



Thermo Scientific

MABPac HIC-10

Product Manual

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Product Manual

for

MABPac HIC-10

MABPac HIC-10, 5 μm , 4.6 $\times$ 100 mm (P/N: 088480)

MABPac HIC-10, 5 μm , 4.6 $\times$ 250 mm (P/N: 088481)

MABPac HIC-10, 5 μm , 4.6 $\times$ 10 mm Guard Cartridges 2/pk (P/N: 088482)

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Safety and Special Notices

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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.

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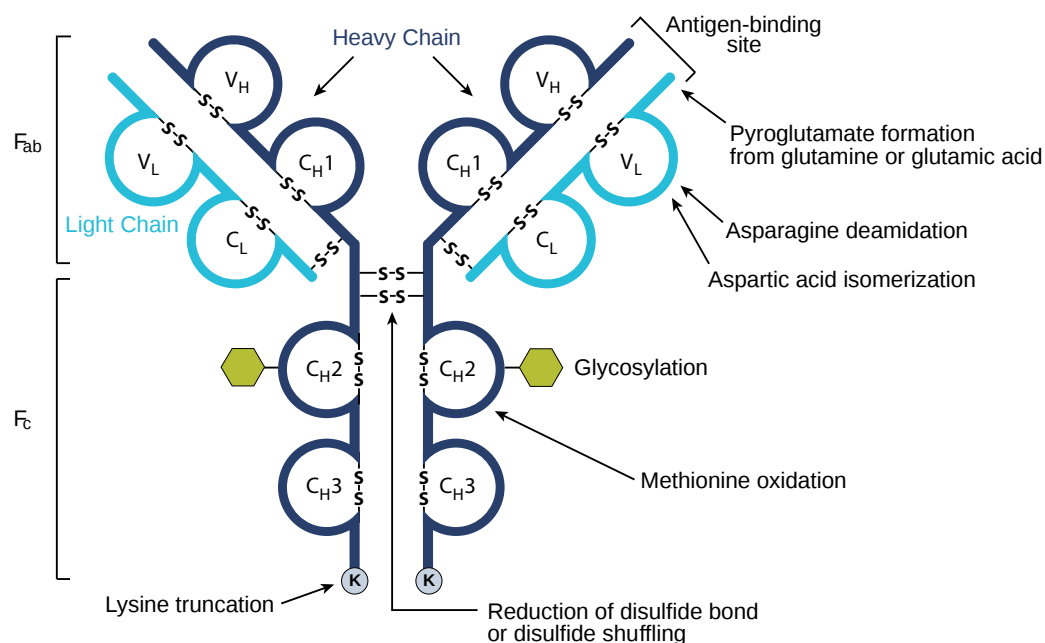
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1. Introduction

The MAbPac HIC-10 column is a high resolution silica based HIC column designed for separation of mAbs and related biologics. The unique chemistry provides high resolution, excellent bio-compatibility, and selectivity different from other HIC columns on the market.

Monoclonal antibodies are the most successful and prominent class of biotherapeutics, currently accounting for approximately one third of total biologics sales. With high specificity and excellent biocompatibility, they are expected to rise at the fastest rate within the total biologics market. Monoclonal antibodies have demonstrated effectiveness against autoimmune disorders, cardiovascular diseases, infectious diseases, and cancer. The proliferation of monoclonal antibody therapeutics and their susceptibility to various biochemical modifications has increased the emphasis on characterization of these highly heterogeneous products for their safety and efficacy (Figure 1).

Figure 1: Structure of IgG and typical forms of heterogeneity



Hydrophobic interaction chromatography (HIC) is a technique for separation of proteins including monoclonal antibodies by hydrophobicity. The HIC mobile phase usually consists of a salting-out agent, which at high concentration, retains the protein by increasing hydrophobic interaction between the protein and the stationary phase. Bound proteins are eluted by decreasing the salt concentration. HIC has been widely used as an orthogonal method to size exclusion chromatography and ion exchange chromatography for the characterization of mAb heterogeneity. Analysis of succinimides, antibody fragments, oxidated mAbs, C-terminal lysine modification, and drug conjugated mAbs were successfully carried out to monitor the stability and in some cases the potency of the drug.

