MAbPac RP Columns

065625 Revision 03 • March 2017

Thermo SCIENTIFIC

For Research Use Only. Not for use in diagnostic procedures.

Product Manual

for

MAbPac RP Analytical and Guard Columns

3 × 100 mm, (Item # 088644)
3 × 50 mm, (Item # 088645)
3 × 10 mm, (Item # 088646)
2.1 × 100 mm, (Item # 088647)
2.1 × 50 mm, (Item # 088648)
2.1 × 10 mm, (Item # 088649)
1.0 × 150 mm, (Item # 303184)
1.0 × 100 mm, (Item # 303183)
1.0 × 50 mm, (Item # 303182)

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Revision History:

Revision 2, March, 2017, Added information for 1 mm column format.

Revision 3, October, 2017, Revised information for 1 mm column format.

Safety and Special Notices

Make sure you follow the precautionary statements presented in this guide. The safety and other special notices appear in boxes.

Safety and special notices include the following:

 **SAFETY**	***Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.***
 **WARNING**	***Indicates a potentially hazardous situation which, if not avoided, could result in damage to equipment.***
 **CAUTION**	***Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. Also used to identify a situation or practice that may seriously damage the instrument, but will not cause injury.***
 **NOTE**	***Indicates information of general interest.***
IMPORTANT	***Highlights information necessary to prevent damage to software, loss of data, or invalid test results; or might contain information that is critical for optimal performance of the system.***
Tip	***Highlights helpful information that can make a task easier.***

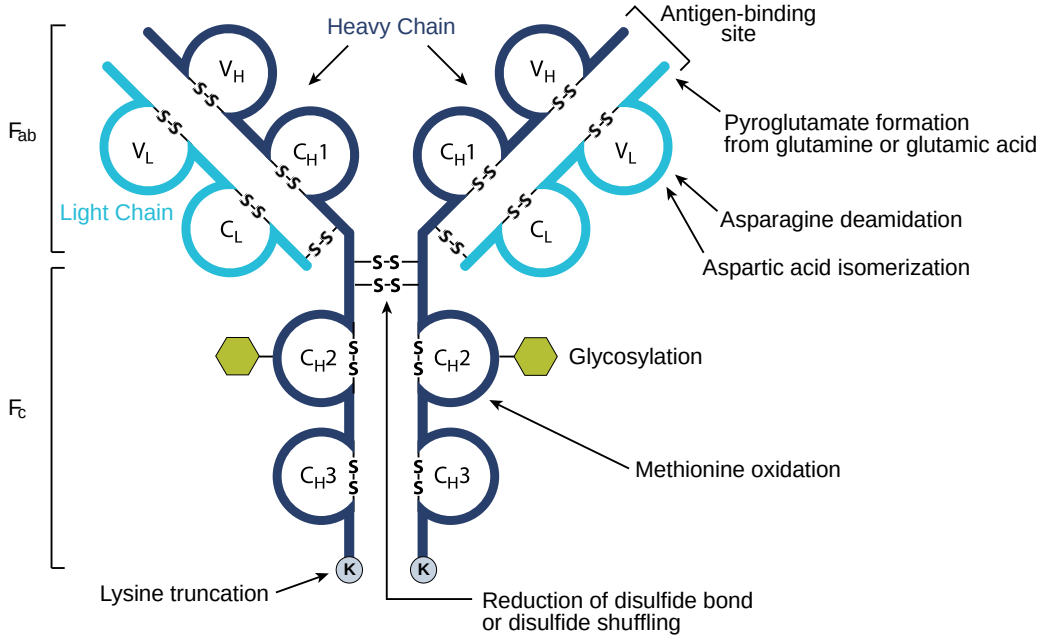
Contents

1. Introduction

1.1 Introduction to the MAbPac RP Column

MAbPac RP is a reverse phase (RP) column specifically designed for separation of intact monoclonal antibodies (mAbs) and mAb fragments (figure 1). The stationary phase is designed to be compatible with mass spectrometry friendly organic solvent such as acetonitrile and isopropanol and low pH eluents containing trifluoroacetic acid or formic acid. The MAbPac RP is based on supermacroporous 4 µm polymer particles that are stable at extreme pH (0 – 14) and high temperature (up to 110 °C). Particles are inherently hydrophobic so there is no bonded phase, alkyl ligand required for reversed-phase separations. The large pore size polymeric resin enables efficient separation of protein molecules with very low carry over.

Figure 1 – Structure of IgG and typical forms of heterogeneity



1.2 MAbPac RP Operating Limits and Specifications

1.2.1 Operating Conditions

Parameter	Recommendation
HPLC systems	Thermo Scientific™ Vanquish™ Horizon UHPLC system with a 35 µL gradient mixer and 75 µm I.D. connection tubing for the 1.0 mm I.D. columns Thermo Scientific™ Vanquish™ Horizon UHPLC system or Thermo Scientific™ Vanquish™ Flex system for the 2.1 mm and 3.0 mm I.D. columns
Flow Rate Range (recommended)	500 – 1,000 µL/min for the 3.0 mm I.D. columns 300 – 600 µL/min for the 2.1 mm I.D. columns 75 – 150 µL/min for the 1.0 mm I.D. columns
Shipping Solution / Long Term Storage Solution	MeCN/H_2O (50:50 v/v)
Typical buffers	**LC/UV experiment** Mobile phase A: H_2O/TFA (99.9:0.1 v/v) Mobile phase B: MeCN/H_2O/TFA (90:9.9:0.1 v/v/v) **LC/MS experiment** Mobile phase A: H_2O/FA/TFA (99.88:0.1:0.02 v/v/v) Mobile phase B: MeCN/H_2O/FA/TFA (90:9.88:0.1:0.02 v/v/v/v)
Solvents Compatibility	Up to 100% acetonitrile, isopropanol, methanol
Temperature Range	up to 110 °C
Pressure Limit	4,000 psi
pH Range	0 – 14 for 3.0 mm and 2.1 mm I.D. columns, 1 – 7 for 1 mm I.D. columns

WARNING

Do NOT flow hydrofluoric acid into the 1 mm I.D. columns.
Do NOT flow hot phosphoric acid into the 1 mm I.D. columns.

NOTE

Assistance is available for any problem during the shipment or operation of Thermo Scientific columns at techsupport.ccs@thermofisher.com

1.2.2 Physical Characteristics

Substrate:	Supermacroporous polymer
Particle size:	4 µm

1.3 Formats of the MAbPac RP Columns

Currently, MAbPac RP size exclusion columns are available in 3.0 mm, 2.1 mm, and 1.0 mm diameter formats.

Product Description	Part Number
MAbPac RP, 4µm, Analytical column 3.0 × 100 mm	088644
MAbPac RP, 4µm, Analytical column 3.0 × 50 mm	088645
MAbPac RP, 4µm, Guard 3.0 × 10 mm	088646
MAbPac RP, 4µm, Analytical column 2.1 × 100 mm	088647
MAbPac RP, 4µm, Analytical column 2.1 × 50 mm	088648
MAbPac RP, 4µm, Guard 2.1 × 10 mm	088649
MAbPac RP, 4µm, Analytical column 1.0 × 150 mm	303184
MAbPac RP, 4µm, Analytical column 1.0 × 100 mm	303183
MAbPac RP, 4µm, Analytical column 1.0 × 50 mm	303182

2. Getting Started; Step-By-Step Procedure

Thermo Fisher Scientific recommends that you perform an efficiency test on your MAbPac RP column before use. The purpose of column performance validation is to ensure no damage has occurred during shipping. Steps 1 – 5 below outline the necessary steps to perform this validation test. Test the column using the conditions described on the Quality Assurance (QA) report 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, plumbing, operating environment, reagent quality, column conditioning, and operator technique.

