Thermo Scientific

MAbPac HIC-Butyl

Product Manual

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Thermo SCIENTIFIC

Product Manual

for

MAbPac HIC-Butyl

MAbPac HIC-Butyl, 5 µm, 4.6 × 100 mm (P/N: 088558)
MAbPac HIC-Butyl, 5 µm, 4.6 × 10 mm Guard Cartridges 2/pk (P/N: 088559)

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

Revision History:

Revision 01, January, 2015, Original Publication.

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.***

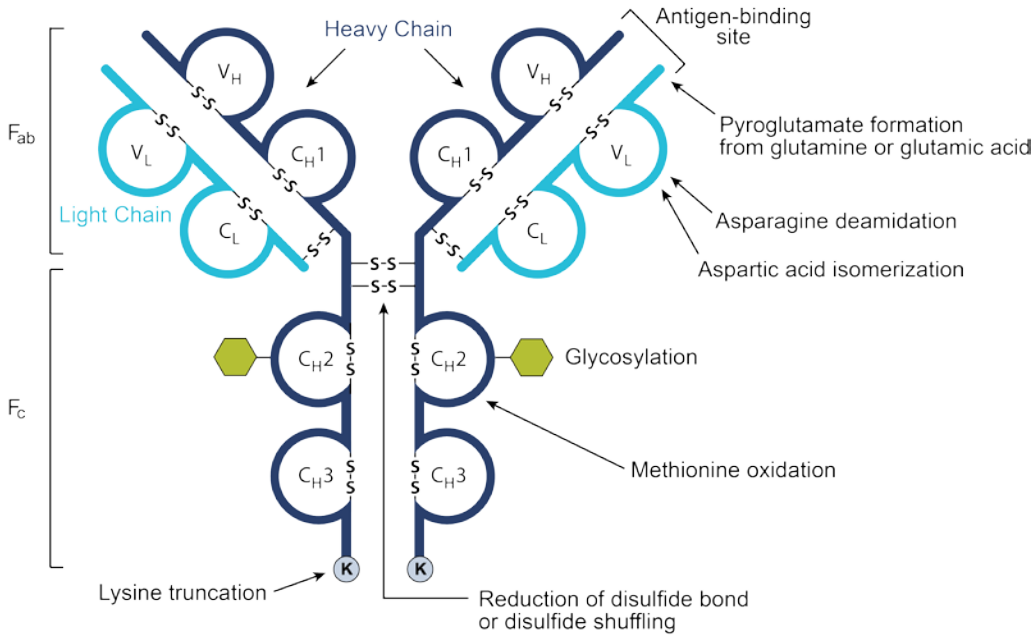
Contents

1. Introduction

Thermo Scientific™ MAbPac™ HIC-Butyl is a high resolution polymer based hydrophobic interaction chromatography (HIC) column designed for the separation of mAbs and ADCs. Hydrophilic, non-porous polymer resin is functionalized with butyl groups that provide hydrophobic interaction with analytes. The density of the butyl group was optimized to provide high resolution separation and biocompatibility.

Monoclonal antibodies (mAbs) have proved to be one of the most successful classes of biotherapeutics. The FDA has approved an increasing number of mAbs for clinical uses against cancer, autoimmune disorder, Crohn's disease and rheumatoid arthritis among other diseases. Recombinant mAbs are subject to a variety of biochemical modifications during processing, delivery and storage (Figure 1). Due to the potential impact of these modifications on mAb safety and efficacy, thorough characterization of mAb products has become increasingly important.

Figure 1: Structure of IgG and Typical Forms of Heterogeneity



Hydrophobic interaction liquid chromatography (HIC) separates proteins and mAb molecules in order of increasing hydrophobicity. Analytes bind to the weakly hydrophobic stationary phase in the presence of high salt concentration and elute off the column as the salt concentration decreases. In contrast to reverse phase liquid chromatography, HIC typically preserves the biological activity of the protein, which is useful for downstream functional analysis such as binding and cell-based potency assays. In addition, HIC typically provides separation with little carryover. Due to these benefits, HIC is not only used for analysis of mAb variants but also has been widely used as a purification method for mAb products.