1.1 Main Features

- Unique chemistry designed for optimal selectivity for mAbs
- Excellent bio-compatibility and extremely low carryover
- 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:	2.0-8.0
Temperature:	5 °C 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	0.4-1.0	1.5	6,000
4.6 × 250 mm	0.4-1.0	1.5	8,000
4.6 x 10 mm	0.4-1.0	2.0	6,000

1.3 Physical Data

Column chemistry:	Proprietary alkyl amide
Substrate:	Ultra-pure silica
Particle size:	5 µm
Surface area:	20 m ² /g
Pore size:	1,000 Å

2. Installation

It is recommended that you run the column performance test upon receiving a new MAbPac HIC-10 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, Dionex 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 phenanthrene 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 – Condition the column

Slowly ramp up the flow rate to 1 mL/min. If possible, set the flow ramps up and down to 1 mL/min. Wash the column with mobile phase B for at least 10 minutes at 1 mL/min (or 8 column volumes).

Step 6 – 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 1-6, 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

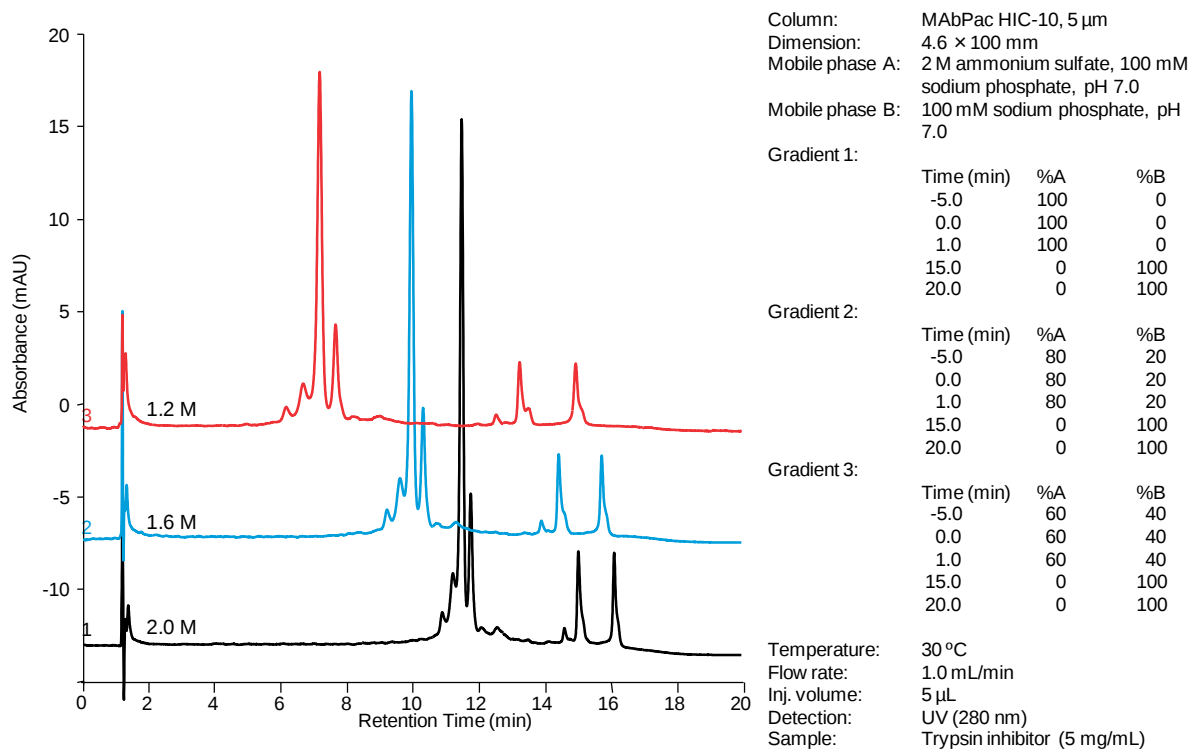
3.1 Mobile Phase

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.

