Thermo Scientific

# MAbPac Protein A

## Column Product Manual

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Thermo SCIENTIFIC

# Product Manual

# for the

# MAbPac Protein A Column

4 x 35 mm (P/N 082539)

***For Research Use Only. Not for Use in Diagnostic Procedures.***

Revision History:

Revision 01, May, 2013, 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

## 1.1 Principles of Affinity Chromatography

Affinity chromatography is used for sample purification based on the reversible interactions between the target molecules and the specific ligands immobilized on a chromatographic matrix. The process usually consists of three steps:

1. Bind: Samples containing the target molecules are loaded onto the column and allowed to bind.
2. Wash: Components in the sample that do not bind to the ligand are washed off the column, while the target molecules are retained.
3. Elute: Specific components in an eluting solution are used to break the target-ligand interactions and then elute the target molecules.

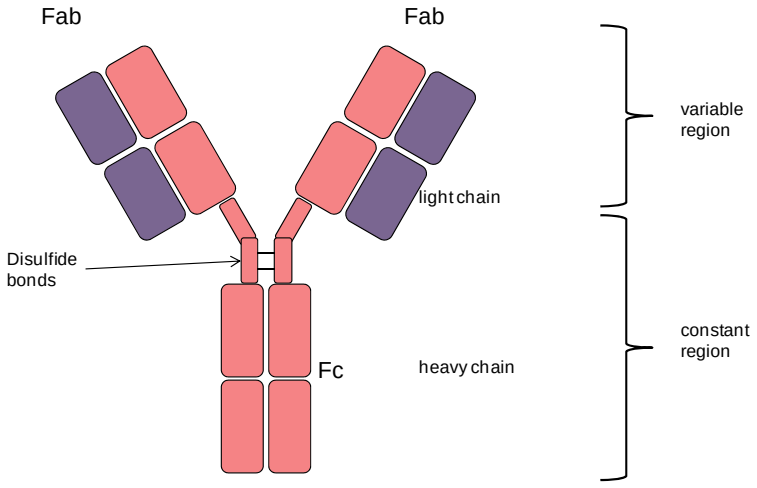
The column can then be equilibrated to remove the competing elution components.

## 1.2 Protein A

Staphylococcal protein A (SPA) plays an important role in immunology and biochemistry, owing to its specific interaction with the $F_c$ part of immunoglobulin G (IgG) from many mammals. SPA is a cell wall associated protein domain exposed on the surface of the Gram-positive bacterium *Staphylococcus aureus*. SPA consists of three different regions; S, being the signal sequence that is processed during secretion, five homologous IgG binding domains E, D, A, B, and C, and a cell-wall anchoring region XM. SPA and the smaller ligands derived from SPA have been used widely for the affinity purification of antibodies.

Recombinant Protein A (rSPA), is expressed in *E. coli* as opposed to the native protein extracted from *Staphylococcus aureus*. rSPA is a 45 kDa protein containing the same amino acid sequence and molecular mass as the native Protein A sourced from *S. aureus*. rSPA is used as the affinity ligand for the Thermo Scientific™ MAbPac™ Protein A column.

Figure 1 Schematic of an antibody showing the fragment crystallizable region (Fc) and the fragment antigen binding region



## 1.3 Hydrophilic Particle Platform

The Thermo Scientific™ MAbPac™ Protein A column is a specially developed polymeric support with the Protein A ligand bonded to the surface. The polymer base material is a novel non-porous polymeric resin consisting of a hydrophobic divinylbenzene core coated with a hydrophilic surface. The recombinant protein A ligands are covalently attached onto the hydrophilic resin surface through their amine groups. The functionalized resin is packed into a 4 × 35 mm PEEK column body.

The MAbPac Protein A column is specifically designed to provide fast monoclonal antibody (MAb) titer analysis of harvest cell cultures (HCC). The HPLC compatibility of this column in combination with low back pressure and high efficiency, allows automation, providing higher throughput and more accurate analysis. The column format allows rapid automation of loading, binding, elution and collection using Thermo Scientific biocompatible systems.

