Thermo Scientific

MAbPac HIC-20

Product Manual

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Thermo SCIENTIFIC

Product Manual

for

MAbPac HIC-20

MAbPac HIC-20, 5 µm, 4.6 × 100 mm (P/N: 088553)
MAbPac HIC-20, 5 µm, 4.6 × 250 mm (P/N: 088554)
MAbPac HIC-20, 5 µm, 4.6 × 10 mm Guard Cartridges 2/pk (P/N: 088555)

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

Revision History:

Revision 01, October, 2014, 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.***

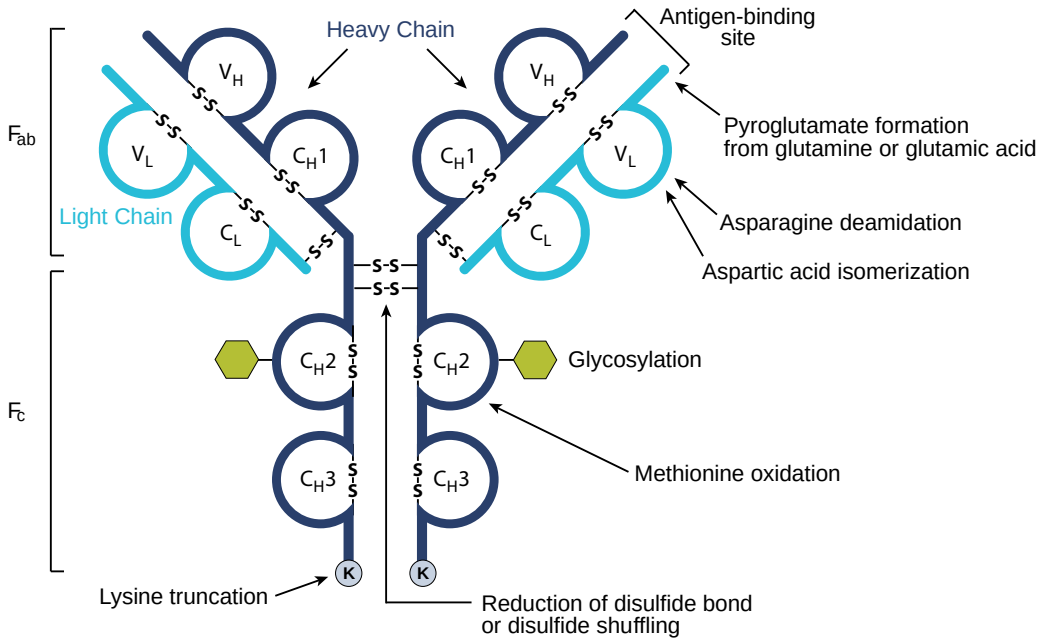
Contents

1. Introduction

Thermo Scientific™ MAbPac™ HIC-20 is a high resolution silica based HIC column designed for the separation of mAbs and mAb variants. Its unique, proprietary column chemistry provides high resolution, rugged stability, and desired selectivity for the analysis of mAbs and related variants.

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

- Excellent separation of mAb fragments and oxidized mAb variants
- Unique chemistry designed for optimal selectivity for mAbs and mAb variants
- High resolution and high efficiency
- Compatible with both organic solvent and aqueous mobile phase
- Rugged column stability

1.2 Specifications and Operating Conditions

pH range:	2.0 – 9.0
Temperature:	up to 60 °C
Aqueous compatibility:	0 – 100 % aqueous mobile phase
Organic compatibility:	Compatible with common HPLC solvents

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	6,000
4.6 × 250 mm	0.5-1.0	1.5	8,000
4.6x10 mm	0.5 – 1.0	2.0	6,000

1.3 Physical Data

Column chemistry:	Proprietary alkyl amide
Substrate:	Ultra-pure silica
Particle size:	5 µm
Pore size:	1,000 Å

2. Installation

It is recommended that you run the column performance test upon receiving a new MAbPac HIC-20 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 us for replacement.