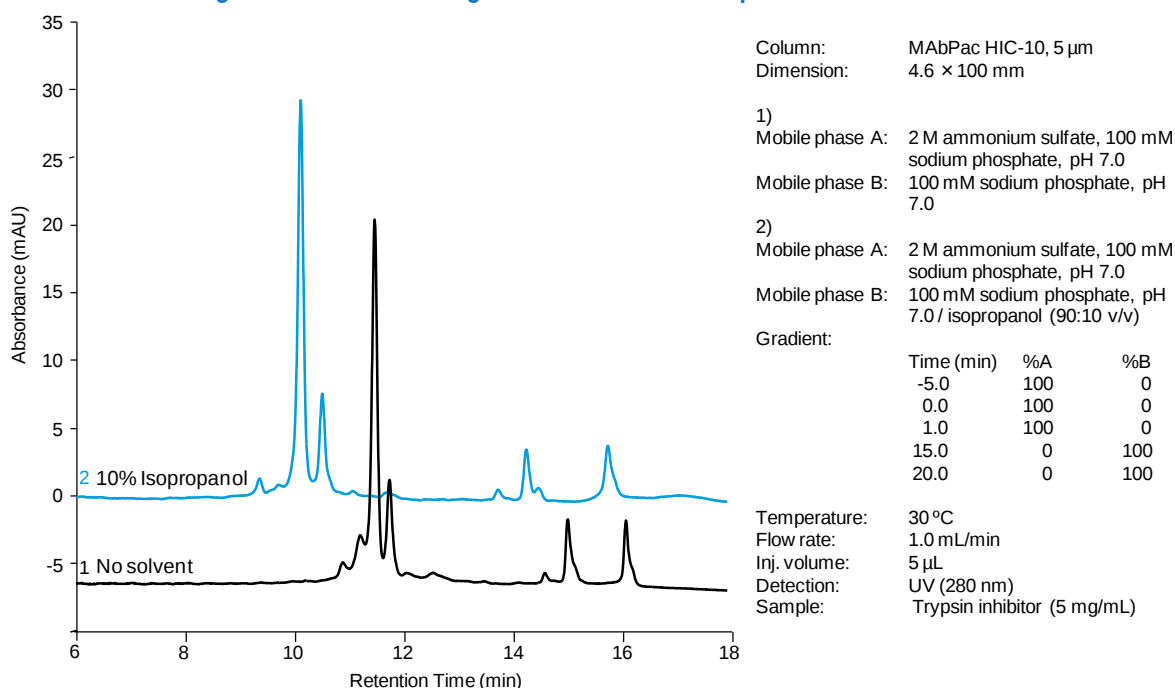
Figure 2: Optimization of the starting salt concentration



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 isopropanol to mobile phase B is demonstrated with a trypsin inhibitor sample in Figure 3.

Figure 3: Addition of organic solvent in mobile phase B



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 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 ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) 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 ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) 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 ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) 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 ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) 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. However 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.

4. Column Care

4.1 Guard Cartridges

Guard cartridges (P/N: 088480) help protect analytical columns from “dirty” samples. They also protect against fouling by contaminated mobile phases. Replace periodically for better column lifetime. Note that the guard cartridge requires the cartridge holder (P/N 069580).

4.2 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

4.3 Operating pH Range: pH 2 to 8

The typical pH range for most applications is between 5 and 7, but the column can be used between pH 2 and 8..

4.4 Operating Temperature: 5 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.

4.5 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.

4.6 Column Washing Procedure

1. Wash the column with DI water 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 DI water 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.



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.

5. Applications

5.1 Proteins

The MAbPac HIC-10 provides high-efficiency, high resolution protein separation within 10 minutes as shown in Figure 4. 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).