## 1.4 Column Parameters:

| | |
|---|---|
| Column Dimension: | 4×35 mm |
| Ligand: | Protein A |
| Resin particle size: | 12±1.5 µm |
| Binding capacity | ~3.5mg IgG/g resin |
| Dynamic binding capacity | 100 µg IgG/column at 2 mL/min flow rate |

## 1.5 Typical Operating Parameters

| | |
|---|---|
| Operating Flow range: | 0.5 to 2.5 mL/min |
| Recommended flow: | 2.0 mL/min |
| Eluent pH range: | pH 2.5-7.5 |
| Operating Temperature: | ≤ 30°C |
| Pressure: | ≤ 1000 psi |
| Organic Solvent Limit: | 20% Acetonitrile |

**CAUTION**

***If acetonitrile is used, the concentration must be kept constant to ensure prolonged column life,. Sudden pressure surges should be avoided as they may result in column damage.***

# 2. Installation

The MAbPac Protein A column can be used on any Bio HPLC system that is compatible with high salt aqueous buffers, although a Thermo Scientific HPLC system is highly recommended. The common set-up of a chromatography system for this column should contain a gradient pump, an autosampler, a thermal compartment, and a detector. A fraction collector is beneficial when collection of eluted samples is needed.

## 2.1 Detection

For immunoglobulin (IgG), UV absorbance at 280nm or 214nm is commonly used for detection in chromatography analysis. The UV detector should be connected immediately after the MAbPac Protein A column to avoid band broadening caused by dispersion. UV detectors from various manufacturers can be used. Various cell volumes and path lengths are available to suit micro, analytical or semi-preparative applications. We recommend using Thermo Scientific analytical flow cell (cell volume: 11 µL; cell material: PEEK; path length: 10 nm; pressure limit: 5 MPa; p/n 6074.0200) at 2 mL/min flow rate.

## 2.2 Pumps

Gradient LC pumps from any manufacturer can be used. The pump should have at least two channels to allow step or linear gradient elution of IgG samples from the MAbPac Protein A column. Due to the use of chloride salts Thermo Scientific recommends the use of bio inert systems to prevent column damage due to metal (from stainless steel) leaching.

## 2.3 Injectors

An autosampler is used to inject samples into the system. A manual injector can be used if an autosampler is not available.

## 2.4 Column Oven

Optimal reproducibility of results is achieved by regulating the temperature of the column using a column oven during chromatography. Therefore, a column oven is highly recommended. This can also be beneficial for temperature sensitive samples.

# 3. Operation

## 3.1 Eluent

The MAbPac Protein A column can be used with most common buffers and eluents used in biological analysis, provided that they are compatible with Protein A and the samples. Phosphate buffer is commonly used for Protein A affinity analysis. High concentration salt such as 0.15M NaCl in the eluents can be used to help prohibit non-specific binding of protein samples to the column. Although the following eluents are recommended for applications in this manual, the optimal buffer can vary for different applications. Method development is needed for different applications to find the optimal conditions.

### 3.1.1 Suggested Buffer Systems

Eluent A: 50mM sodium phosphate, 150 mM NaCl, pH 7.5

Eluent B: 50mM sodium phosphate, 150 mM NaCl, pH 2.5

Or

Eluent A: 50mM sodium phosphate, 150 mM NaCl, 5% acetonitrile, pH 7.5

Eluent B: 50mM sodium phosphate, 150 mM NaCl, 5% acetonitrile, pH 2.5

Or

Eluent A: 1X PBS, pH 7.5 (made from 10X PBS, Fisher Cat # M6506)

Eluent B: 1X PBS+ 30 mM HCl, pH 1.9

All chemicals should be at least ACS reagent grade. Deionized water with resistance of 18.2 mΩ-cm should be used. All eluents need to be filtered through 0.2µm filters before use.

**CAUTION**

***If acetonitrile is used, the concentration must be kept constant to ensure prolonged column life. Sudden pressure surges should be avoided as they may result in column damage.***

## 3.2 Column Installation

Install the column on the LC instrument in the correct flow direction as indicated on the column label.