1.1 Main Features

- Optimal selectivity for antibody-drug conjugates (ADCs)
- High resolution and high efficiency
- Stable at wide range of pH (2-12)
- Rugged column packing

1.2 Specifications and Operating Conditions

pH range:	2.0 – 12.0
Temperature:	10 °C to 60 °C
Aqueous compatibility:	50 – 100 % aqueous mobile phase
Organic compatibility:	Compatible with common HPLC solvents up to 50%

Dimension (mm)	Recommended Flow Rate (mL/min)	Maximum Flow rate (mL/min)	Maximum Pressure (psi)
4.6 × 100 mm	0.5 – 1.0	1.5	4,000

1.3 Physical Data

Column chemistry:	Butyl
Substrate:	Hydrophilic, non-porous polymer
Particle size:	5 µm

2. Installation

It is recommended that you run the column performance test upon receiving a new MAbPac HIC-Butyl column. The purpose of such test is to ensure no damage has occurred during shipping. Steps 1-6 below outline the necessary steps to perform this validation test. Test the column using the conditions described on the Quality Assurance Report (QAR) enclosed in the column box.

Step 1 – Visually inspect the column

Report any damage to Thermo Fisher Scientific. Depending on the nature of the damage, we may request that you ship the damaged column back to Thermo Scientific for replacement.

Step 2 – Prepare mobile phase and standard injection solution

To obtain reliable, consistent and accurate results, use HPLC grade solvents and Type 1 reagent grade water with a specific resistance of 18.2 megohm-cm or greater filtered through a 0.22 µm filter.

NOTE

If your LC system does not have an integrated degasser, degas all mobile phases individually. Whenever applicable, degas the aqueous component and solvent component separately before mixing them together. Excessive purging or degassing of mobile phases should be avoided because it may change mobile phase composition.

Mobile Phase (1 L): 50 mM Sodium phosphate, 300 mM NaCl, pH 6.8

1. Dissolve following components in 990 ± 2 g of DI water.
   a. 3.00 ± 0.05 g of NaH_2PO_4 (sodium phosphate monobasic: MW 119.98)
   b. 8.95 ± 0.10 g of $Na_2HPO_4 \cdot 12H_2O$ (sodium phosphate dibasic: MW 358.14)
   c. 17.53 ± 0.20 g of NaCl (sodium chloride MW 58.4)
2. Check the pH (should be 6.50 – 6.60)
3. Filter the solution with 0.22 µm filter.

Standard Injection Solution

1. Prepare 1 mg/mL solution of formamide in DI water.

Step 3 – Setup the LC system

The column can be used on any LC system that is equipped with a LC pump, a column oven, an injector (or an autosampler), and a UV detector. The system should be thoroughly primed before use.

Step 4 – Condition the column

Slowly ramp up the flow rate to 0.2 mL/min. If possible, set the flow ramps up and down to 1 mL/min[2]. Wash the column with mobile phase for 20 minutes at 0.2 mL/min.

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 should be made until the reproducible retention is obtained.

NOTE

Due to various reasons, such as difference of LC systems, system plumbing, operating environment, and reagent quality, oven temperature control, you may observe slightly different retention times from those in the enclosed QAR test.

Step 7 – 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.

WARNING

Wash the system thoroughly with water after analysis. High salt concentration may clog your system if left overnight.

NOTE

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

3. Considerations in Method Development

3.1 Mobile phase

Type of salt, concentration of salt in the starting mobile phase, addition of organic solvent and pH are important parameters in Hydrophobic Interaction Chromatography (HIC).

3.1.1 Type of salt and concentration in the starting mobile phase

Most commonly used salts in HIC are ammonium sulfate, sodium chloride and ammonium acetate. We recommend 2 M ammonium sulfate as a starting point (see section 3.1.4). If the retention time is long, decrease the salt concentration of the starting mobile phase. If the peaks elute early and are broad, increase the concentration of the salt. The starting salt concentration can affect the resolution. Decrease in ammonium sulfate concentration, resulted in higher resolution. Decreasing the initial salt concentration can be achieved by simply adjusting the gradient.

3.1.2 Organic solvent

Addition of organic solvent may improve the resolution especially for large hydrophobic proteins or monoclonal antibodies. The most commonly used organic solvents are isopropanol and acetonitrile.

3.1.3 Mobile phase pH

The pH of the mobile phase affects the charge of the protein which contributes to the overall hydrophobicity of the sample. Therefore adjusting the pH may result in a different separation profile.