Figure 4: Separation of standard protein mixture

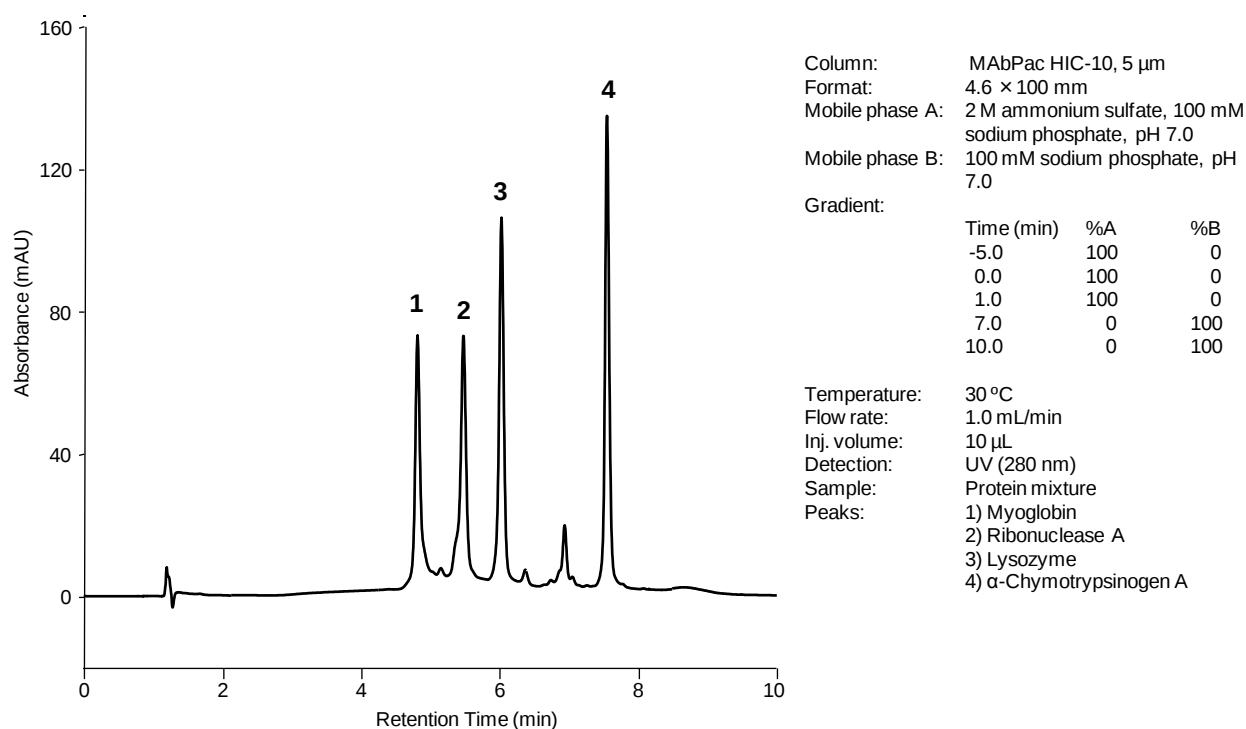
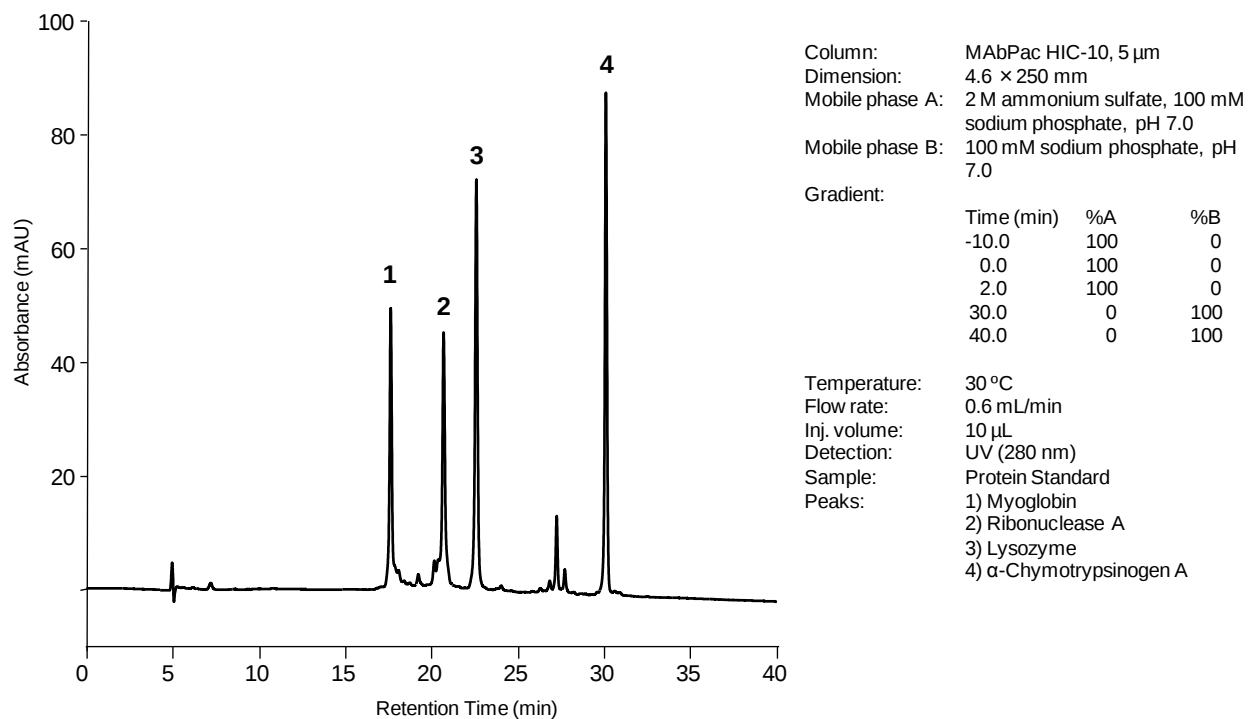