### 3.2.1 Column Start-up

The column is shipped in the recommended eluent A (50mM sodium phosphate, 150 mM NaCl, pH 7.5) with 0.1% sodium azide. After the column is installed, gradually increase the flow rate to 2 mL/min over 4 minutes (0.5mL/min/min). Pump at least 20 mL of eluent A through the column to remove the sodium azide and equilibrate the column.

### 3.2.2 Common Gradient and Equilibration

A step gradient with phosphate buffer is usually used to purify IgG samples on the Protein A column. IgG has much higher affinity to Protein A at higher pH (7.0-7.5) than at lower pH (2.5-3.0). After IgG elution, wash the column with eluent A and the column can be recovered. Equilibration time is flow rate dependant. The column should be equilibrated with **at least 10 column volumes (2 min at 2 mL/min)** eluent A prior to next sample loading.

### 3.2.3 Sample Preparation

Samples containing proteases may cause cleavage of the Protein A conjugated to the surface resulting in column deterioration and degreased capacity.. Any protease should either be removed from the samples or protease inhibitors added to the sample to avoid any unnecessary reduction in performance.

### 3.2.4 Flow Rate

The MAbPac Protein A column can be used at a flow rate up to 2.5 mL/min. The recommended flow rate is 2 mL/min. Higher flow rates will generate higher column pressure and may also decrease the binding efficiency of the samples to the protein A. Figure 2 shows that as you increase the flow rate, there is little effect on the amount of IgG that binds to the column. The decrease in total area is due to the use of the same data collection rate of the detector (Table 1).

