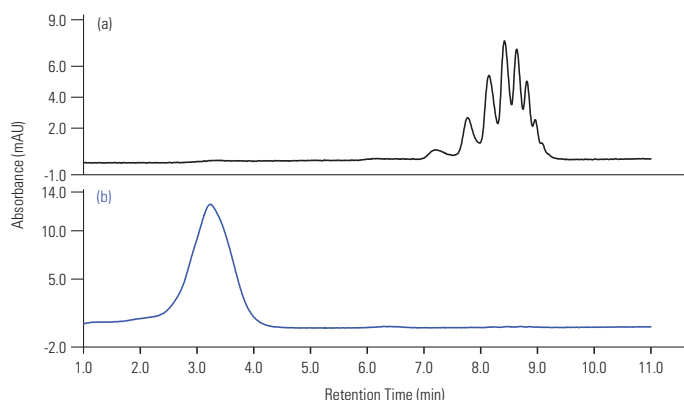


Column:	MAbPac RP, 4 $\mu$ m		
Format:	2.1 $\times$ 50 mm		
Mobile phase A:	H <sub>2</sub> O/TFA (99.9 : 0.1 v/v)		
Mobile phase B:	MeCN/ H <sub>2</sub> O/TFA (90: 9.9 : 0.1 v/v/v)		
Gradient:	Time (min)	%A	%B
	0.0	65	35
	0.5	65	35
	4.5	45	55
	5.0	45	55
	5.5	65	35
	6.0	65	35
Flow Rate:	0.6 mL/min		
Temperature:	80 °C		
Inj. Volume:	2 $\mu$ L		
Detection:	UV (280 nm)		
Sample:	Trastuzumab-MMAE		

Figure 7. MMAE modified trastuzumab ADC.

## Analysis of PEGylated Protein

Protein PEGylation technology has been used successfully to increase the circulating half life and decrease antigenicity of protein drugs. Most PEGylated proteins contains multiple PEGylated forms. Figures 8a and 8b illustrate the separation of a PEGylated protein and its de-PEGylated form on a 2.1 × 50 mm MAbPac RP column. At least eight PEGylated forms are resolved based on the degree of PEGylation.



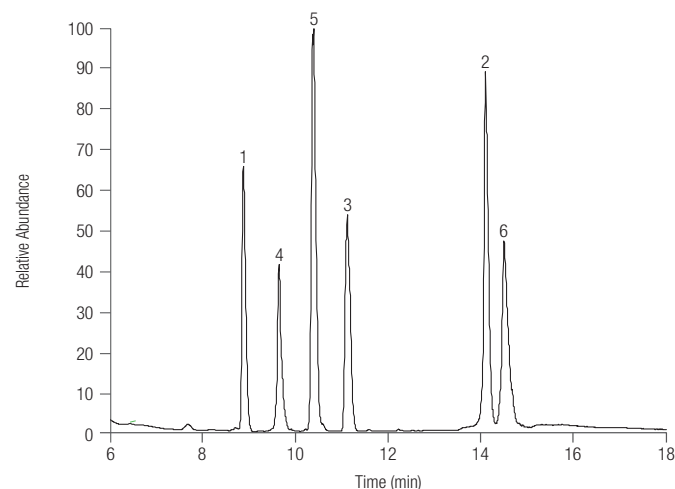
Column:	MAbPac RP, 4 μm		
Format:	3.0 × 50 mm		
Mobile phase A:	H <sub>2</sub> O/TFA (99.9 : 0.1 v/v)		
Mobile phase B:	MeCN/ H <sub>2</sub> O/TFA (90: 9.9 : 0.1 v/v/v)		
Gradient:	Time (min)	%A	%B
	0.0	55	45
	1.0	55	45
	11.0	25	75
	12.0	25	75
	14.0	55	45
	15.0	55	45
Temperature:	80 °C		
Flow rate:	0.5 mL/min		
Inj. volume:	10 μL		
Detection:	UV (280 nm)		
Sample:	(a) Pegylated protein (11 mg/mL)		
	(b) De-pegylated protein (1.24 mg/mL)		

Figure 8. PEGylated protein.