Step 2 – Prepare mobile phase

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)

0.1 M Ammonium acetate, pH 5.4 / Acetonitrile (70:30 v/v)

1. Mix 50 mL of 2 M ammonium acetate solution, pH 5.4 (Fisher Scientific P/N NC0230496, Thermo Scientific P/N 033440) with 650 mL DI water.
2. Add 300 mL of acetonitrile to make up to 1 L.
3. Degas by sonication for 5 minutes.

Step 3 – Prepare Standard Injection Solution

0.025 mg/mL cytosine, 0.025 mg/mL phenanthrene in mobile phase

1. Prepare 1 mg/mL stock solution of cytosine in DI water.
2. Prepare 1 mg/mL stock solution of phenanthrene in acetonitrile.
3. Mix 25 µL of 1mg/mL cytosine, 25 µL of 1 mg/mL phenenthrene and 950 µL of mobile phase to make 1 mL standard injection solution.

Step 4 – 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 5 – Verify the performance of the column

Perform the column performance test using the conditions described in the Quality Assurance Report (QAR) 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 in 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 6 – Conditioning

If the column performance is satisfactorily confirmed in Step 1-6, condition the column before real sample analysis. For conditioning, inject high concentration (e.g. 10 mg/mL) of ovalbumin, BSA, or the protein of choice (dissolved in mobile phase A).

Inject ≥750 µg of protein.

Step 7 – Real sample analysis

After equilibrating the column with concentrated ovalbumin sample, the column is ready for real 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

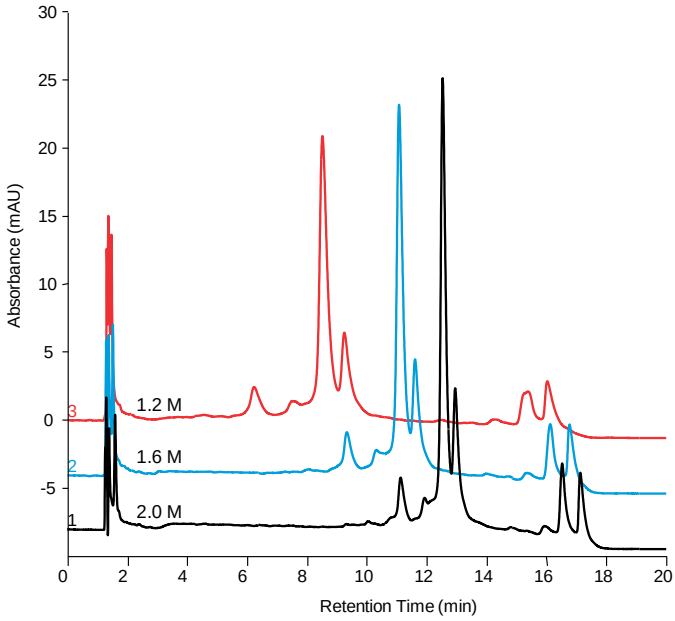
3.1 Mobile phase

Type of salt, concentration of salt in the starting mobile phase, addition of organic solvent and pH are important parameters in 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 as shown in Figure 2. Decrease in ammonium sulfate concentration, resulted in higher resolution. Decreasing the initial salt concentration can be achieved by simply adjusting the gradient.

Figure 2. Optimization of the starting salt concentration



Column:	MAbPac HIC-20, 5 µm
Dimension:	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 1:

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

Gradient 2:

Time (min)	%A	%B
-5.0	80	20
0.0	80	20
1.0	80	20
15.0	0	100
20.0	0	100

Gradient 3:

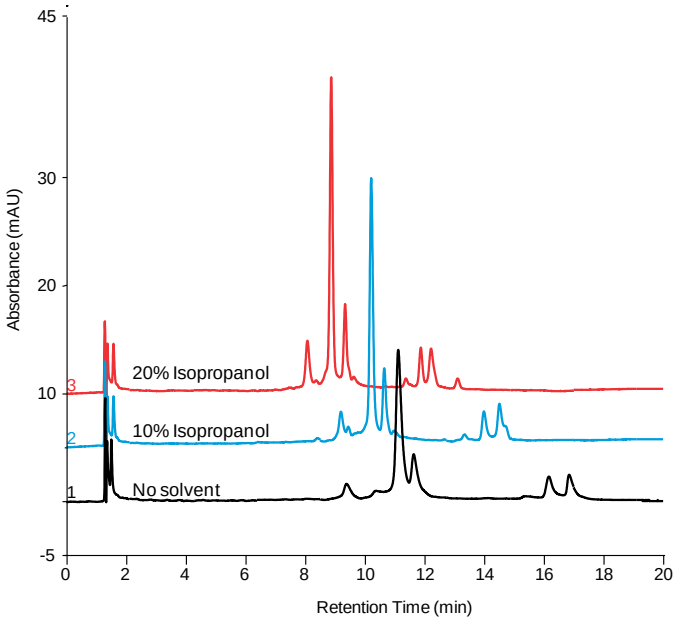
Time (min)	%A	%B
-5.0	60	40
0.0	60	40
1.0	60	40
15.0	0	100
20.0	0	100

Temperature:	30 °C
Flow rate:	1.0 mL/min
Inj. volume:	10 µL
Detection:	UV (280 nm)
Sample:	Trypsin inhibitor (5 mg/mL)

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. The effect of adding isoproanol to mobile phase B is demonstrated with a trypsin inhibitor sample in Figure 3.

Figure 3. Addition of organic solvent in mobile phase B



Column: MAbPac HIC-20, 5 µm
Dimension: 4.6 × 100 mm

1)
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0

2)
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0 / isopropanol (90:10 v/v)

3)
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 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: 30 °C
Flow rate: 1.0 mL/min
Inj. volume: 5 µL
Detection: UV (280 nm)
Sample: Trypsin inhibitor (5 mg/mL)

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 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 are 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. However in some cases, slower flow rate may improve the resolution.

3.3 Temperature

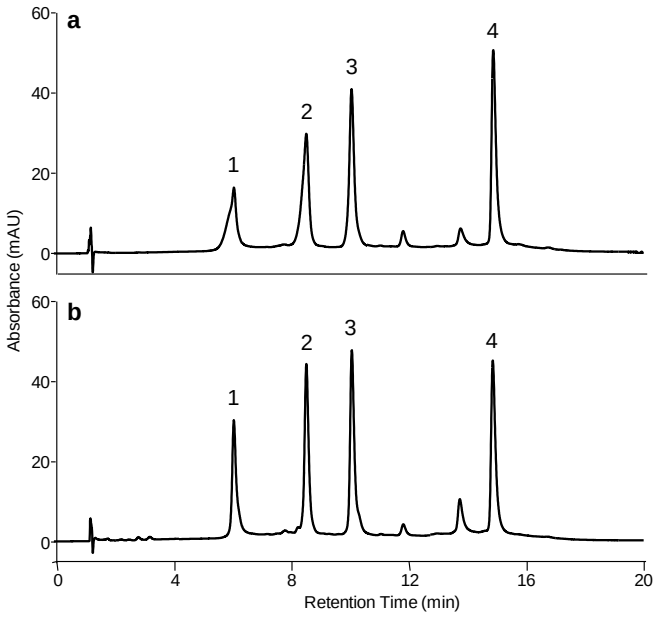
Temperature affects the hydrophobicity of the protein. In general, the hydrophobicity of the protein increases as you increase the temperature. However since the extent of hydrophobicity change may be different between proteins, temperature may affect the selectivity and the resolution. Therefore, increase 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 4. When the sample is dissolved in DI water (Figure 4a), the early eluting protein peaks (peak 1 & 2) are broader and fronting is observed 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 4. Effect of sample matrix on peak shape



Column:	MAbPac HIC-20, 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: Room temperature between 10 and 30 °C.
Short term storage (overnight): Low salt concentration mobile phase
Long term storage: 0.1 M ammonium acetate, pH 5.4 / acetonitrile (50:50 v/v) solution

WARNING

Do NOT store the column in pure water. It is critical to store the column in 0.1 M ammonium acetate, pH 5.4 / acetonitrile (50:50 v/v) solution for long term storage.