2.1 Step 1 – Visually inspect the column

Report any visible damage upon receiving the column to Thermo Fisher Scientific immediately. Depending upon the nature of the damage, we may request that you return the damaged column back to us for a replacement column.

2.2 Step 2 – Mobile Phase Selection

The MAbPac RP column can be used with a variety of mass spectrometry friendly organic solvents such as acetonitrile and isopropanol. In general we recommend adding 0.1% TFA to the mobile phase for good separation. When the HPLC system is coupled to mass spectrometry, it is recommended to reduce the TFA content to 0.02% to avoid ion suppression.

2.3 Step 3 – Set up the LC system

Use a standard LC system equipped with a LC pump, a column oven, a UV detector (210 - 220 nm and/or 280 nm) and an injector (or an autosampler). It is highly recommended that the system be optimized for low dead volume; usage of small internal diameter tubing (such as 100 µm) and a proper detector flow cell (such as the 2.5 µL semi-micro flow cell) is required for best results. The system should be thoroughly primed before use. It is recommended the column is run at high temperature (70 to 80 °C) to achieve better separation of mAb and mAb fragments.

Analytical format MAbPac RP columns in 2.1 mm ID and 3.0 mm ID format can be used with HPLC system equipped with either 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 µL/min and therefore should be operated with a high pressure mixing pump such as the Vanquish Horizon UHPLC system to avoid excessive gradient delay. The 1.0 mm ID MAbPac RP is designed to be used with high resolution mass spectrometer such as Thermo Scientific™ Q Exactive™ BioPharma for accurate intact mass detection and top-down protein sequencing.

2.4 Step 4 – Condition the column

Set the pressure limit on the pump to ≤ 4000 psi (276 bar). Slowly ramp up the flow rate: 0.5 mL/min for 3.0 mm ID column, 0.3 ml/min for 2.1 mm ID column, or 0.1 ml/min for 1.0 mm ID column. If possible, set 1-minute flow ramps up and down. Wash the column with mobile phase for 20 minutes.

2.5 Step 5 – Verify the performance of the column

Perform the column performance test using the conditions described in the Quality Assurance Report and compare the result with the one in the report. After the column is fully equilibrated, multiple injections of protein samples should be made until the reproducible results are obtained.

NOTE

Due to various reasons, such as differences in LC systems, mobile phases, etc, you may observe somewhat different separation from that in the report.

2.6 Step 6 – Real sample analysis

Once the column performance is satisfactorily confirmed in Step 1-5, the column is ready for real sample analysis. Equilibrate the column with the desired mobile phase before sample analysis.

NOTE

It is recommended that the column performance test be performed periodically to monitor the condition of the column.

3. Column Care

3.1 Column storage

The column can be stored in the mobile phase for short-term storage. For long-term storage (more than 5 days), it is recommended to store the column in a solution containing 90% acetonitrile in deionized water.

3.2 Operating pH range: pH 0 to 14 for 3.0 mm and 2.1 mm I.D. columns, pH 1 to 7 for 1.0 mm I.D. columns

WARNING

Do NOT flow hydrofluoric acid into the 1 mm I.D. columns.
Do NOT flow hot phosphoric acid into the 1 mm I.D. columns.

3.3 Operating temperature limit: up to 110 ºC

MAbPac RP column is stable at high temperature up to 110 ºC. The typical operating temperature for mAb and mAb fragment separation is between 70 ºC to 80 ºC.

3.4 Pressure limit: 4000 psi

The back pressure of the column is strongly correlated to the column temperature and flow rate.

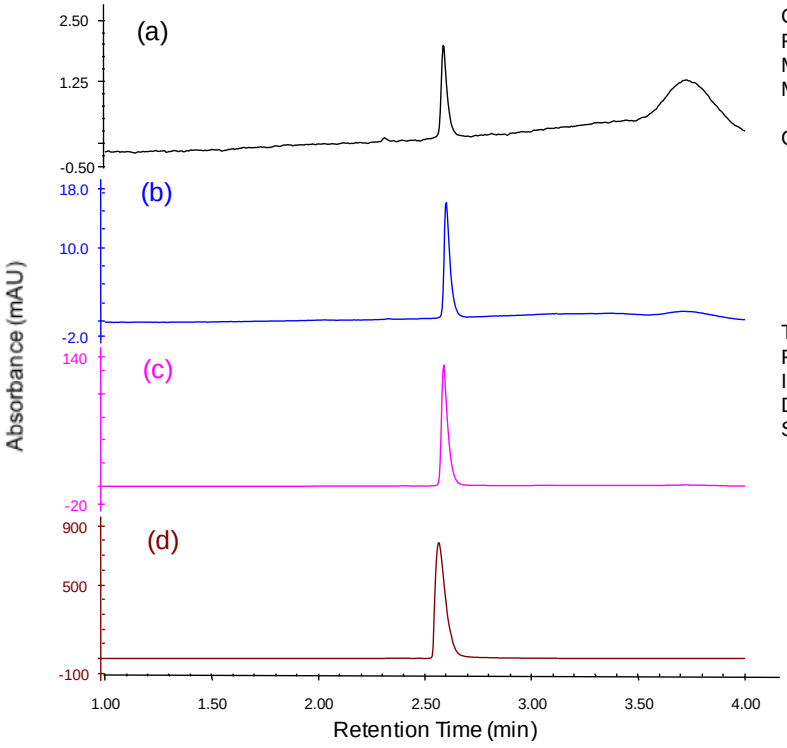
3.5 Flow rate

Please refer to operating condition table (section 1.2) for recommended flow rate at 80 ºC.

3.6 Loading capacity

The loading capacity of MAbPac RP column spans at least three orders of magnitude. Figure 2 shows a mAb analyzed on MAbPac RP at four different sample loadings: 0.02 µg, 0.2 µg, 2 µg, and 20 µg. The peak area to sample load is plotted and linear fit yields a R^2 equal to 1. This correlation allows the MAbPac RP column to be used for quantitation of mAb over a wide range of concentrations.

Figure 2 – Loadability



Column:	MAbPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	H_2O/TFA (99.9 : 0.1 v/v)
Mobile phase B:	MeCN/ H_2O/TFA (90: 9.9 :0.1 v/v/v/)
Gradient:	

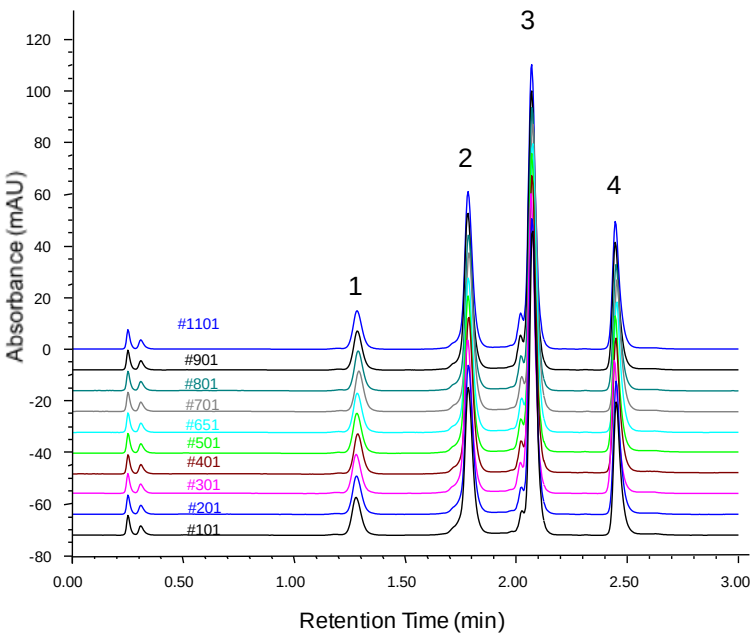
Time (min)	%A	%B
-1	85	15
0.0	85	15
2.5	50	50
2.7	50	50
2.8	85	15
3.0	85	15

Temperature:	80 °C
Flow rate:	0.6 mL/min
Inj. volume:	1 µL
Detection:	UV (280 nm)
Sample:	a. mAb (0.02 mg/mL) b. mAb (0.2 mg/mL) c. mAb (2 mg/mL) d. mAb (20 mg/mL)

3.7 Ruggedness

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 3 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 Figure 3.