## Top-down LC/MS/MS Analysis of Intact Protein Standards

Thermo Scientific™ Pierce™ Intact Protein Mix consists of six recombinant proteins: IGF-I LR3 (9 kDa), thioredoxin (12 kDa), protein G (21 kDa), carbonic anhydrase II (29 kDa), protein AF (51 kDa), and exo klenow (68 kDa). These proteins are selected to satisfy the following criteria: 1) evenly covering a MW range of 10kD–66kD, 2) presenting mostly clean, modification and adduct-free ESI spectra, and 3) having ESI charge state distributions covered a wide *m/z* range from 500–2,000. A 1 mm ID MAbPac RP column was chosen to analyze these proteins because it provides higher sensitivity than larger

internal diameter columns, when sample size is limited. Figure 9a shows baseline separation of all six proteins using an acetonitrile/H<sub>2</sub>O/formic acid mobile phase. Top-down MS/MS spectra were acquired on a Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer using top 3–5 DDA method. OT MS1 data was acquired at resolution settings of 15 at *m/z* 200 and OTMS2 at a resolution of 120K at *m/z* 200. Figure 9b shows deconvolution results from Thermo Scientific™ Protein Deconvolution™ 4.0 software and Figure 9c shows top-down results from ProSight PD 1.1 node in Proteome Discoverer 2.1 software.



Column:	MAbPac RP, 4 μm		
Format:	1.0 × 150 mm		
Mobile phase A:	H <sub>2</sub> O/FA (99.9 : 0.1 v/v)		
Mobile phase B:	MeCN/FA (99.0: 0.1 v/v)		
Gradient:	Time (min)	%A	%B
	0.0	90	10
	1.0	80	20
	15.0	55	45
	16.0	10	90
	20.0	10	90
	20.1	90	10
	25.0	90	10
Temperature:	60 °C		
Flow Rate:	0.1 mL/min		
Inj. Volume:	1 μL		
MS Detection:	Positive-ion mode		
Mass Spec:	Q Exactive HF		
Sample:	Pierce Intact Protein Standard Mix, 500 ng/μL (p/n A35526)		
	1. IGF-I LR3 (9 kDa)		
	2. Thioredoxin (12 kDa)		
	3. Protein G (21 kDa)		
	4. Carbonic Anhydrase II (29 kDa)		
	5. Protein AF (51 kDa)		
	6. Exo Klenow (68 kDa)		

Figure 9a. Total ion chromatogram of the LC/MS analysis of the Intact Protein Standard Mix.

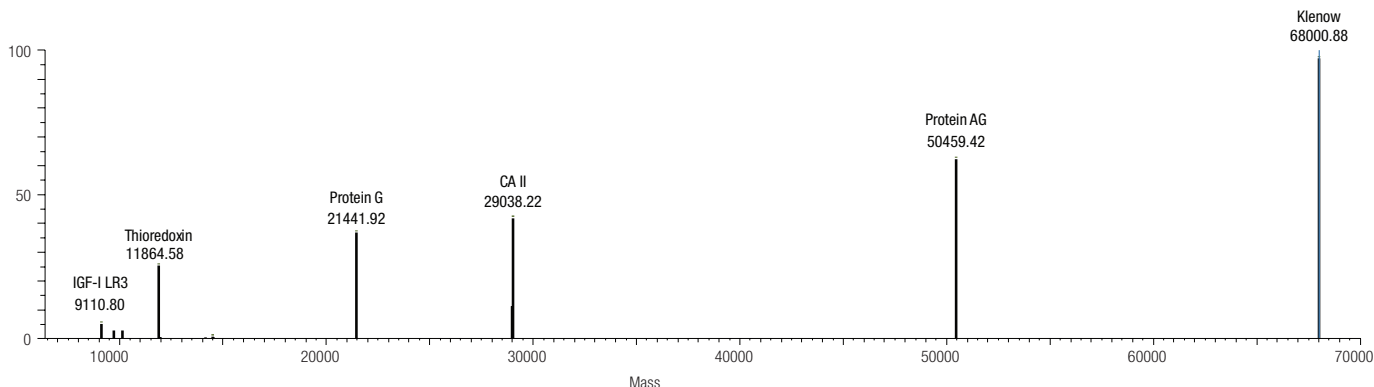


Figure 9b. Average masses for all six proteins in the Intact Protein Standard Mix.