3.1.4 Mobile phase recommendations

Use these conditions as a starting point and further adjust if needed.

Mobile phase Formula 1 (for less hydrophobic samples)	
Mobile phase A	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B	100 mM sodium phosphate, pH 7.0

Mobile phase Formula 2 (for more hydrophobic samples)	
Mobile phase A	1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / isopropanol (95:5 v/v)
Mobile phase B	50 mM sodium phosphate, pH 7.0 / isopropanol (80:20 v/v)

3.1.5 Mobile phase preparation

Like any HPLC application detected by UV, the mobile phase should have a low UV background. Make sure the salt, water and the organic solvent do not absorb UV and free of UV absorbing impurities.

3.1.6 Mobile phase formula 1

3.1.6.1 Mobile Phase A (1 L)

2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0

Dissolve 13.8 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$) and 264.2 g of ammonium sulfate in 800 mL of DI water, adjust the pH to 7.0 with 50% NaOH solution and bring the volume to 1000 mL with DI water. Filter the mobile phase through a 0.22 µm filter.

3.1.6.2 Mobile Phase B (1 L)

100 mM sodium phosphate, pH 7.0

Dissolve 13.8 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$) in 900 mL of DI water, adjust the pH to 7.0 with 50% NaOH solution and bring the volume to 1000 mL with DI water. Filter the mobile phase through a 0.22 µm filter.

3.1.7 Mobile phase formula 2

3.1.7.1 Mobile Phase A (1 L)

1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / isopropanol (95:5 v/v)

Dissolve 6.55 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$) and 188.30 g of ammonium sulfate in 750 mL DI water, adjust the pH to 7.0 using 50% NaOH solution. Bring the volume to 950 mL with DI water. Then bring the volume to 1000 mL with isopropanol. Filter the mobile phase through a 0.22 µm filter.

3.1.7.2 Mobile Phase B (1 L)

50 mM sodium phosphate, pH 7.0 / isopropanol (80:20 v/v)

Dissolve 5.52 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$) in 750 mL DI water, adjust the pH to 7.0 using 50% NaOH solution. Bring the volume to 800 mL with DI water. Then bring the volume to 1000 mL with isopropanol. Filter the mobile phase through a 0.22 µm filter.

3.2 Gradient and flow rate

The slope of gradient affects the resolution. Start with 30-minute gradient and adjust the gradient time based on the resolution of the chromatogram. The standard flow rate for 4.6 mm i.d. columns is 1 mL/min. In some cases, slower flow rate may improve the resolution.

3.3 Temperature

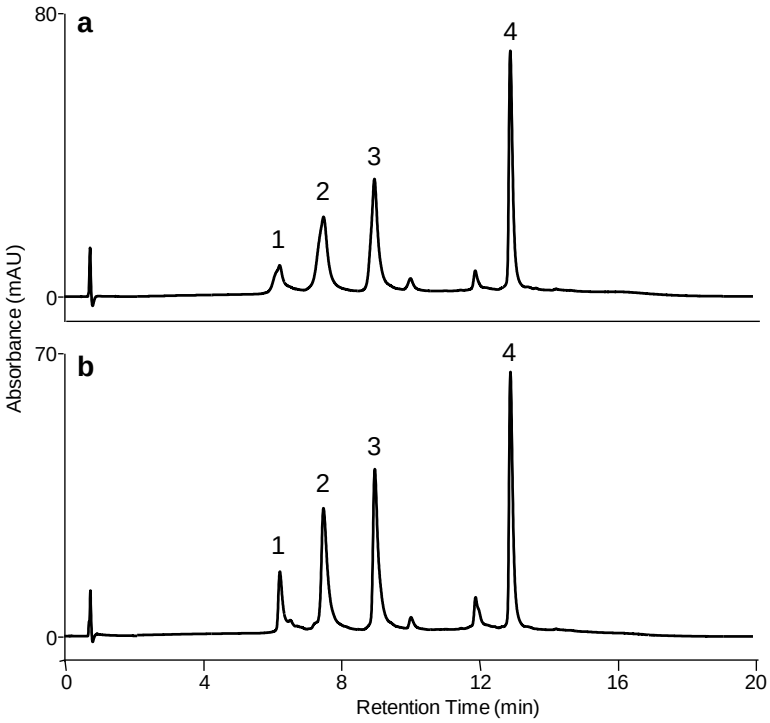
Temperature affects the hydrophobicity of the protein. In general, the hydrophobicity of the protein increases as you increase the temperature. However since the extent of hydrophobicity change may be different between proteins, temperature may affect the selectivity and the resolution. Therefore, increase or decrease in temperature may be used to optimize the separation depending on the property of the analytes.