Figure 5: Ultra-high resolution separation of standard protein mixture

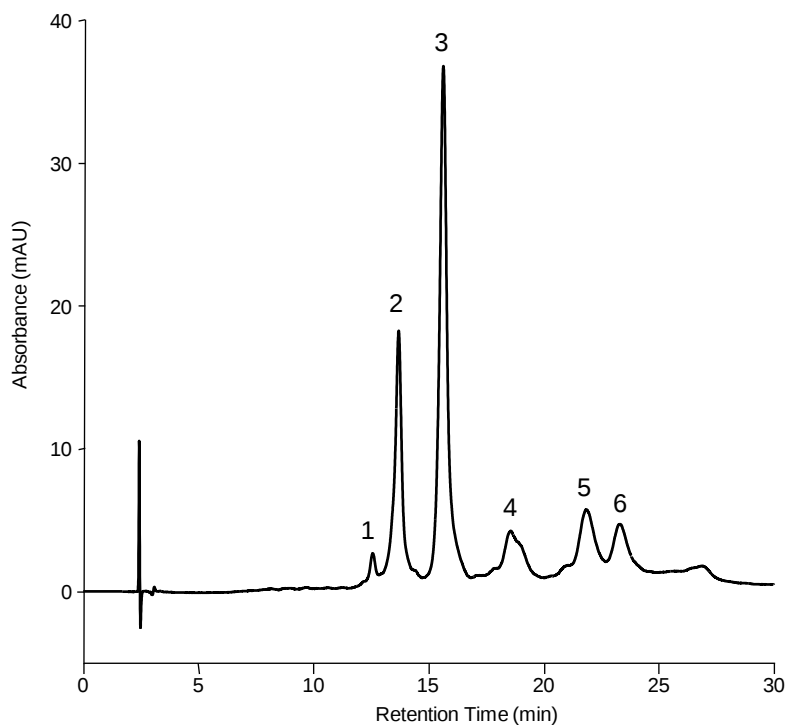
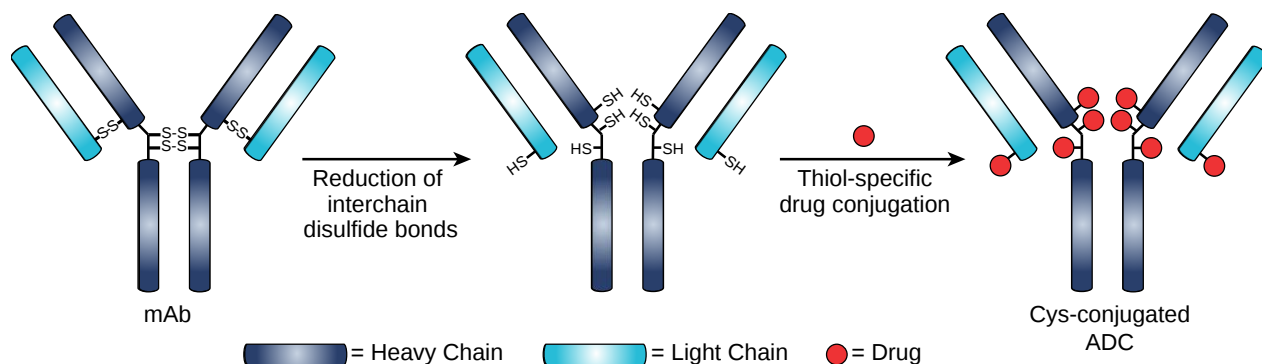