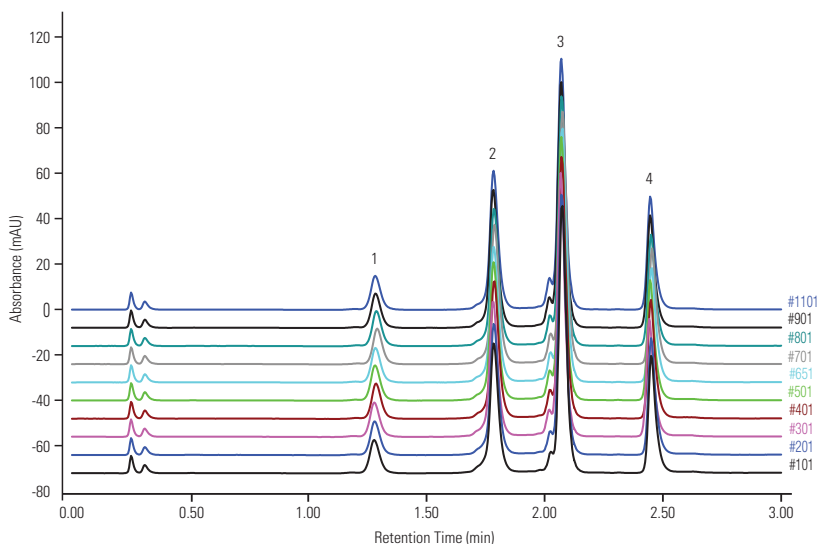
#	Checked	Master	Accession	Description	Coverage	# Peptides	# PSMs	# Unique Peptides	# Protein Groups	# AAs	MW [kDa]	calc. pI	# Peptides ProSightPD Absolute Mass Search
1	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	ProteinG	ProteinG Immunoglobulin G-binding protein G	100%	2	17	2	1	198	21.4	4.75	2
2	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	rCA	rCA Carbonic anhydrase 2	100%	1	9	1	1	260	29.1	7.12	1
3	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	M_trx	Thioredoxin, mitochondrial	99%	1	2	1	1	108	12.0	5.08	1
4	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	ProteinAG	ProteinAG Immunoglobulin G-binding protein A	100%	1	8	1	1	455	50.6	4.77	1
5	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	IGF_LR3	Insulin-like growth factor I	100%	1	2	1	1	83	9.1	8.28	1
6	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Klenow	Klenow DNA polymerase [ECO:0000256]	100%	1	4	1	1	605	68.0	6.11	1

Figure 9c. List of identified proteins using Proteome Discoverer 2.1 software.

### Excellent Reproducibility and Chemical Stability

Column ruggedness is a critical characteristic for accurate and reproducible results, as well as good column lifetime. MAbPac RP columns are packed using a carefully developed packing protocol to ensure excellent packed bed stability, column efficiency and peak asymmetry. Figure 10 demonstrates that the

excellent performance of the MAbPac RP is maintained throughout 1,000 runs at 80 °C providing consistent retention time, peak shape, and peak efficiency, with minimal increase in column backpressure. The RSDs of retention time from four protein peaks are tabulated in Table 1.



Column:	MAbPac RP, 4 µm		
Format:	2.1 × 50 mm		
Mobile phase A:	H <sub>2</sub> O/TFA (99.9 : 0.1 v/v)		
Mobile phase B:	MeCN/ H <sub>2</sub> O/TFA (90: 9.9 :0.1 v/v/v)		
Gradient:	Time (min)	%A	%B
	-1	80	20
	0.0	80	20
	2.5	50	50
	2.7	50	50
	2.8	80	20
	3.0	80	20

Temperature: 80 °C  
 Flow Rate: 0.6 mL/min  
 Inj. Volume: 1 µL  
 Detection: UV (280 nm)

Sample:

- Ribonuclease A (0.5 mg/mL)
- Cytochrome C (0.5 mg/mL)
- Lysozyme (0.5 mg/mL)
- mAb (1 mg/mL)

Peaks	1	2	3	4
RSD of Retention Time	0.535	0.262	0.225	0.123

Figure 10. Excellent reproducibility.