Figure 3 – Excellent reproducibility



Column:	MAbPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	H_2O/TFA (99.9 : 0.1 v/v)
Mobile phase B:	MeCN/ H_2O/TFA (90: 9.9 :0.1 v/v/v)
Gradient:	

Time (min)	%A	%B
0.0	80	20
2.5	50	50
2.7	50	50
2.8	80	20
4.0	80	20

Temperature:	80 °C
Flow rate:	0.6 mL/min
Inj. volume:	1 µL
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)

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

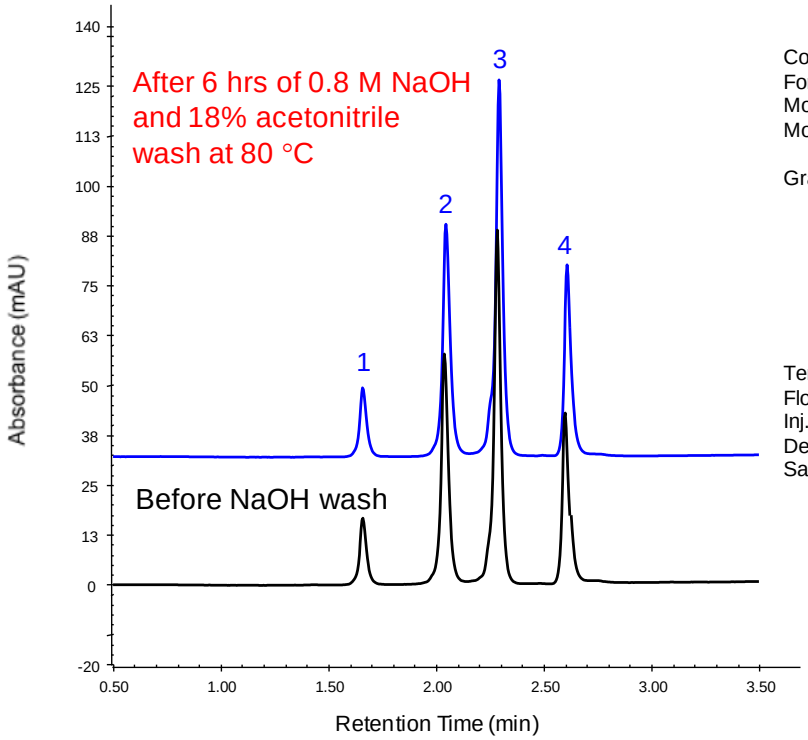
3.8 Column washing procedure

To elute very hydrophobic/sticky protein, increase the mobile phase organic content up 90% (such as 90% acetonitrile) and wash the column for 10-20 min. MAbPac RP chemistry (PS-DVB) is stable under a wide pH range. If needed, the 3.0 and 2.1 mm I.D. columns can be washed with a mixture of organic solvent and high pH eluent. Figure 4 shows that MAbPac RP, 2.1x50 mm column maintains its performance after 6 hours of wash with 0.8 M NaOH and 18% acetonitrile at 80 °C. Compared to silica based RP columns, the MAbPac RP has chemical stability, especially under alkaline condition and provides a great advantage for column cleaning and removal of protein carryover. However for the 1 mm I.D. column, do NOT wash the column with high pH eluent.

To eliminate metal contamination, wash the column in the following sequence:

1. 100 mM NH_4OAc (pH 5.0-5.4) for 10 column volumes
2. 100 mM Sodium Pyrophosphate solution (pH 6.5; adjust pH using phosphoric acid) for 100 column volumes
3. 100 mM NH_4OAc (pH 5.0-5.4) for 10 column volumes
4. MeCN/100 mM NH_4OAc (90:10; v/v) for 20 column volumes
5. MeCN/H_2O (50:50; v/v) for 10 column volumes

Figure 4 – Superior chemical stability



Column:	MAbPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	H_2O/TFA (99.9 : 0.1 v/v)
Mobile phase B:	MeCN/ H_2O/TFA (90: 9.9 :0.1 v/v/v)
Gradient:	

Time (min)	%A	%B
0.0	85	15
2.5	50	50
2.7	50	50
2.8	85	15
5.0	85	15

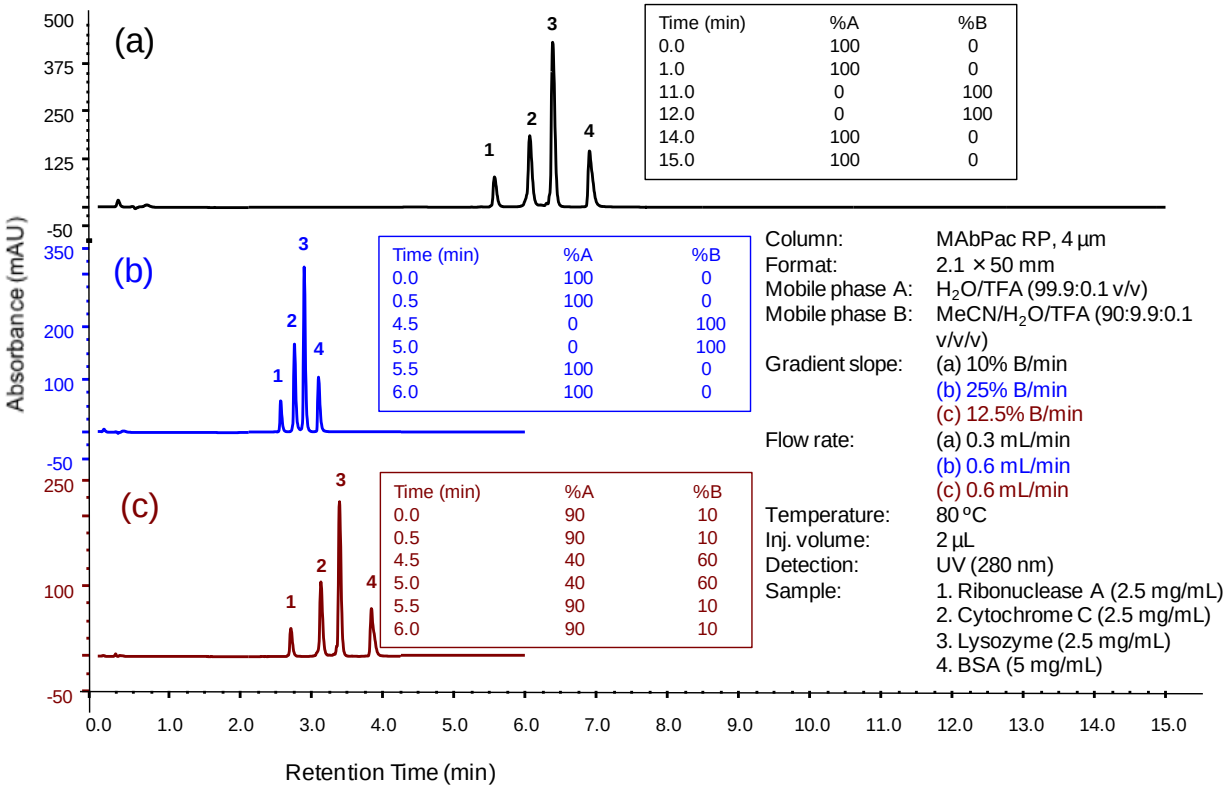
Temperature:	80 °C
Flow rate:	0.6 mL/min
Inj. volume:	1 µL
Detection:	UV (280 nm)
Sample:	1. Ribonuclease A (0.5 mg/mL) 2. Cytochrome C (0.5 mg/mL) 3. Lysozyme (0.5 mg/mL) 4. mAb (1 mg/mL)