4.2 Operating pH range: pH 2 to 9

The typical pH range for most applications is between 5 and 7, but the column can be used between pH 2 and 9.

4.3 Operating temperature: up 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 0.4-1.0 mL/min. Flow rate up to 1.5 mL/min is acceptable provided that the pressure limit (6,000 psi for 100 mm column and 8,000 for 250 mm column) 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

WARNING

When switching from aqueous to organic phase, or organic to aqueous phase, make sure that the flow rate is equal to or less than 0.2 mL/min.

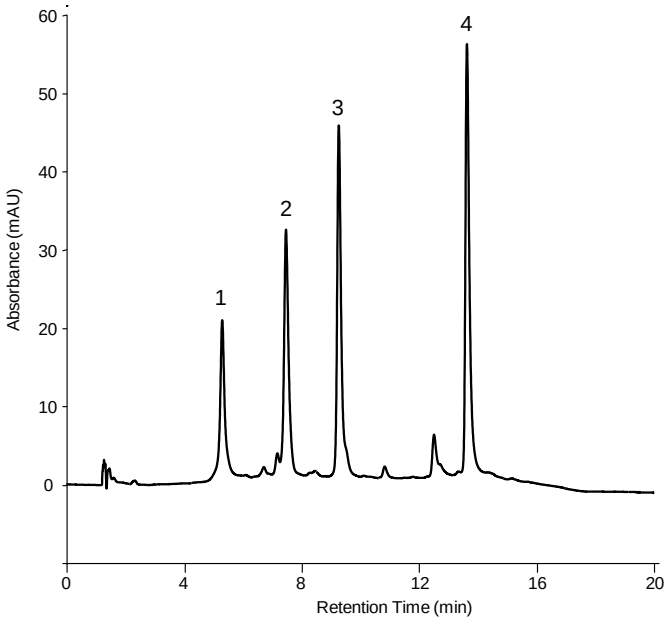
1. Wash the column with 0.05% acetic acid for 30 column volumes at 0.2 mL/min.
2. Wash the column with isopropanol for 30 column volumes at 0.2 mL/min.
3. Wash the column with 0.05% acetic acid again for 10 column volumes at 0.2 mL/min.
4. If problem persists, reverse the column direction and repeat above process.
5. If the issue still persists, replace with a new column.
6. After washing the column, inject 250 µg of protein of choice to condition the column before running your sample.

5. Applications

5.1 Proteins

The MAbPac HIC-20 provides high-efficiency, high resolution protein separation within 20 minutes as shown in Figure 5. Such high resolution allows for integration and fraction collection. The ultra-high resolution can be achieved on a 4.6 × 250 mm column.

Figure 5. Separation of standard protein mixture



Column:	MAbPac HIC-20, 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:	