3.4 Sample matrix

It is highly recommended to use the starting mobile phase to dissolve the sample. This ensures proper binding for the early eluting samples. The effect of sample matrix on peak shape is demonstrated in Figure 2. When the sample is dissolved in DI water (Figure 2a), the early eluting protein peaks (peak 1, 2 & 3) are broader compared to the sample that is dissolved in 0.5X mobile phase A (Figure 4b). In many cases, directly dissolving the sample in high salt concentration mobile phase causes precipitation of the protein. Therefore we recommend the following procedure.

If the sample is solid, dissolve the sample in water or appropriate buffer then dilute the sample in half with the starting mobile phase. If your sample is already in a solution, simply dilute the sample in half with the starting mobile phase.

Figure 2: Separation of Standard Protein Mixture



Column:	MAbPac HIC-Butyl, 5 µm
Format:	4.6 × 100 mm
Mobile phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B:	100 mM sodium phosphate, pH 7.0
Gradient:	

Time (min)	%A	%B
-5.0	100	0
0.0	100	0
1.0	100	0
15.0	0	100
20.0	0	100

Temperature:	30 °C
Flow rate:	1.0 mL/min
Inj. volume:	10 µL
Detection:	UV (280 nm)
Sample:	Protein mixture
Sample matrix:	a) Water b) 1 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0
Peaks:	1) Myoglobin 2) Ribonuclease A 3) Lysozyme 4) α-Chymotrypsinogen A

4. Column Care

4.1 Column storage

Storage temperature: Between 10 and 30 °C
Short term storage (overnight): Mobile phase or DI water
Long term storage: 20% ethanol or methanol in DI water

4.2 Operating pH range: pH 2 to 12

The typical pH range for most applications is between 5 and 7, but the column can be used between pH 2 and 12.

4.3 Operating temperature: 10 to 60°C

The typical temperature for routine analysis in HIC mode is between 20 and 40°C. At lower temperature make sure the pressure limit is not exceeded. To extend the column lifetime, elevated temperature (>40°C) is not recommended.

4.4 Flow rate and pressure

The typical flow rates for 4.6 mm i.d. columns are 0.5-1.0 mL/min. Flow rate up to 1.5 mL/min is acceptable provided that the pressure limit (4,000 psi) is not exceeded. In general, higher salt concentration and addition of isopropanol exhibits higher pressure at a given flow rate. It is important not to expose the column to pressure surge.

4.5 Column washing procedure

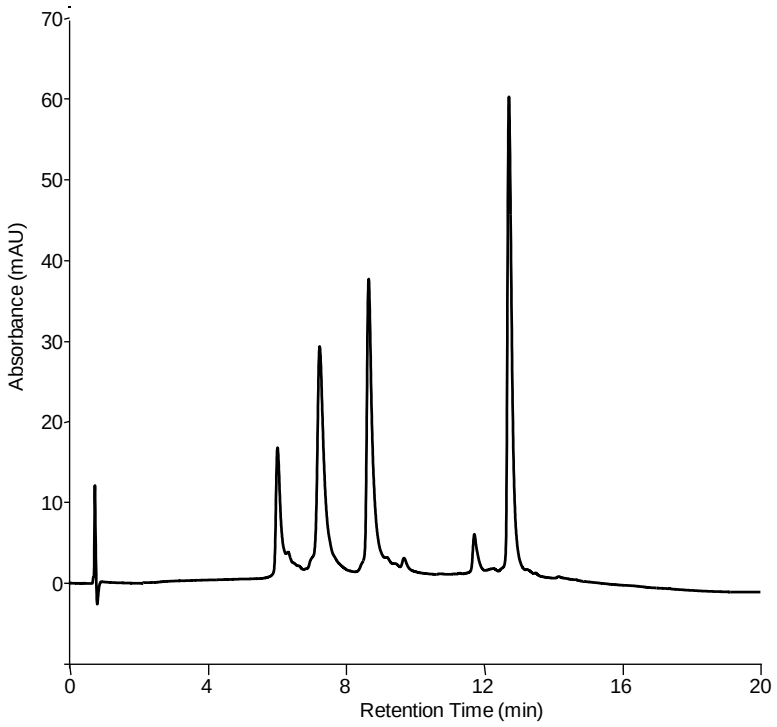
1. Wash the column with DI water for 30 column volumes.
2. Inject 200 µL of 0.1 M NaOH three times.
3. Wash the column with DI water for 20 column volumes
4. If problem persists, inject 200 µL of 20% acetic acid (aqueous) three times.
5. Wash the column with DI water for 20 column volumes
6. Test the column for performance
7. If the issue still persists, replace with a new column.