Figure 2 Effect of Flow Rate on IgG Binding

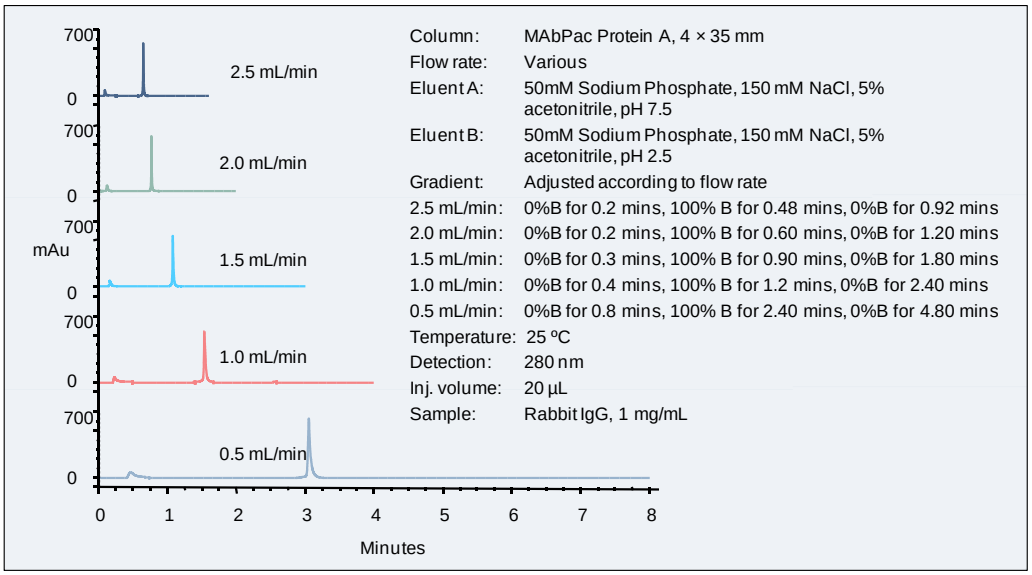


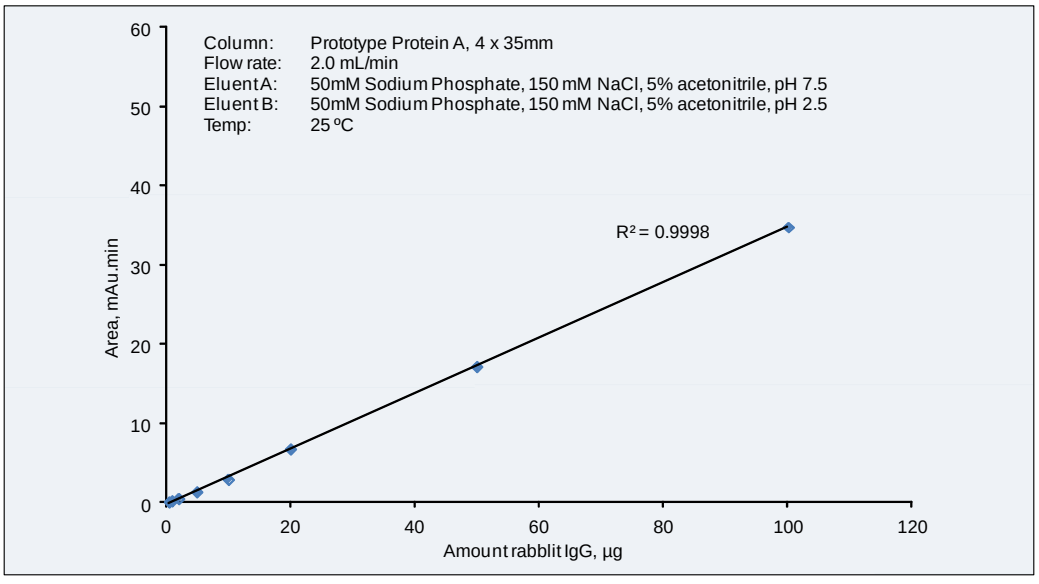
Table 1 Effect of Flow Rate on IgG Peak Area

| Flow Rate (mL/min) | Total Area (mAu./min) | Unbound Area (mAu./min) | Unbound Relative Area, % | IgG Area (mAu./min) | IgG Relative Area |
|---|---|---|---|---|---|
| 2.5 | 7.381 | 1.196 | 16.20 | 6.185 | 83.80 |
| 2.0 | 8.826 | 1.419 | 16.08 | 7.407 | 83.92 |
| 1.5 | 12.111 | 2.068 | 17.08 | 10.043 | 82.92 |
| 1.0 | 17.895 | 2.937 | 16.41 | 14.958 | 83.59 |
| 0.5 | 34.219 | 5.582 | 16.31 | 28.637 | 83.69 |

### 3.2.5 Loading Capacity

The dynamic loading capacity of rabbit IgG, isolated from pooled normal serum, is no less than 100 µg on the MAbPac Protein A column, analyzed at 2 mL/min. Figure 3 shows the linearity of area to sample load for rabbit IgG when loaded onto the MAbPac Protein A column. This correlation allows the MAbPac Protein A column to be used for quantitation of MAb in harvest cell culture over a wide range of concentrations.

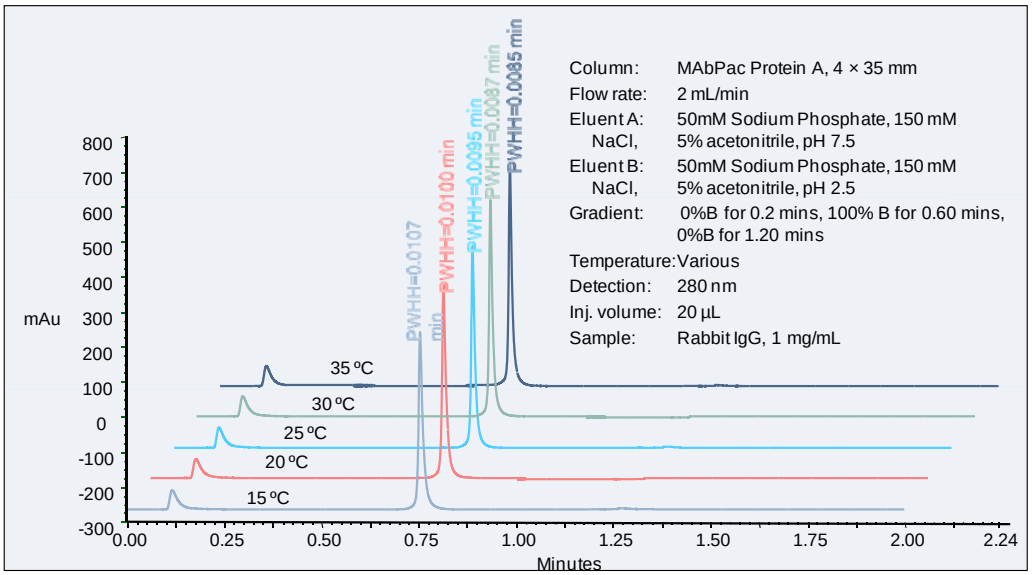
Figure 3 Area Dependence on Rabbit IgG Loading