4. Example Applications

4.1 Optimization for protein separation

The MAbPac RP column is designed for high resolution and fast separation of proteins. The speed and resolution of the separation can be optimized by adjusting both gradient slope and flow rate. The supermacroporous nature of the resin provides low back pressure characteristic and enables high flow rate. Figure 5 illustrates the separation of four standard proteins: ribonuclease A, cytochrome C, lysozyme, and BSA. In figure 5a, the four proteins were baseline separated using a 10-min gradient method with a gradient slope of 10% B per min. The total run time was 15 minutes. By doubling the flow rate, a 4-min gradient method with a gradient slope of 25% B per min (figure 5b) can separate the same four proteins, although the resolution is slightly lower than the separation achieved by the 10-min gradient method. By doubling the flow rate and reducing the total gradient range (10% B to 60% B), slightly better separation was achieved using a 4-min gradient method with a gradient slope of 12.5% B per min (figure 5c).

Figure 5 – Impact of flow rate and gradient slope on protein separation

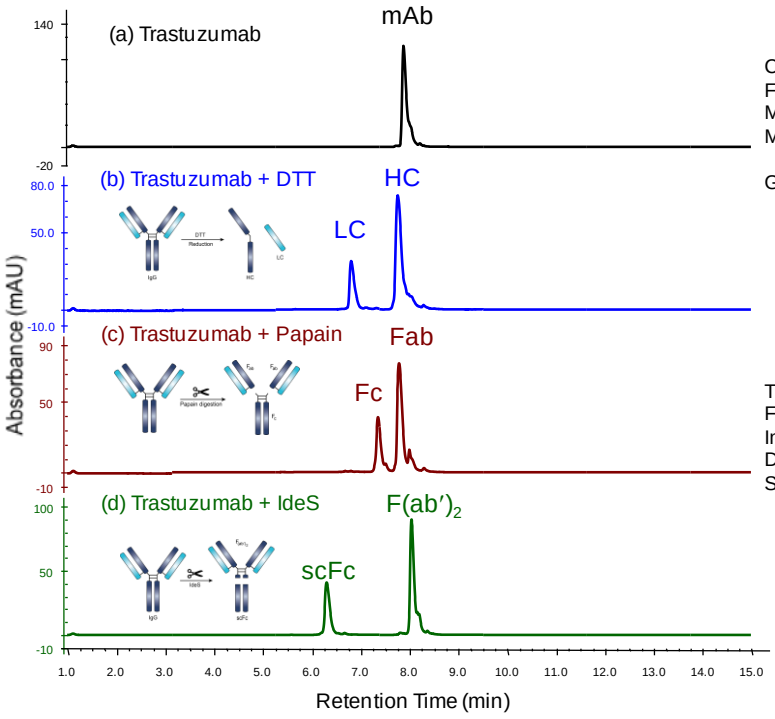


4.2 Separation of mAb and mAb fragments

Monoclonal antibodies are heterogeneous (Figure 1). 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 time consuming. A simpler and direct way to analyze the mAb variants and locate the modifications is to measure mAb fragments. Light chain (LC) and heavy chain (HC) are generated by the reduction of mAb, Fc and Fab fragments are generated by papain digestion. scFc and F(ab′)$_2$ fragments are generated by IdeS digestion. Figure 6 shows the analysis of Trastuzumab intact molecule and fragments. LC and HC (Figure 6b), Fc and Fab (Figure 6c), scFc and F(ab′)$_2$ (Figure 6d) are baseline separated using a MAbPac RP column with a 10-min gradient. Similar experiments have been carried out for Rituximab, Infliximab, and Bevacizumab. In all cases, mAb fragments have been successfully separated (data not shown).

Figure 6 – Analysis of Trastuzumab and fragments using MAbPac RP.

(a) Trastuzumab; (b) Trastuzumab LC and HC; (c) Trastuzumab Fc and Fab fragments; (d) Trastuzumab scFc and F(ab′)$_2$ fragments.



Column:	MAbPac RP, 4 µm
Format:	3 × 50 mm
Mobile phase A:	H_2O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
Mobile phase B:	MeCN/ H_2O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)
Gradient:	

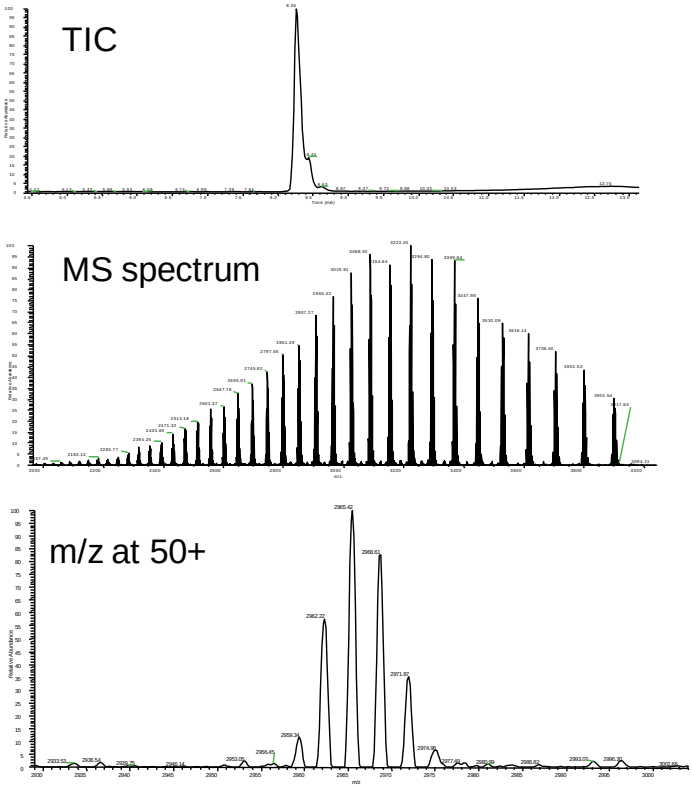
Time (min)	%A	%B
0.0	80	20
1.0	80	20
11.0	55	45
12.0	55	45
14.0	80	20
15.0	80	20

Temperature:	80 °C
Flow rate:	0.5 mL/min
Inj. volume:	5 µL
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)

4.3 LC/MS analysis of mAb

High resolution mass spectrometers such as Thermo QExactive 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 7** 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 .