5. Applications

5.1 Proteins

The MAbPac HIC-Butyl provides high-efficiency, high resolution protein separation within 20 minutes as shown in Figure 2. Such high resolution allows for integration and fraction collection.

Figure 3: Separation of Standard Protein Mixture



Column:	MAbPac HIC-Butyl, 5 µm
Format:	4.6 × 100 mm
Mobile phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B:	100 mM sodium phosphate, pH 7.0
Gradient:	

Time (min)	%A	%B
-5.0	100	0
0.0	100	0
1.0	100	0
15.0	0	100
20.0	0	100

Temperature:	30 °C
Flow rate:	1.0 mL/min
Inj. volume:	10 µL
Detection:	UV (280 nm)
Sample:	Protein mixture
Peaks:	1) Myoglobin 2) Ribonuclease A 3) Lysozyme 4) α-Chymotrypsinogen A

5.2 Antibody-Drug Conjugates

Antibody-drug conjugate (ADC) is an emerging class of biotherapeutics due to its high selectivity and high potency. ADCs are mAbs attached to cytotoxic drugs by chemical linkers with labile bonds. The antibody part of the ADC specifically recognizes a tumor marker and once the ADC is internalized into the target cell, the toxic drug is released which in turn kills tumor cells. There are two most common methods to attach the cytotoxic drug. The first method is to attach the drug to free cysteine residues after reduction. And the second method is to attach the drug to lysine residues that are positioned throughout the antibody. These conjugation methods yield heterogeneous mixtures of ADC variants since there is more than one cysteine or lysine residues present in an antibody molecule. The heterogeneity of ADC molecules may potentially cause safety and efficacy issues when administered into patients. Therefore purification and full characterization of ADCs is critical during development and production. Since the attachment of a drug molecule alters the hydrophobicity of the mAb, hydrophobicity based HPLC methods such as reverse phase chromatography and HIC are commonly used to characterize ADCs. Opposed to reverse phase chromatography, HIC does not denature the protein analytes. Therefore HIC is ideal for further functional analysis of separated ADC molecules.

Figure 4 shows the separation of a cysteine-conjugated ADC mimic sample on the MAbPac HIC-Butyl column. The ADC mimics were conjugates between a drug mimic and mAb via the sulfhydryl group of interchain cysteine residues which results in a mixture of drug-loaded antibody species with 0 to 8 drugs (Figure 4a). The unmodified mAb and ADCs with DAR values ranging from 2 to 8 are well resolved by the MAbPac HIC-Butyl column.

Figure 5 shows the separation of a lysine-conjugated ADC mimic sample on the MAbPac HIC-Butyl column. The drug mimic is conjugated to lysine residues of the mAb. Typically lysine-linked ADCs are highly heterogenic due to multiple lysine residues within a mAb. MAbPac HIC-Butyl column is able to provide a reasonable separation of this complex mixture based on the hydrophobicity of the species.

Figure 4A: Schematic Representation of Conjugation of Drug Mimic via Interchain Cysteine Residues