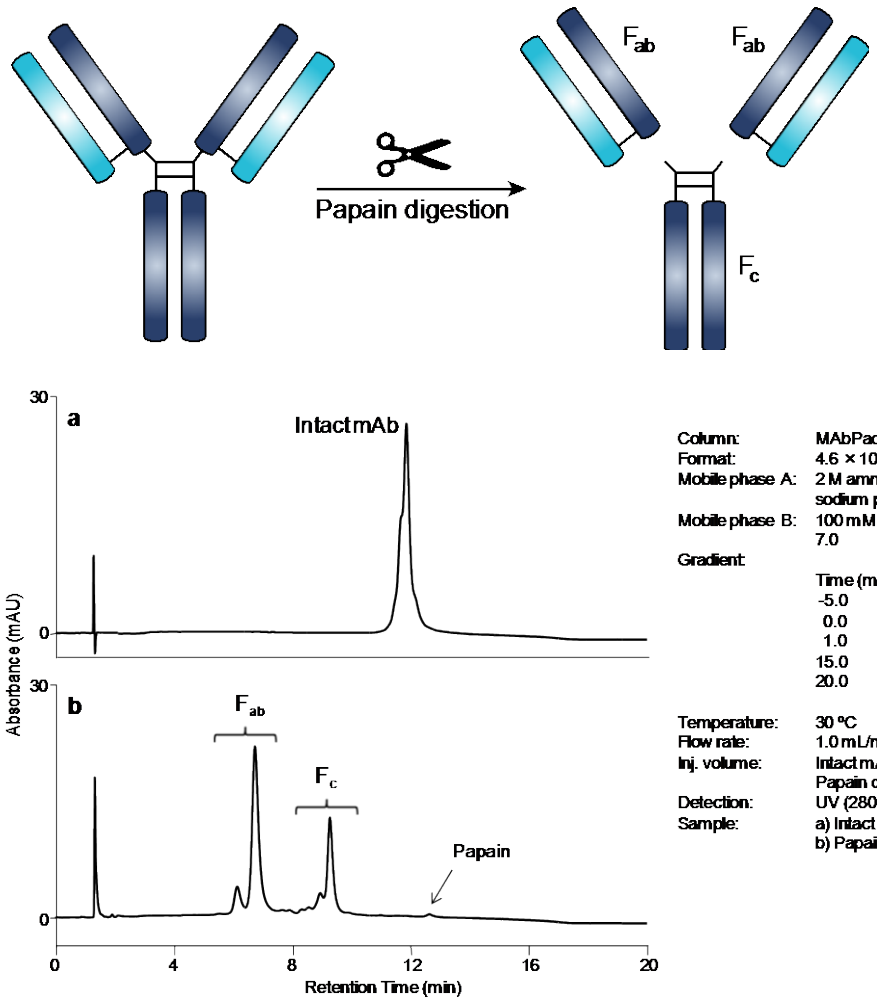
Time (min)	%A	%B
-5.0	100	0
0.0	100	0
1.0	100	0
15.0	0	100
20.0	0	100

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 Fragments

Analysis of antibody fragments is important for both characterization of Fab or Fc based biotherapeutics and localization of the sources of heterogeneities on a monoclonal antibody molecule. HIC can provide the resolution required for the separation of Fab and Fc fragments and their hydrophilic or hydrophobic variants. Figure 6 shows a comparison of an intact mAb and its papain digest on MAbPac HIC-20. The MAbPac HIC-20 column efficiently separates Fab and Fc fragments and further separates variants of these fragments. These variant peaks imply oxidation or other modifications in these fragments.

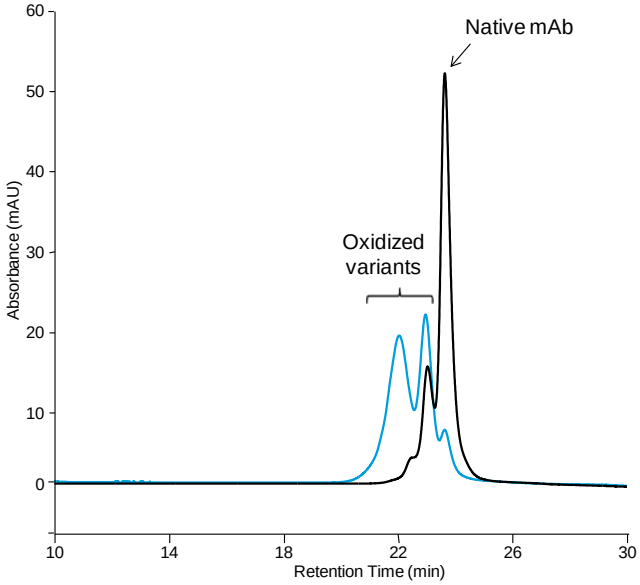
Figure 6. Separation of F_{ab} and F_c fragments



5.3 Oxidized mAb

Monoclonal antibodies are susceptible to oxidation during storage and delivery. Oxidation of methionine or tryptophan residues have been linked to decreased or loss of bioactivity of mAb therapeutics. Therefore it is critical to monitor oxidation to confirm the stability and clinical efficacy of mAb products. MAbPac HIC-20 column was able to differentiate oxidized mAb from the native mAb as shown in Figure 7.