Figure 7 – LC/MS analysis of Trastuzumab on MAbPac RP



Column:	MAbPac RP, 4 µm
Format:	3 × 50 mm
Mobile phase A:	H_2O/FA/TFA (99.88:0.1:0.02 v/v/v)
Mobile phase B:	MeCN/ H_2O/FA/TFA (90:9.88 :0.1:0.02 v/v/v/v)
Gradient:	

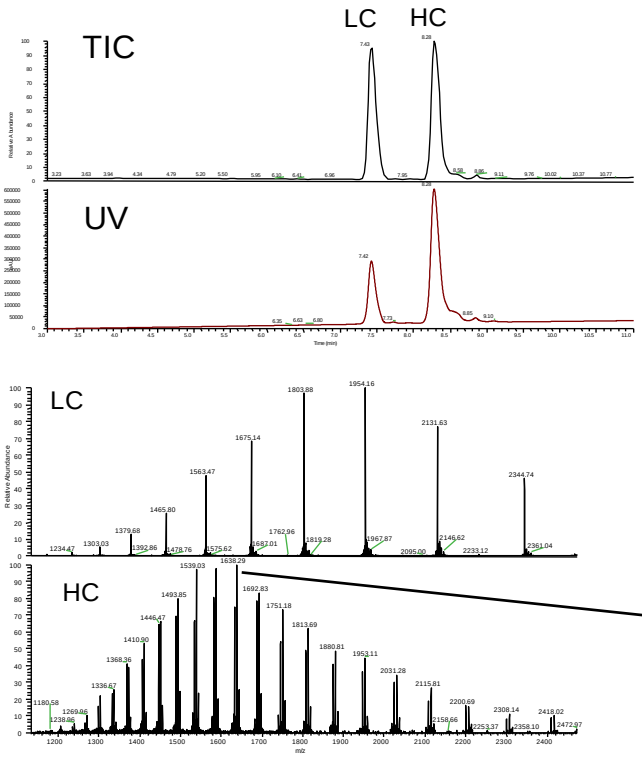
Time (min)	%A	%B
0.0	80	20
1.0	80	20
11.0	55	45
12.0	55	45
14.0	80	20
15.0	80	20

Temperature:	80 ºC
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)

4.4 LC/MS analysis of mAb fragments

During characterization, mAb is often reduced to LC and HC. Mass spectrometry analysis of these fragments can quickly review and localize the modifications. **Figure 8** 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.

Figure 8 – LC/MS analysis of Trastuzumab light chain (LC) and heavy chain (HC) on MAbPac RP



Column:	MAbPac RP, 4 µm
Format:	3 × 50 mm
Mobile phase A:	H_2O/FA/TFA (99.88:0.1:0.02 v/v/v)
Mobile phase B:	MeCN/ H_2O/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)

4.5 LC/MS analysis of oxidized mAb

Methionine (Met) oxidation is one of the critical quality attributes required to be closely monitored. The two Met residues in the CH2-CH3 domain interface of recombinant humanized and fully human IgG1 antibodies were found susceptible to oxidation. It is desirable to monitor the progress of the Met oxidation without complete digestion of mAb. A workflow was designed to first reduce mAb and then further digest it with IdeS resulting in smaller (25 kDa) fragments (Figure 9). Figure 10a shows that oxidized HC and non-oxidized HC can be barely separated chromatographically, with 0.02% TFA in the mobile phase. However, the high resolution orbitrap instrument can clearly resolve the oxidized (Figure 10b) and non-oxidized HC (Figure 10c) at *m/z* 1633.54 and 1633.06 respectively. Further digestion of the HC by IdeS resulted in two smaller fragments: scFc and Fd′. Figure 11a shows the baseline separation of scFc, LC, and Fd′. In addition, oxidized and non-oxidized scFc fragments are better separated than the oxidized and non-oxidized HC. The +10 charge state of the oxidized scFc and non-oxidized scFc are shown in Figure 11b (at *m/z* 2525.60) and in Figure 11c (at *m/z* 2524.08). Separation of these oxidized and non-oxidized fragments can be improved by increasing the mobile phase TFA concentration from 0.02% to 0.1% (Figure 12).

Figure 9 – mAb reduction and IdeS digestion flowchart.

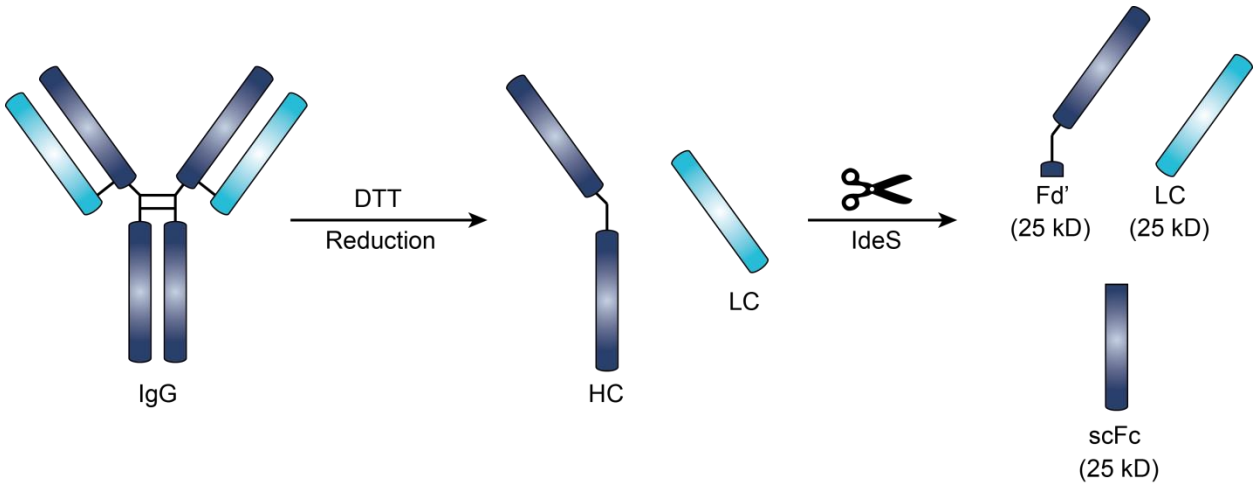
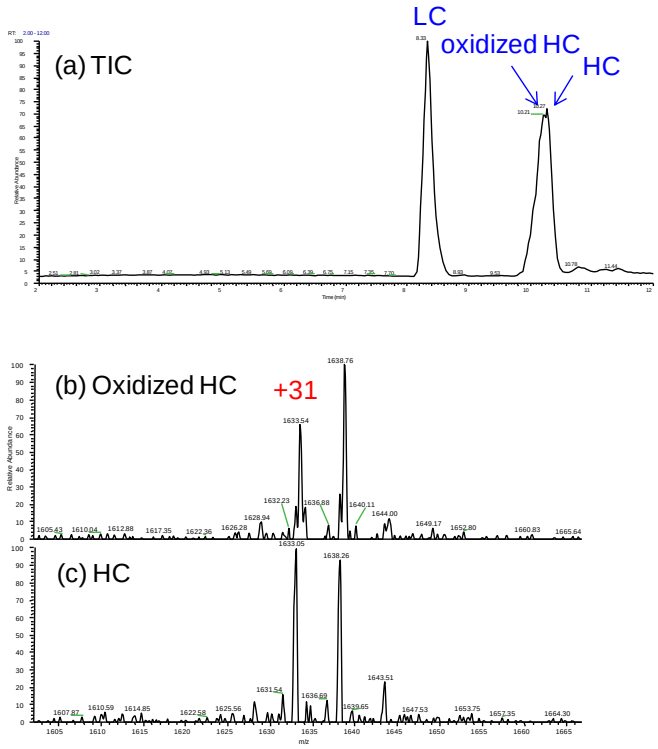


Figure 10 – LC/MS analysis of Trastuzumab LC and HC. (a) total ion current (TIC); (b) mass spectrum of oxidized HC; (c) mass spectrum of non-oxidized HC.