### 3.2.6 Column Temperature

It is recommended to use the MAbPac Protein A column at temperatures no higher than 35˚C. To ensure data consistency and to prolong the column lifetime, it is recommended to use a column oven to control the temperature to 25˚C. Figure 4 shows how rabbit IgG is minimally affected by temperature. Proteins with different binding association may be affected differently.

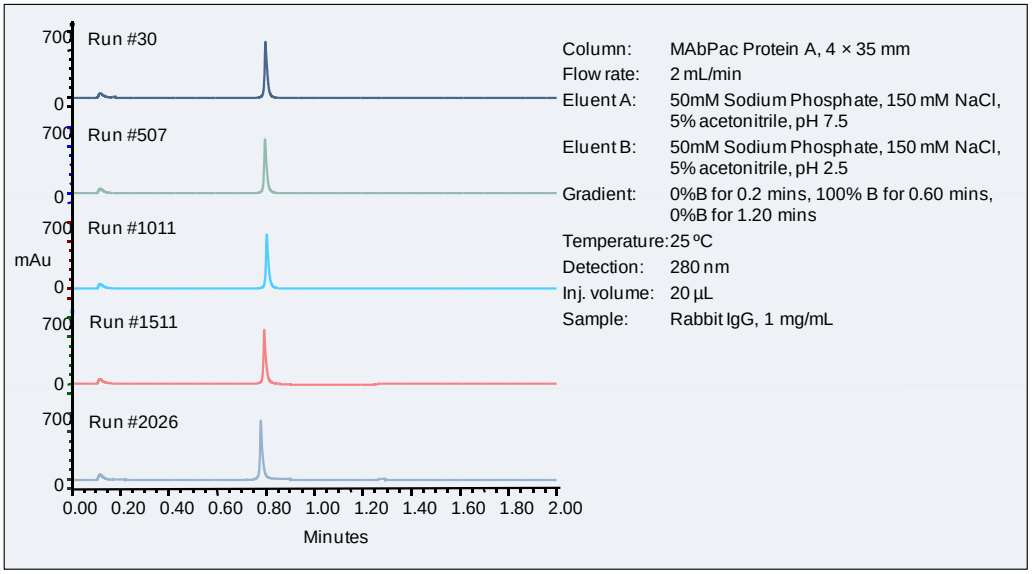
Figure 4 Temperature Effect on Binding Efficiency



### 3.2.7 Column Ruggedness

The MAbPac Protein A column has been tested for continuously for 2,000 cycles. Every hundred cycles, a set of calibration standards (from 0.01 mg/mL to 5 mg/mL) was analyzed. As shown in Figure 5, the retention time, peak area, and peak area of IgG remain the same. In the upper range, there is no loss of binding capacity and in the lower range sensitivity is maintained. It is critical to maintain the acetonitrile content throughout the entire analysis. Cycling the acetonitrile content in the eluent may cause polymer swelling and shrinking and therefore reduce the lifetime of the column.

Figure 5 20 µg Rabbit IgG Loaded onto MAbPac Protein A Column for 2,000 Injections at 2 mL/min.