Figure 7. Comparison of untreated mAb and H_2O_2 oxidized mAb



Column: MAbPac HIC-20, 5 µm
Format: 4.6 × 250 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:

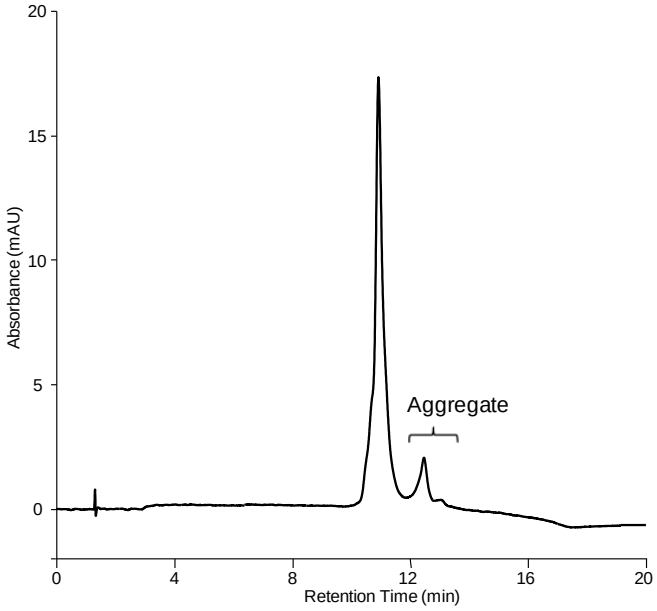
Time (min)	%A	%B
-6.0	50	50
0.0	50	50
2.0	50	50
30.0	0	100
35.0	0	100

Temperature: 30 °C
Flow rate: 0.5 mL/min
Inj. volume: Untreated mAb: 20 µL (1.25 mg/mL)
Oxidized mAb: 20 µL (1.25 mg/mL)
Detection: UV (280 nm)
Sample: Untreated mAb
H_2O_2 oxidized mAb

5.4 Antibody Aggregates

Protein and antibody aggregates are formed either during product expression in cell culture, downstream processing or storage. These aggregates may cause undesirable immune reactions which affect the safety of the drug. SEC is the most widely used technique for the detection and quantification of protein aggregates in biological drug products. However several researchers have reported the use of HIC for the removal of protein aggregates. Figure 8 demonstrates the separation of monoclonal antibody aggregates from the non-aggregated form on the MAbPac HIC-20 column. In HIC, aggregates typically elute later than the main peak due to the increased hydrophobicity.

Figure 8. Separation of monoclonal antibody aggregate



Column:	MAbPac HIC-20, 5 µm
Format:	4.6 × 100 mm
Mobile phase A:	2.0 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	60	40
0.0	60	40
1.0	60	40
15.0	0	100
20.0	0	100

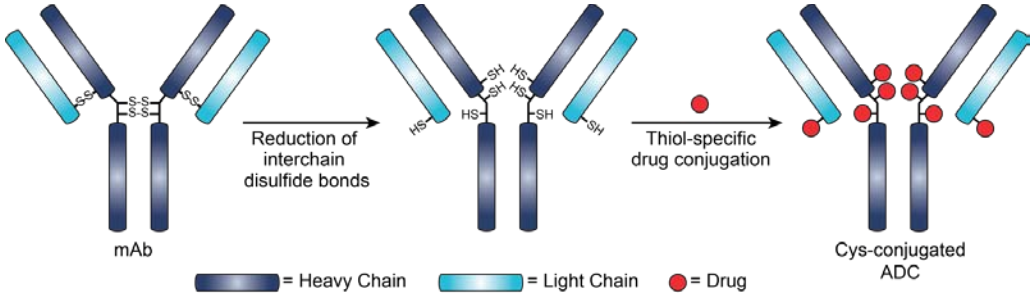
Temperature:	30 °C
Flow rate:	1.0 mL/min
Inj. volume:	2 µL (4 mg/mL)
Detection:	UV (280 nm)
Sample:	mAb