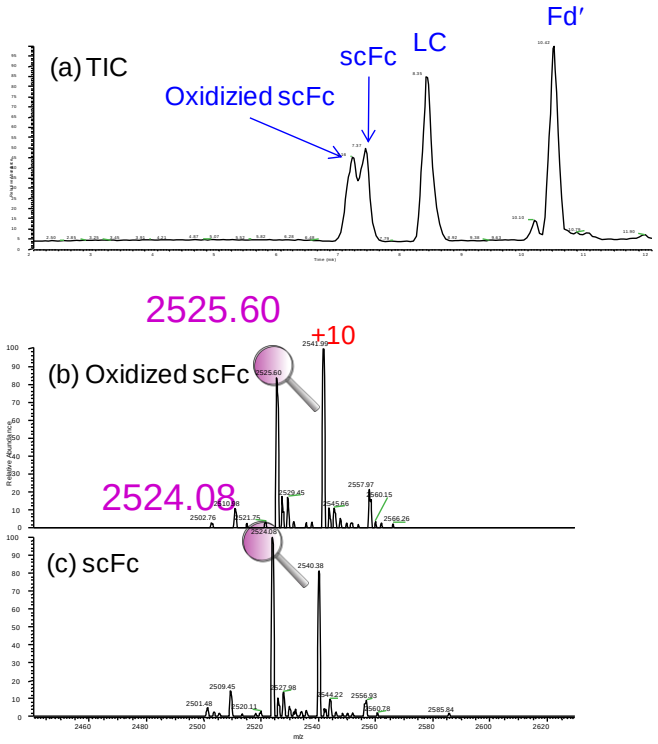
Column:	MAbPac RP, 4 µm
Format:	3 × 50 mm
Mobile phase A:	H_2O/FA/TFA (99.88:0.1:0.02 v/v/v)
Mobile phase B:	MeCN/ H_2O/FA/TFA (90:9.88 :0.1:0.02 v/v/v/v)
Gradient:	

Time (min)	%A	%B
0.0	75	25
1.0	75	25
11.0	63	37
12.0	63	37
14.0	75	25
15.0	75	25

Temperature:	80 °C
Flow rate:	0.5 mL/min
Inj. volume:	2 µL
MS Detection:	positive-ion mode
Mass spec:	Q Exactive™ Plus
Sample:	oxidized trastuzumab, reduced by DTT (2 mg/mL)

Figure 11 – LC/MS analysis of Trastuzumab scFc, LC, and Fd′.

(a) total ion current (TIC); (b) mass spectrum of oxidized scFc; (c) mass spectrum of non-oxidized scFc.



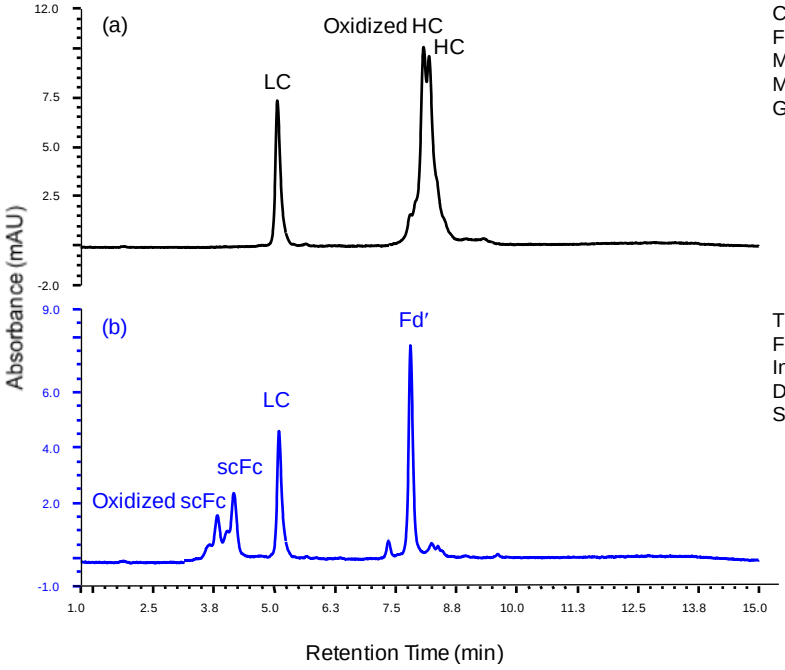
Column:	MAbPac RP, 4 µm
Format:	3 × 50 mm
Mobile phase A:	H_2O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
Mobile phase B:	MeCN/ H_2O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)
Gradient:	

Time (min)	%A	%B
0.0	75	25
1.0	75	25
11.0	63	37
12.0	63	37
14.0	75	25
15.0	75	25

Temperature:	80 °C
Flow rate:	0.5 mL/min
Inj. volume:	2 µL
MS Detection:	positive-ion mode
Mass spec:	Q Exactive™ Plus
Sample:	Oxidized trastuzumab, reduced by DTT and digested by IdeS (1 mg/mL)

Figure 12 – Separation of Trastuzumab fragments with mobile phases containing 0.1% TFA.

(a) Trastuzumab LC and HC; (b) Trastuzumab scFc, LC, and Fd′.



Column:	MAbPac RP, 4 µm
Format:	3 × 50 mm
Mobile phase A:	H_2O/TFA (99.9:0.1 v/v)
Mobile phase B:	MeCN/H_2O/TFA (90:9.9:0.1 v/v/v)
Gradient:	

Time (min)	%A	%B
0.0	70	30
1.0	70	30
11.0	60	40
12.0	60	40
14.0	70	30
15.0	70	30

Temperature:	80 °C
Flow rate:	0.5 mL/min
Inj. volume:	2 µL
Detection:	UV (280 nm)
Sample:	(a) Trastuzumab + DTT (2 mg/mL) (b) Trastuzumab + DTT + IdeS (1 mg/mL)

4.6 Analysis of Antibody-Drug Conjugate (ADC)

Antibody-drug conjugates have 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 13, ADCs were prepared by enzymatically activating mAb Fc domain glycans with azides using the mutant beta-galactosyltransferase enzyme. The azide-activated antibodies were then conjugated with dibenzocyclooctyne6 (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 14).