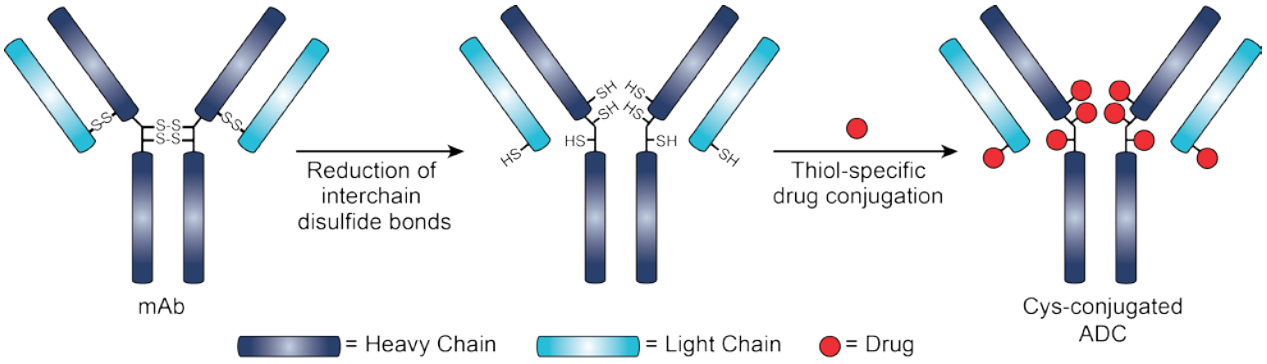
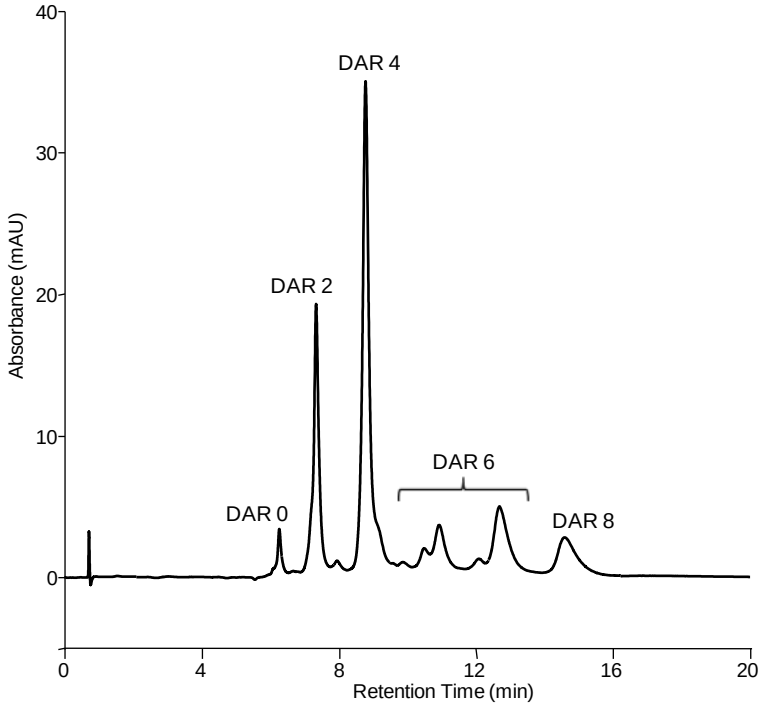