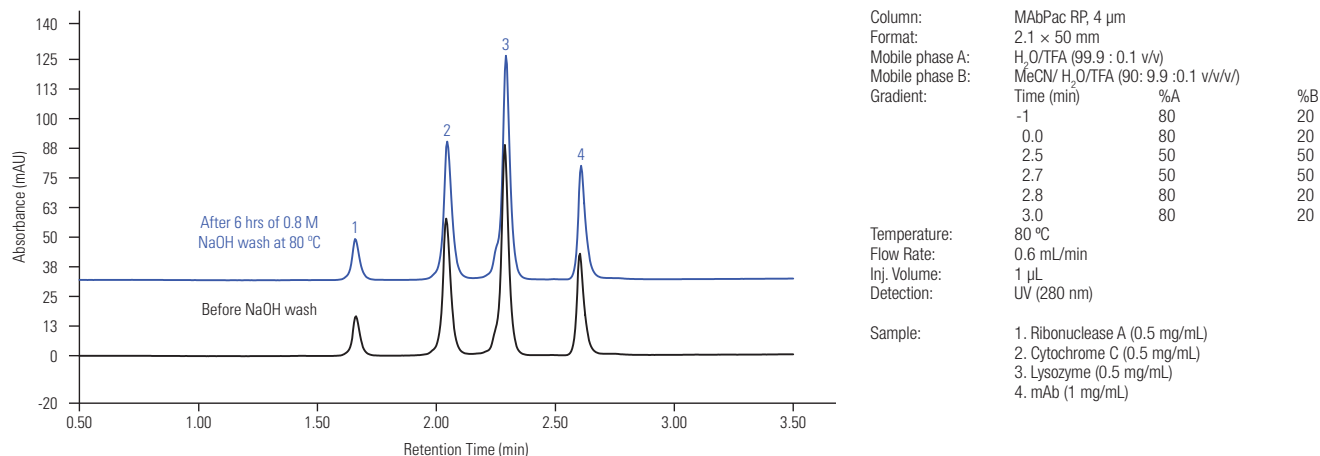


MABPac RP chemistry (PS-DVB) is stable under a wide pH range. Figure 11 shows that MABPac RP column maintains its performance after 6 hours of wash with 0.8 M NaOH at 80 °C. Compared to silica based RP

columns, such chemical stability, especially under alkaline condition provides a great advantage for column cleaning and removal of protein carryover.

**Table 1. Reproducibility of MABPac RP column.**

Run Sequence	Ribonuclease A			Cytochrome C			Lysozyme			mAb		
	Ret. Time (min)	PWHH (min)	Area	Ret. Time (min)	PWHH (min)	Area	Ret. Time (min)	PWHH (min)	Area	Ret. Time (min)	PWHH (min)	Area
101	1.28	0.046	0.732	1.78	0.038	2.632	2.08	0.034	4.812	2.45	0.033	1.973
201	1.28	0.045	0.729	1.78	0.038	2.715	2.07	0.035	4.871	2.45	0.033	2.051
301	1.28	0.044	0.735	1.78	0.038	2.794	2.07	0.035	4.942	2.44	0.033	2.069
401	1.29	0.043	0.734	1.79	0.038	2.798	2.07	0.035	4.896	2.45	0.033	1.996
501	1.28	0.044	0.743	1.78	0.038	2.849	2.07	0.035	4.941	2.45	0.033	2.044
601	1.28	0.044	0.726	1.78	0.038	2.688	2.07	0.034	4.692	2.45	0.033	2.041
701	1.29	0.043	0.737	1.79	0.038	2.829	2.08	0.036	4.807	2.45	0.033	2.042
801	1.29	0.043	0.724	1.78	0.038	2.799	2.07	0.035	4.708	2.45	0.034	1.914
901	1.28	0.044	0.723	1.78	0.038	2.801	2.07	0.035	4.681	2.45	0.033	1.99
1001	1.28	0.044	0.729	1.78	0.038	2.826	2.07	0.035	4.748	2.45	0.033	2.018
1101	1.3	0.043	0.72	1.79	0.038	2.786	2.08	0.035	4.634	2.45	0.033	1.894
<b>RSD</b>	<b>0.535</b>	<b>2.150</b>	<b>0.935</b>	<b>0.262</b>	<b>0.000</b>	<b>2.414</b>	<b>0.225</b>	<b>1.545</b>	<b>2.272</b>	<b>0.123</b>	<b>0.911</b>	<b>2.844</b>