Figure 13 – Site-selective antibody-drug conjugates (ADCs)

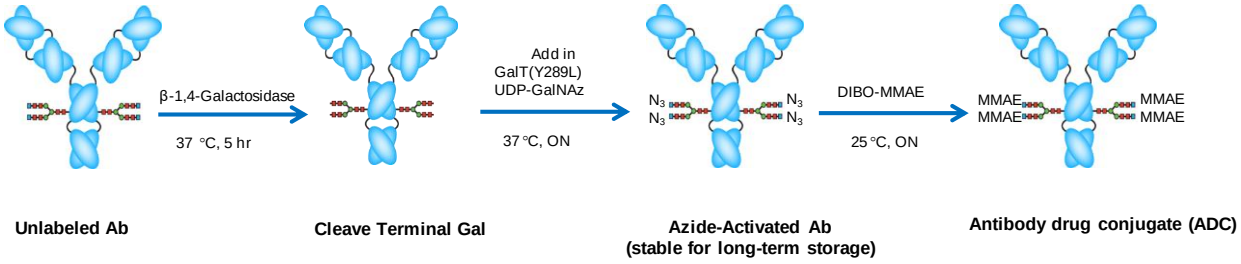
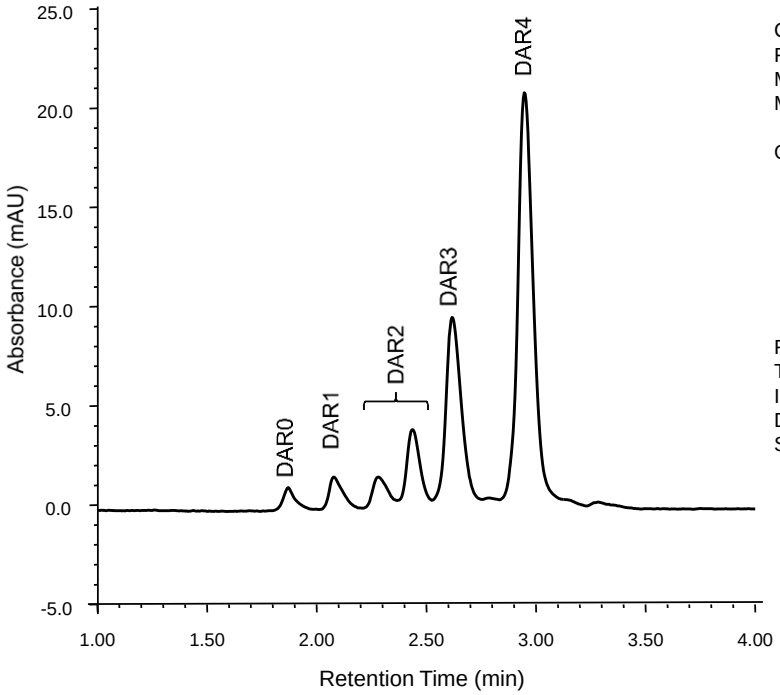


Figure 14 – Site-selective antibody-drug conjugates (ADCs)



Column:	MAbPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	H_2O/TFA (99.9 : 0.1 v/v)
Mobile phase B:	MeCN/ H_2O/TFA (90: 9.9 :0.1 v/v/v)
Gradient:	

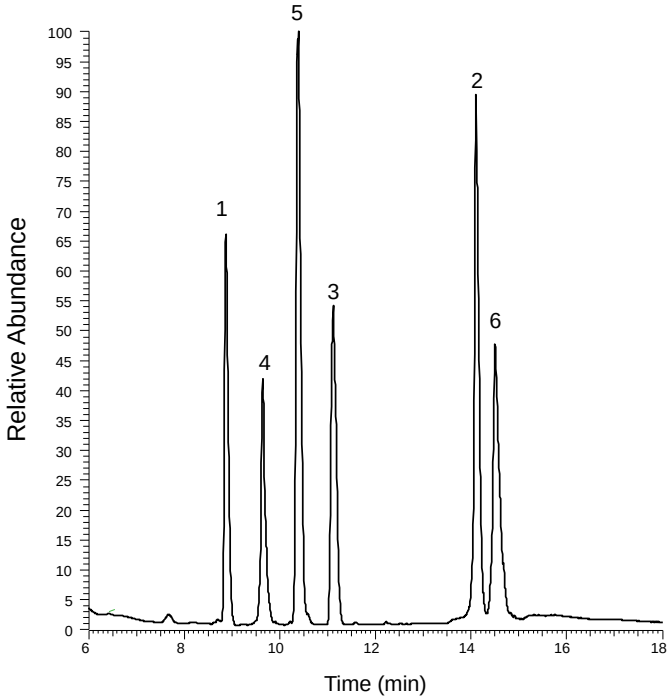
Time (min)	%A	%B
0.0	65	35
0.5	65	35
4.5	45	55
5.0	45	55
5.5	65	35
6.0	65	35

Flow rate:	0.6 mL/min
Temperature:	80 ºC
Inj. volume:	2 µL
Detection:	UV (280 nm)
Sample:	Trastuzumab-MMAE

4.7 Top-down LC/MS/MS Analysis of Intact Protein Mix

ThermoFisher Pierce Intact Protein Mixconsists of sixrecombinant 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. 1 mm ID MAbPac RP column is chosen to analyze these proteins because it provides higher sensitivity. Figure 15 shows baseline separation of all six protein using an acetonitrile/H_2O/formic acid mobile phase. Top-down MS/MS spectra were acquired using Top 3-5 DDA methods. 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. Top panel shows deconvolution results from Protein Deconvolution 4.0 software and Bottom panel shows top down results from ProSight PD 1.1 node in Proteome Discoverer 2.1 software.

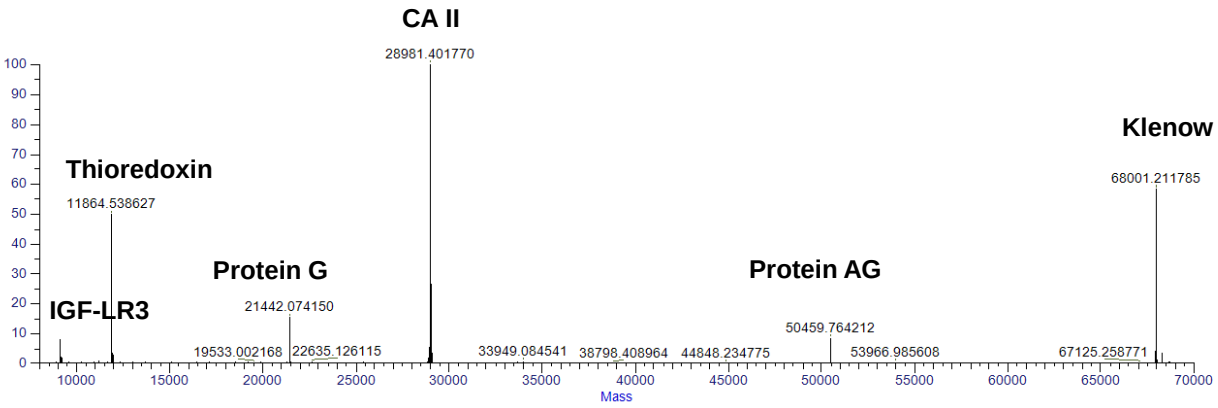
Figure 15 – LC/MS/MS Analysis of Intact Protein Mix