### 3.2.8 pH range

It is recommended to use the MAbPac Protein A column at pH 2.5 – pH 7.5.

**CAUTION**

***Note: Although the MAbPac Protein A column can be used at slightly higher or lower pH, such as pH 8.0 and pH 2.0, the column degrades faster outside the recommended range. For best lifetime of the column, it is recommended to avoid extreme pH and use it within the recommended pH range.***

### 3.2.9 Column Storage

To maintain column lifetime, the MAbPac Protein A column should to be stored at 2-8°C when not in use. For short term storage (<2 days), the column may be left in eluent A at room temperature. For long term storage (>2 days), the column should to be stored in the recommended eluent A with 0.1% sodium azide at 2-8˚C.

# 4. Example Applications

**NOTE**

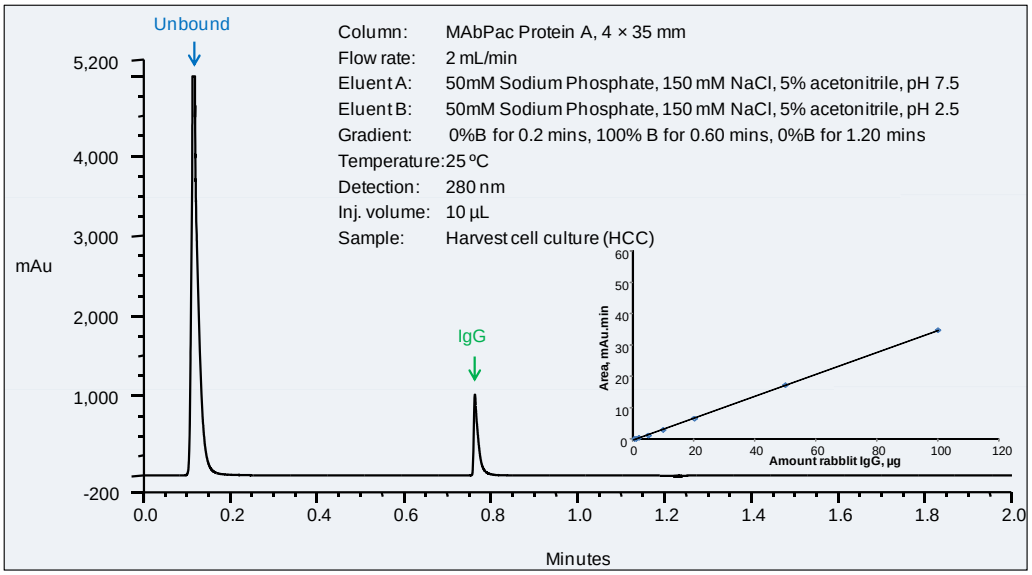
***For assistance, visit Unity Lab Services online at www.unitylabservices.com.***
***From the U.S., call the Customer Care Center for Dionex Products at 1-800-346-6390.***
***Outside the U.S., call the nearest Thermo Fisher Scientific office.***

The chromatograms in this section were obtained using columns that reproduced the Quality Assurance Report on optimized Bio HPLC systems. Different systems will differ slightly in performance due to slight differences in column sets, system void volumes, liquid sweep-out times of different components and laboratory temperatures.

## 4.1 Determination of MAb Titer of Harvest Cell Culture

The MAbPac Protein A column is used to measure the monoclonal antibody (MAb) titer from harvest cell culture (HCC). In the chromatogram below, 10 µL of HCC sample was injected onto MAbPac Protein A column. A large peak elutes in the intial (binding) portion of the method and represents unbound material. The MAb was released using a low pH wash at pH 2.5. The MAb titer is determined using a calibration curve previously generated and the integrated IgG peak area.

Figure 6 Analysis of HCC on the MAbPac Protein A (4 × 35 mm) column



# 5. Troubleshooting

The purpose of the Troubleshooting Guide is to help solve operating problems that may arise while using MAbPac Protein A column.

## 5.1 High Backpressure

If the system pressure is very high, it is advisable to find out what is causing the high system pressure.