**Figure 11. Superior chemical stability.**

## HPLC system consideration

Analytical format MAbPac RP columns in 2.1 mm ID and 3.0 mm ID format can be used with an HPLC system equipped with either a high pressure mixing pump (HPG), or low pressure mixing pump (LPG) to achieve high resolution separation. Low flow MAbPac RP columns, in 1.0 mm ID, are normally operated in the flow rate range of 75 to 150  $\mu\text{L}/\text{min}$  and therefore should be operated with a high pressure mixing pump to avoid excessive gradient delay. Figure 12 demonstrates that the excellent

separation of the four protein standards is achieved on a 1  $\times$  100 mm MAbPac RP column using a Thermo Scientific™ Vanquish™ Horizon UHPLC system with a 35  $\mu\text{L}$  gradient mixer. The gradient delay observed here is  $\sim$  2.5 min.

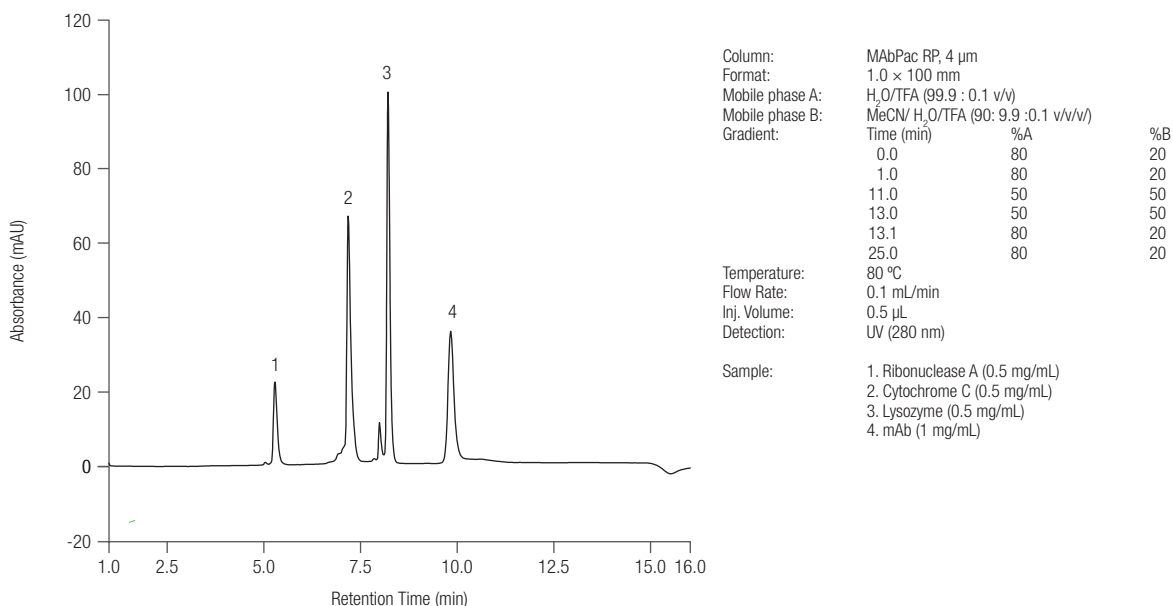


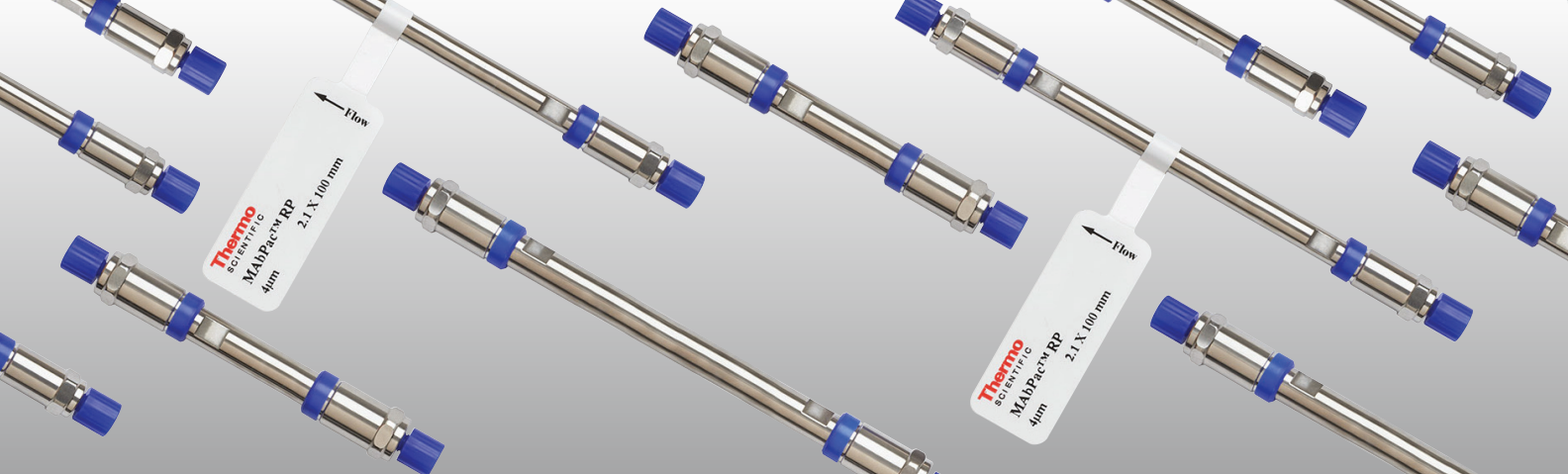
Figure 12. Separation of intact proteins/mAb using 1mm ID format column.

## Consistent Manufacturing

Each MAbPac RP column is manufactured according to stringent specifications to ensure column-to-column reproducibility. Each column is shipped with a test chromatogram.