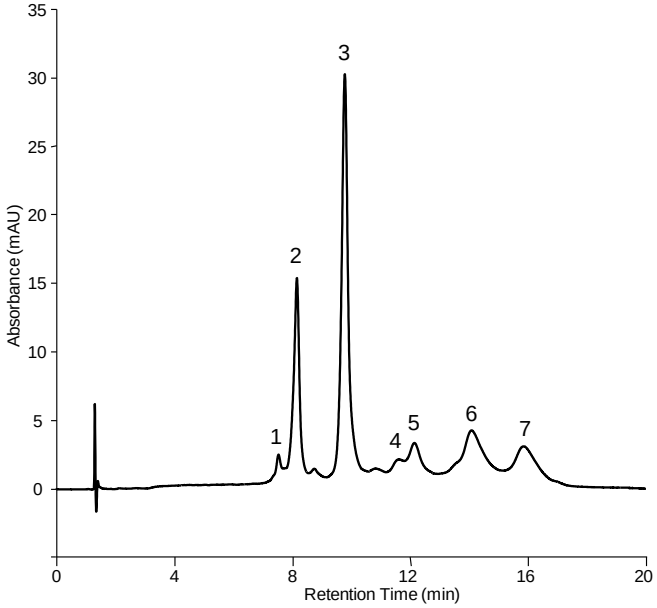
5.5 Antibody-Drug Conjugates

Antibody-drug conjugates (ADCs) are a rapidly growing class of pharmaceutical drugs that target cancer cells. Antibody-drug conjugates utilize the exquisite selectivity of the antibody to achieve targeted delivery of cytotoxic drugs. The conjugation of drug to the antibody often results in an ADC molecule that is heterogeneous with respect to both the distribution and loading of cytotoxic drugs on the mAb. The number of drugs attached to the mAb has been shown to directly affect the safety and the efficacy of the drug. Unconjugated mAb has significantly lower potency while the ADCs with high drug load are subject to rapid renal clearance. Therefore it is critical to fully characterize and monitor the heterogeneity of ADCs during development and production.

Hydrophobic interaction chromatography is ideal for the separation of ADCs since attachment of cytotoxin alters the hydrophobicity of the antibody. The least hydrophobic unconjugated antibody elutes first and as the number of attached drugs increases the elution time of each ADC increases as well. Therefore HIC is often used to characterize the distribution of ADC molecules with different drug-to-antibody ratios (DARs). Figure 9 shows the separation of a cysteine-conjugated ADC mimic sample on the MAbPac HIC-20 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 9). The unmodified mAb and ADCs with DAR values ranging from 2 to 8 are well resolved by the MAbPac HIC-20 column. Best peak shape and separation were achieved using 5 % IPA in mobile phase A and 20% IPA in mobile phase B at 25°C.

Figure 9. Separation of cysteine-conjugated ADC mimic





Column:	MAbPac HIC-20, 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
Peaks:	1) Unconjugated mAb 2) DAR 2 3) DAR 4 4) DAR 6 5) DAR 6 6) DAR 6 7) DAR 8

6. Frequently Asked Questions

6.1 What factors do I need to consider for method development using MAbPac HIC-20

Type and concentration of salt used, pH of the mobile phase, addition of organic solvent to the mobile phase 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.

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-20 column?

The column can be stored in the low salt concentration mobile phase for short period of time (e.g., overnight). For longer storage, store the column in 0.1 M ammonium acetate, pH 5.4 / acetonitrile (50:50 v/v) solution.

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?

Wash the column with DI water for 30 column volumes at 0.2 mL/min flow rate. Then wash the column with isopropanol for 30 column volumes at 0.2 mL/min. Finally wash the column with DI water again for 10 column volumes at 0.2 mL/min. If problem persists, reverse the column direction and repeat above process. 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 the recovery is low?

Inject ≥75 µL of 10 mg/mL (≥750 µg) of ovalbumin, BSA or protein of your choice (dissolved in mobile phase A) and then run your sample. The column requires equilibration with high concentration of protein before first time use and after stored in long term storage solution.

6.7 What should I do if I see carryover?

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.