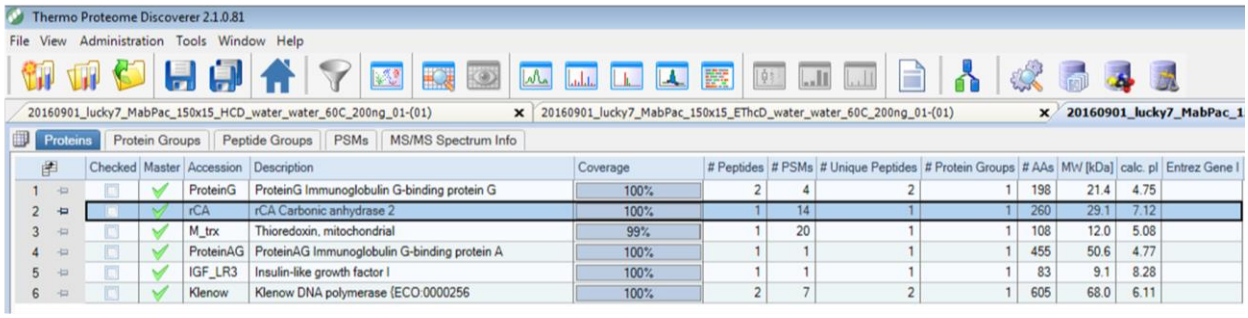


Column:	MAbPac RP, 4 µm
Format:	1.0 × 150 mm
Mobile phase A:	H_2O/FA (99.9 : 0.1 v/v)
Mobile phase B:	MeCN/FA (99.0: 0.1 v/v)
Gradient:	

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 ºC
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) 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)





	Checked	Master	Accession	Description	Coverage	# Peptides	# PSMs	# Unique Peptides	# Protein Groups	# AAs	MW [kDa]	calc. pI	Entrez Gene I
1			ProteinG	ProteinG Immunoglobulin G-binding protein G	100%	2	4	2	1	198	21.4	4.75	
2			rCA	rCA Carbonic anhydrase 2	100%	1	14	1	1	260	29.1	7.12	
3			M_trx	Thioredoxin, mitochondrial	99%	1	20	1	1	108	12.0	5.08	
4			ProteinAG	ProteinAG Immunoglobulin G-binding protein A	100%	1	1	1	1	455	50.6	4.77	
5			IGF_LR3	Insulin-like growth factor I	100%	1	1	1	1	83	9.1	8.28	
6			Klenow	Klenow DNA polymerase (ECO:0000256	100%	2	7	2	1	605	68.0	6.11	

5. Frequently Asked Questions

5.1 What factors do I need to consider for developing a high throughput method using MAbPac RP

Flow rate, column operating temperature, and gradient slope should be considered (see section 4.1). MAbPac RP is a polymer column which is stable at extreme high temperature and extreme pH. If higher flow rate is desired, operating temperature can be increased to decrease the column back pressure.

5.2 What is the recommended operating temperature for MAbPac RP?

For small proteins such as cytochrome C and lysozyme, 30 °C is sufficient. For large proteins such as mAb, 70 °C to 80 °C is recommended in order to decrease the strength of secondary interactions between mAb and the stationary phase and obtain decent peak shape.

5.3 What is the carryover on MAbPac RP?

In general, the carryover is less than 0.7% for mAb. For example, mAb peak area is determined by injecting 25 µg of mAb onto a 3.0 × 50 mm MAbPac column; the carry over peak area is determined from the blank run immediately following the mAb injection. The carryover is calculated using the following equation: $Area_{carryover}/Area_{mAb}$ x 100%.

mAbs are large and very hydrophobic biomolecules. In order to achieve low carryover for mAb analysis, such as less than 1%, the pore size of the column should be larger than the conventional RP column and the stationary phase is optimized. MAbPac RP column is designed for low carryover and high resolution mAb analysis.

5.4 What is the recommended maximum flow rate?

At 80 °C, the maximum flow rate for the 1.0 × 50 mm ID column is 0.15 ml/min.

At 80 °C, the maximum flow rate for the 2.1 × 50 mm ID column is 0.6 ml/min.

At 80 °C, the maximum flow rate for the 3.0 × 50 mm ID column is 1 ml/min.

The column pressure should not exceed 4,000 psi in order to avoid compressing the resin bed.

5.5 What is the recommended TFA concentration for protein separation?

0.1% TFA if the column is not coupled to the mass spectrometer and 0.02% for LC/MS experiment. 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).

5.6 How to mitigate carryover issue?

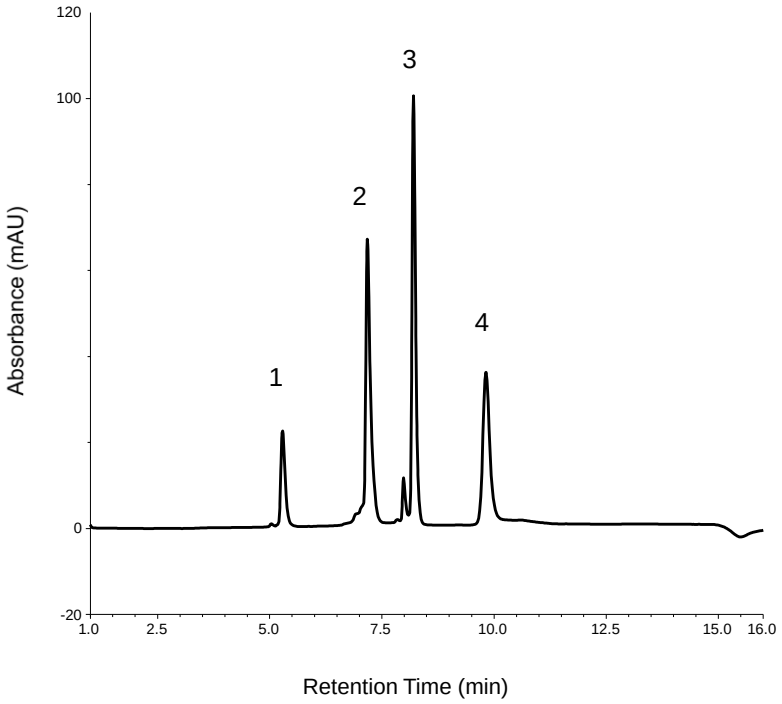
The system may not be clean or previous sample may not have completely eluted from the column. Wash the system and the column with high organic eluent, such as 90% acetonitrile. If needed, 100 mM NaOH can be added to the high organic wash (for 3.0 mm and 2.1 mm I.D. columns. Do

not use NaOH solution for 1 mm I.D. columns). In addition, a blank can be added in between samples.

5.7 How to optimize the HPLC system configuration to achieve the best separation using the 1.0 mm ID MAbPac RP column?

The recommended flow rate for the 1.0 mm ID column is between 75 µL to 150 µL. In order to avoid excessive gradient delay, the high pressure pump Vanquish Horizon system with the default 35 µl gradient mixer (25 µL capillary mixer + 10 µL static mixer) is recommended. If Vanquish Horizon system is not available, Vanquish Flex Binary system with 200 µL gradient mixer (50 µL capillary mixer + 150 µL static mixer) will be the alternative choice. In addition, 75 µm I.D. connection tubing could reduce the system dead volume. Figure 16 shows that excellent separation of the four protein standards is achieved on a 1 × 100 mm MAbPac RP using a default configuration of Vanquish™ Horizon UHPLC system. The gradient delayed observed here is ~ 2.5 min.

Figure 16 – Separation of intact proteins/mAb using 1 mm ID format column



HPLC:	Thermo Scientific™ Vanquish™ Horizon UHPLC system
Column:	MAbPac RP, 4 µm
Format:	1.0 × 100 mm
Mobile phase A:	H_2O/TFA (99.9 : 0.1 v/v)
Mobile phase B:	MeCN/ H_2O/TFA (90: 9.9 :0.1 v/v/v/)
Gradient:	

Time (min)	%A	%B
0.0	80	20
1.0	80	20
11.0	50	50
13.0	50	50
13.1	80	20
25.0	80	20

Temperature:	80 ºC
Flow rate:	0.1 mL/min
Inj. volume:	0.5 µL
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)