To find out which part of the chromatographic system is causing the problem, disconnect the pump eluent line from the injection valve and turn the pump on. Watch the pressure. It should not exceed 50 psi (0.34 MPa). Continue adding the system components (injection valve, column, and detector) one by one, while watching the system pressure. The pressure should increase up to a maximum of ~1200 psi (8.3 MPa) at a flow rate of 1.0 mL/min when the column is connected. No other components should add more than 100 psi (0.69 MPa) of pressure. Refer to the appropriate manual for cleanup or replacement of the problem component.

### 5.1.1 System Flow Path:

Find out what part of the system is causing the high pressure. It could be a piece of tubing that has plugged or whose walls have collapsed, an injection valve with a plugged port, a column with particulates plugging the bed support (frit), a plugged high pressure in-line filter, or the detector cell.

### 5.1.2 Clogged Column Bed Support (Frit) Assemblies:

If the column inlet frit or the media is determined to be the cause of the high back pressure, clean the column in the reversed direction, or regenerate the columns using the methods described in Section 6.

### 5.1.3 Flow Rate:

Make sure that the pump is set to the correct eluent flow rate. Higher than recommended eluent flow rates will cause higher pressure. Measure the actual pump flow at various flow rates, if necessary by collecting the flow of deionized water into a pre-weighed graduated cylinder. Calculate the flow rate based on the collected volume of deionized water.

## 5.2 High Background or Noise:

### 5.2.1 Contamination of Eluents:

Make sure that all eluents are made correctly, and from chemicals with the recommended purity.

Make sure that the deionized water used to prepare the reagents has a specific resistance of 18.2 megohm-cm.

### 5.2.2 Contaminated Column:

Remove the column from the system. If the background noise decreases, then the column itself is the cause of the high background. Clean the column as instructed in Section 6- Column Cleanup.

### 5.2.3 Contaminated Hardware:

To eliminate the hardware as the source of the high background signal, remove the column and pump deionized water with a specific resistance of 18.2 megohm.cm through the system. The background signal should be less than 0.1 mAu at $UV_{280}$. If it is not, check the detector cell by injecting deionized water directly into it. See the appropriate detector manual for further details.

### 5.2.4 Air Bubbles in Detector:

If the pump loses prime, or a large injector loop filled with air is activated, air can enter the system. The air can dissolve at high pressure, but comes out of solution when it elutes from the column (low back pressure), and become trapped in the detector cell. Ensure that the pump is not pumping air, and that the injection system is working properly, and then clear the bubble by applying increased back pressure to the detector cell.

### 5.2.5 Low Capacity

If no binding or sudden loading capacity loss is observed, check the following:

#### 5.2.5.1 Eluents:

Improper eluent concentration or pH may be the problem. Remake the eluent as required for your application. Ensure that the water and chemicals used are of the required purity.

#### 5.2.5.2 Samples:

Column overloading may be the problem. If the sample contains more than one species that will bind to the column they will be in competition for binding sites. Reduce the amount of sample injected onto the column by either diluting the sample or injecting a smaller volume onto the column.

### 5.2.6 Column Equilibration:

The column may not be equilibrated well. Equilibrate the column by running through eluent A at 1ml/min for at least 15min.

### 5.2.7 Flow Rate:

Flow rate can affect binding efficiency and release. During loading, at high flow rates the target protein may pass through the column before having the chance to bind to the column. Reduce the flow rate. During elution, if flow rate used is too low then the released protein may not have time to elute from the column within the specified analysis time. Increase analysis time or increase the flow rate.

### 5.2.8 Temperature:

High temperatures can be detrimental to the column. Use the column at temperatures under 30˚C with a thermostatted column oven.

## 5.3 Low Sample Recovery

The sample may bind to the column too strongly. Try washing the column with eluting buffer at pH 2. For more washing options, see Section 6 - Column Cleanup.