5.2 Antibody-Drug Conjugates

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 often used 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 6 shows the separation of a cysteine-conjugated ADC mimic sample on the MAbPac HIC-10 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 6). The unmodified mAb and ADCs with DAR values ranging from 2 to 8 are well resolved by the MAbPac HIC-10 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 6: Separation of cysteine-conjugated ADC mimic



Column: MAbPac HIC-10, 5 μ m
 Format: 4.6 $\times$ 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:

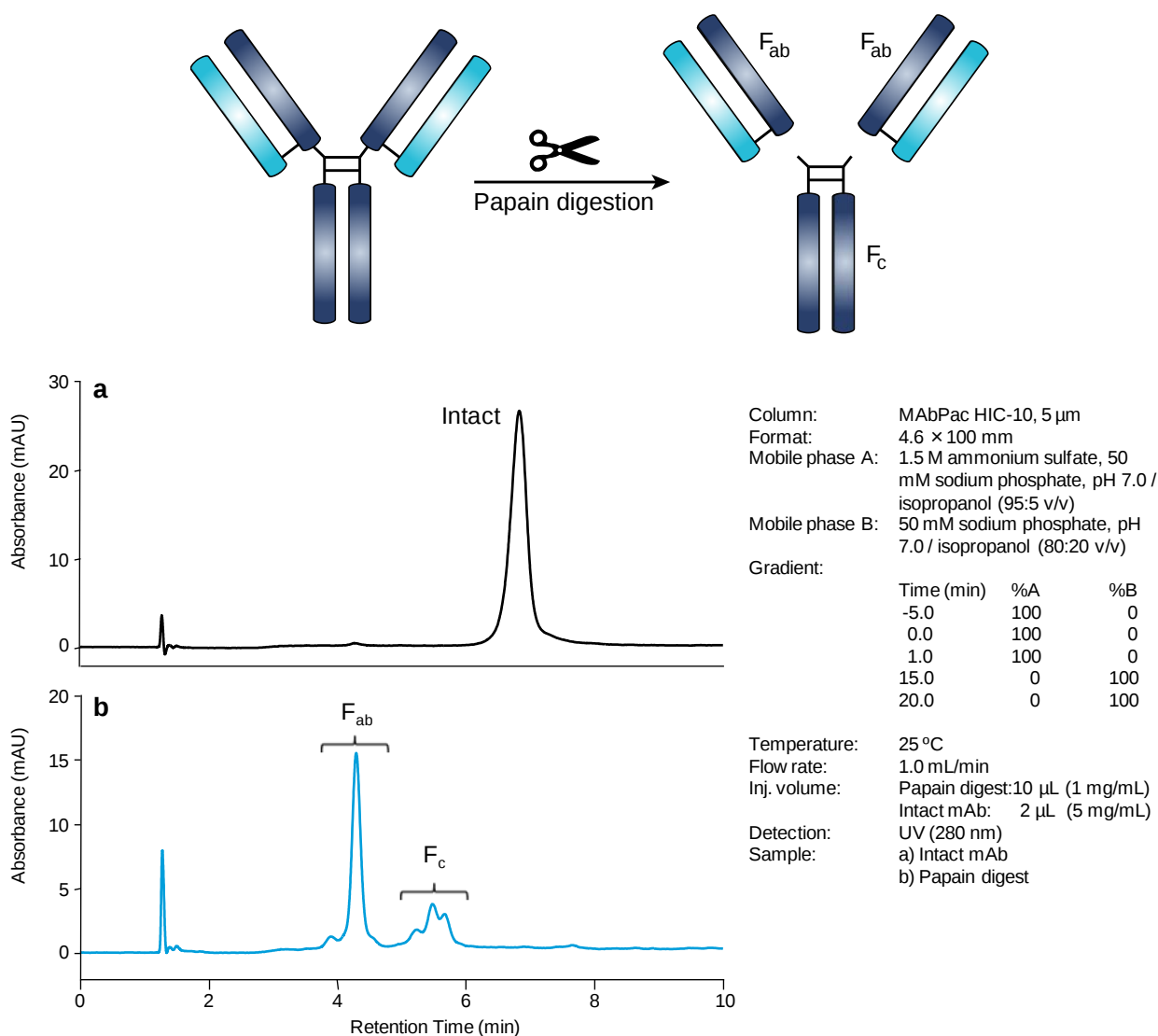
Time (min)	%A	%B
-5.0	100	0
0.0	100	0
1.0	100	0
30.0	0	100
35.0	0	100