Figure 4B: Separation of Cysteine-Conjugated ADC Mimic

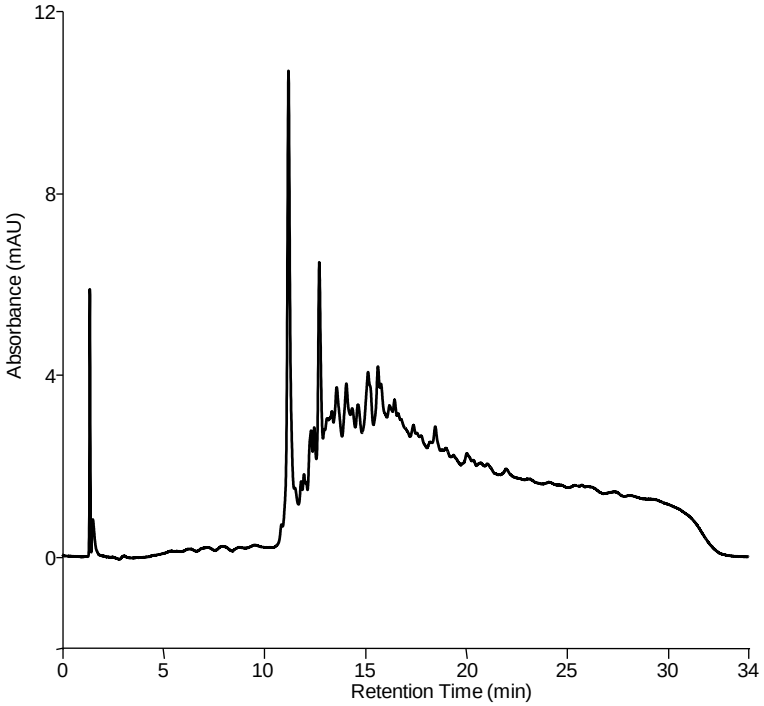


Column:	MAbPacHIC-Butyl, 5 µm
Format:	4.6 × 100 mm
Mobile phase A:	1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / isopropanol (95:5 v/v)
Mobile phase B:	50 mM sodium phosphate, pH 7.0 / isopropanol (80:20 v/v)
Gradient:	

Time (min)	%A	%B
-5.0	100	0
0.0	100	0
1.0	100	0
15.0	0	100
20.0	0	100

Temperature:	25 °C
Flow rate:	1.0 mL/min
Inj. volume:	5 µL (5 mg/mL)
Detection:	UV (280 nm)
Sample:	Cys-conjugated ADC mimic

Figure 5: Separation of Lysine-Conjugated ADC Mimic



Column:	MAbPacHIC-Butyl, 5 µm
Format:	4.6 × 100 mm
Mobile phase A:	1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / isopropanol (95:5 v/v)
Mobile phase B:	50 mM sodium phosphate, pH 7.0 / isopropanol (80:20 v/v)
Gradient:	

Time (min)	%A	%B
-5.0	100	0
0.0	100	0
1.0	100	0
29.0	0	100
34.0	0	100

Temperature:	35 °C
Flow rate:	0.5 mL/min
Inj. volume:	5 µL (5 mg/mL)
Detection:	UV (280 nm)
Sample:	Lys-conjugated ADC mimic

6. Frequently Asked Questions

6.1 What factors do I need to consider for method development using MAbPac HIC-Butyl

Type and concentration of salt used, pH of the mobile phase, addition of organic solvent to the mobile phase, flow rate and temperature should be considered (section 3).

Most common salting out agents that are used in HIC are ammonium sulfate, ammonium acetate and sodium chloride. Resolution may improved by adjusting the starting salt concentration.

The pH of the mobile phase will affect the charge of the protein which contributes to the overall hydrophobicity of the sample. Therefore adjusting the pH may result in a different separation profile.

Typically a larger molecule like monoclonal antibodies and very hydrophobic proteins require some organic solvent in the mobile phase. Adjusting the amount of organic solvent may improve the separation. Most common organic solvents used in HIC are acetonitrile and isopropanol.

For more challenging analytes, reducing the flow rate and increasing the gradient time may improve the resolution.

In some cases, resolution may be improved by simply changing the temperature. Increase or decrease the temperature to get the best resolution.

6.2 How should I store MAbPac HIC-Butyl column?

The column can be stored in DI water for short period of time (overnight). For longer term, store the column in 20% ethanol or methanol in DI water.

6.3 What should I do if the background signal increases significantly?

Take out the column, connect a union and run a blank run to see if the background signal increases. If the background signal still increases, it is most likely that either the system or the mobile phase is not clean. Try washing the HPLC system with ample amount of water and prepare a fresh set of mobile phases. If you do not see background signal increase without the column, wash the column using the procedure described in section 4.5.

6.4 What should I do if the column exhibits excessively high backpressure?

Follow the washing procedure specified in section 4.5. If the issue still persists, replace with a new column.

6.5 What should I do if the peaks are broad?

Peaks may be broad when the sample is not dissolved in the starting mobile phase (high salt mobile phase). If the sample is already dissolved in water or buffer solution, dilute the sample in half with the starting mobile phase and then inject more if necessary. If this does not resolve the problem, increase the concentration of salt in the starting mobile phase or try increasing the temperature.

Peak broadening may also occur due to excessive extra column volume. Reduce the excessive column volume by reducing the length of tubing from the autosampler to the column and from the column to the detector.

6.6 What should I do if I see carryover?

The simplest solution is to add a blank run in between sample runs.

In addition, the system may not be clean or previous sample may not have completely eluted from the column. Wash the system and the column with low salt concentration and re-run the chromatogram.

Another factor that may cause carryover is the autosampler needle. If your autosampler does not wash the needle after injection, you may observe carryover. Change the autosampler settings so that the needle is washed after sample injection.