## 5.4 Decreased Detection Sensitivity:

Detection sensitivity may be caused by sample degradation, column degradation leading to increased peak width (lower peak height), or limitations to light throughput in the absorbance detector (e.g., dirty or improperly installed flow cell, near failure of $D_2$ lamp). Refer to detector manual for clean up procedures. It could also be due to inappropriate detection wavelength. Check wavelength in use.

## 5.5 Small Peak Areas:

Small peak areas can occur when the injection valve is incorrectly installed or controlled. Plumb the injection valve's sample loop so that it is inserted into the flow path when the inject command is issued. At increased flow rates the data collection rate may need to be increased.

## 5.6 Poor Peak Shape:

When using the MAbPac Protein A column to analyze concentrated samples containing particulate matter the column inlet frit may become contaminated. You may see a split elution peak.

Disconnect the column from the detector and attach the column out let to the pump. Allow at least 10 column volumes of eluent to flow through the column before attaching the inlet of the column to the detector. Direct connection may flush debris into the cell and can damage the cell. This procedure can be carried out many times to extend the lifetime of the column.

## 5.7 System Problems:

### 5.7.1 High Detection Background Caused by the System:

A. Verify the problem is not related to the detector or column.
B. Prepare new eluents with freshly filtered deionized water.
C. Rinse all eluent lines with the new eluents (at least 40mL using the priming syringe)
D. If new eluent introduces high background without the column set installed, your deionized water source, or eluent components have become contaminated.

### 5.7.2 No Peaks, Poor Peak Area Reproducibility or Unexpectedly Small Peak Area:

A. Check the position and filling levels of sample vials in the autosampler.
B. Check injector needle height setting.
C. Check each line of the schedule for proper injector parameters. Employ full loop methods if other injection modes (partial loop fill) do not provide acceptable reproducibility.
D. Service the injection valve (check for leaks, rotor fragments, or sediments inside the valve)
E. Check sampling needle for bits of vial septa clogging the flow path.

### 5.7.3 Incorrect or Variable Retention Times:

A. Check your eluent preparation procedure for possible errors.
B. Prime the pump if necessary.
C. Measure the flow rate by weighing out the eluent collected during exactly five minutes of flow. Recalibrate the pump if necessary.
D. Set the eluent composition for 100% for each eluent and draw out at least 40mL of eluent from each of the lines to verify pump is primed.
E. Check and/or service the pump's proportioning valve. With the pumping turned off, the flow through the pump outlet tubing (disconnected from the injector) should be zero in all eluent positions. Check this separately for each eluent line.

For further information on system troubleshooting please refer to the appropriate system component manual.

# 6. Column Cleanup

The column can be cleaned using the following washing options to remove proteins that bind strongly to either through affinity binding or non-specific interactions.

For flow rates higher or lower than 1 mL/min the equivalent number of column volumes should be used based of a column volume of 0.5 mL.

## 6.1 Eluent Wash

A. Prepare eluting buffer, at pH 3 or below. For clean up it is advised to prepare a eluting buffer BELOW the pH of your standard eluting buffer.
B. Flush the column at 1 mL/min for 30 min.
C. Equilibrate the column with eluent A at 1 mL/min for at least 30 min before use.

## 6.2 Salt Wash

A. Prepare eluent containing 1M NaCl, eluent B
B. Flush the column at 1 mL/min for 40-60 min.
C. Equilibrate the column with eluent A at 1ml/min for at least 30 min before use.

## 6.3 Solvent Wash

A. Add 10% Acetonitrile to eluent A.
B. Flush the column at 1 mL/min for 40-60 min.
C. Equilibrate the column with eluent A at 1 mL/min for at least 30 min before use.

**CAUTION**

***Always use high purity grade chemicals and water to prepare the washing solutions. Always filter the washing solution through 0.2µm filters before use.***

**CAUTION**

***Always ensure that the cleanup protocol used does not switch directly between eluents that can react or precipitate when mixed together. Choose a flow rate that will not create higher column back pressure than maximum pressure in column specifications.***

# NOTES

For further information call 1-800-DIONEX-0 (1-800-346-6390)