Temperature: 25 $^{\circ}$ C
 Flow rate: 0.5 mL/min
 Inj. volume: 5 μ L (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 8

5.3 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 7 shows a comparison of an intact mAb and its papain digest on MAbPac HIC-10. The MAbPac HIC-10 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.

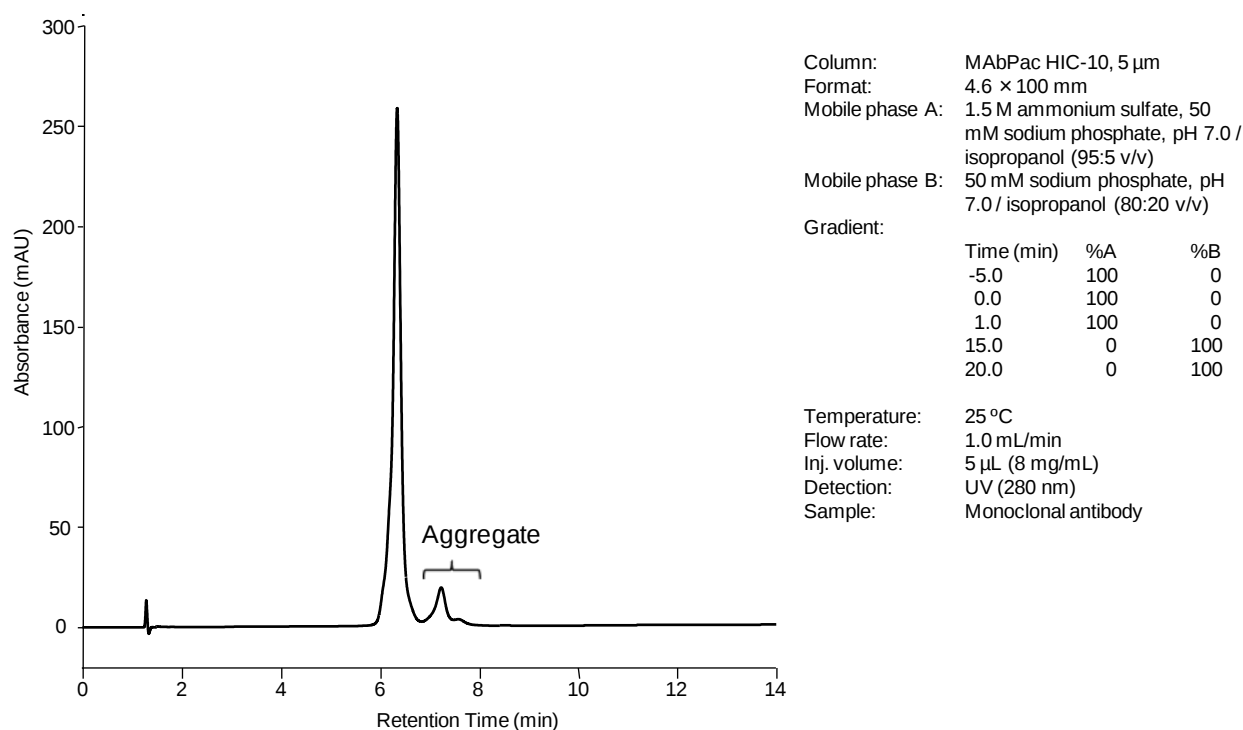
Figure 7: Separation of F_{ab} and F_c fragments



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-10 column. In HIC, aggregates typically elute later than the main peak due to the increased hydrophobicity.