## Physical Data

Chemistry	Phenyl
Polymer Substrate	DVB particles
Particle size	4 $\mu\text{m}$
Pore size	1,500 $\text{\AA}$
Column housing	Stainless steel for the 3.0 and 2.1 mm ID PEEK for the 1.0 mm ID



## Operational Specifications

Column	Column ID (mm)	Flow Rate (mL/min)	Pressure Limit (psi)	Temperature (°C)	pH Range
MAbPac RP	3.0	0.50–1.00	4,000	< 110	0–14
MAbPac RP	2.1	0.30–0.60	4,000	< 110	0–14
MAbPac RP	1.0	0.075–0.15	4,000	< 110	0–14

## Ordering Information

Description	Particle Size (µm)	Part Number
MAbPac RP, Analytical, 3.0 × 100 mm	4	088644
MAbPac RP, Analytical, 3.0 × 50 mm	4	088645
MAbPac RP, Guard, 3.0 × 10 mm	4	088646
MAbPac RP, Analytical, 2.1 × 100 mm	4	088647
MAbPac RP, Analytical, 2.1 × 50 mm	4	088648
MAbPac RP, Guard, 2.1 × 10 mm	4	088649
MAbPac RP, Analytical, 1.0 × 150 mm	4	302598
MAbPac RP, Analytical, 1.0 × 100 mm	4	302695
MAbPac RP, Analytical, 1.0 × 50 mm	4	302597

Find out more at [thermofisher.com/mabpac](http://thermofisher.com/mabpac)

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