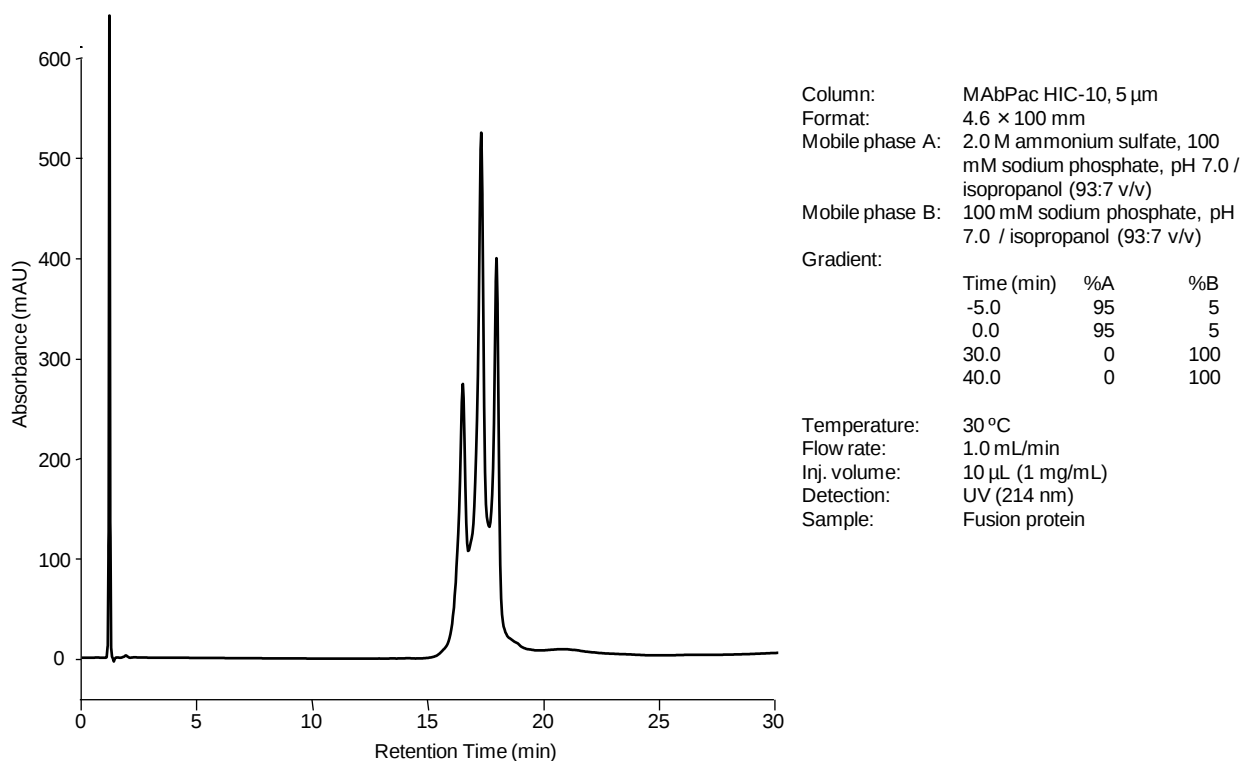
Figure 8: Separation of monoclonal antibody aggregate



5.5 Fusion Proteins

Fusion proteins are proteins created through the joining of two or more genes that originally form separate proteins. Recombinant fusion proteins are commonly used in biological research and therapeutics. For example, GFP proteins have been fused to numerous proteins as a biological tool to localize and monitor the protein of interest in cells. Among fusion protein therapeutics, Fc fusion proteins are the most promising with already six approved Fc-fusion drugs on the market. Researchers have attached Fc region of a monoclonal antibody onto a therapeutic protein to increase its stability and deliverability. Figure 9 shows the separation of a fusion protein containing three fractions: the fusion protein, the fusion protein with loss of several amino acid residues, and the fusion protein with some tertiary structure distortion. All three components were successfully separated on the MAbPac HIC-10 column while other HIC phases failed to resolve them.

Figure 9: Separation of fusion protein



6. Frequently Asked Questions

6.1 What factors do I need to consider for method development using MAbPac HIC-10

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. Commonly used organic solvents for HIC include acetonitrile and isopropanol.

In some cases, resolution may be improved by simply changing the temperature.

6.2 How should I store MAbPac HIC-10 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 4.6.

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 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.