
# ProPac WCX-10 and SCX-10

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Thermo SCIENTIFIC

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

# PRODUCT MANUAL

**FOR**

# PROPAC® WCX-10 AND SCX-10

**PROPAC WCX-10G GUARD COLUMN**
**4 x 50 mm, P/N 054994**
**2 x 50 mm, P/N 063480**

**PROPAC WCX-10 ANALYTICAL COLUMN**
**22 x 250 mm, P/N 088766**
**9 x 250 mm, P/N 063474**
**4 x 250 mm, P/N 054993**
**4 x 150 mm, P/N 088779**
**4 x 100 mm, P/N 088778**
**4 x 50 mm, P/N 074600**
**2 x 250 mm, P/N 063472**

**PROPAC SCX-10G GUARD COLUMN**
**4 x 50 mm, P/N 079930**
**2 x 50 mm, P/N 063462**

**PROPAC SCX-10 ANALYTICAL COLUMN**
**22 x 250 mm, P/N 088769**
**9 x 250 mm, P/N 063700**
**4 x 250 mm, P/N 054995**
**2 x 250 mm, P/N 063456**

# TABLE OF CONTENTS

# 1 INTRODUCTION

## 1.1 FEATURES OF THE PROPAC CATION EXCHANGE COLUMNS

The ProPac protein columns are ion exchange columns designed specifically to provide high-resolution and high efficiency separations of proteins and glycoproteins (pI = 3-10, MW>10,000 DA). The stationary phase is composed of 10-mm non-porous, solvent compatible ethylvinylbenzene-divinylbenzene copolymer beads which are surrounded by a highly hydrophilic, neutral polymer to reduce non-specific interactions between the surface and the biopolymer. For the weak cation exchange column (ProPac WCX-10), the surface is grafted with carboxylic acid groups. For the strong cation exchanger (ProPac SCX-10), the surface is grafted with sulfonic acid groups.

## 1.2 PROPAC WCX/SCX-10 OPERATING LIMITS AND SPECIFICATIONS

### 1.2.1 Operating Conditions

| PARAMETER | RECOMMENDATION |
|---|---|
| Flow Rate: | 2 x 250mm: 0.25 mL<br>4 x 150mm & 4 x 100mm: 1.0 mL/min<br>4 x 250mm: 1.0 mL/min<br>9 x 250mm: 5 mL/min*<br>22 x 250mm: Upto 30 mL/min*<br>(*Maximum pressure 3000 Psi) |
| Shipping Solution / Long Term Storage Solution: | 20 mM $Na_2HPO_4/H_3PO_4$, pH 6.5 wth 0.1% sodium azide |
| Short Term Storage Solution (overnight): | Your low salt concentration eluent (pH 3-10) |
| Typical buffers: | MES and other Good's buffers are preferred. Sodium or potassium salts of phosphate, Tris may be used.<br>***Always maintain a minimum ionic strength of at least 20 mM, to ensure optimum resolution.*** |
| Solvents: | 80% acetonitrile if needed for cleaning.<br>***Avoid using alcohol with the ProPac WCX-10 column.*** |
| Detergent Compatibility | Nonionic, anionic or zwitterionic detergents**.**<br>***Do not use cationic detergents.*** |
| Temperature Range: | Ambient to 60 ºC |

| PARAMETER | RECOMMENDATION |
|---|---|
| Pressure Limit: | 3000 Psi for all the columns:<br>2 x 250mm<br>4 x 50mm<br>4 x 100mm<br>4 x 150 mm<br>4 x 250mm<br>9 x 250mm<br>22 x 250mm |
| pH Range | 2-12 |
| Capacity*<br>Column Volume<br>4 x 250 mm = 3.14 mL<br>9 x 250 mm = 15.7 mL<br>22 x 250 mm = 94.2 mL | ProPac WCX-10 (4 x 250 mm: 6 mg lysozyme/mL column volume<br>ProPac SCX-10 (4 x 250 mm: 3 mg lysozyme/mL column Volume |
| Dynamic capacity for both PP WCX and PP SCX(Suggested Loading amount) | 2 x 250mm: 25 µg<br>4 x 50 mm: 20 µg<br>4 x 100mm: 40 µg<br>4 x 150mm: 60 µg<br>4 x 250mm: 100 µg<br>9 x 250mm: 500 µg<br>22 x 250mm: 3000 µg |

*Capacity is determined as the "breakthrough capacity". Depending on the protein, 10 – 100 µg protein can be injected.

**NOTE**

***Assistance is available for any problem during the shipment or operation: Contact your local Thermo Fisher Scientific office.***

### 1.2.2 Physical Characteristics

| FEATURE | SPECIFICATION |
|---|---|
| Substrate Particle Size | 10 µm |
| Substrate Pore Size | Non-porous |
| Substrate Monomers | ethylvinylbenzene-divinylbenzene |
| Substrate Cross-linking | 55% |
| Mode of Interaction | Cation Exchange |
| Functional Group | WCX-10 - Carboxylic Acid<br>SCX-10 - Sulfonic Acid |

## 1.3 Formats of the ProPac WCX/SCX-10 COLUMNS

ProPac cation exchange columns are available in a variety of formats to suit the need of the application. Sizes range from 2 mm ID columns for analytical work, upto 22 mm ID columns for semi-prep work. The ProPac cation exchange columns are also available in lots selected for the purpose of method validation. Dionex recommends purchasing columns from at least 3 different lots before setting method specifications.

### 1.3.1 ProPac Weak Cation-exchange Columns (Carboxylate Functionality)

| PRODUCT DESCRIPTION | PART NUMBER |
|---|---|
| ProPac WCX-10 Analytical Column (2x250 mm) | 063472 |
| ProPac WCX-10G Guard Column (2x50 mm) | 063480 |
| ProPac WCX-10 Analytical Column (4x250 mm) | 054993 |
| ProPac WCX-10 Analytical Column (4x150 mm) | 088779 |
| ProPac WCX-10HT Analytical Column (4x50 mm) | 074600 |
| ProPac WCX-10G Guard Column (4x50 mm) | 054994 |
| ProPac WCX-10 Analytical Column (9x250 mm) | 063472 |
| ProPac WCX-10 Analytical Column (22x250 mm) | 088766 |
| Lot select column set – 3 columns from 1 resin lot (4x250 mm) | 088767 |
| Lot select column set – 1 column from each of 3 resin lots (4x250 mm) | 088768 |

### 1.3.2 ProPac Strong Cation-exchange Columns (Sulfonate Functionality)

| PRODUCT DESCRIPTION | PART NUMBER |
|---|---|
| ProPac SCX-10 Analytical Column (2x250 mm) | 063456 |
| ProPac SCX-10G Guard Column (2x50 mm) | 063462 |
| ProPac SCX-10 Analytical Column (4x250 mm) | 054995 |
| ProPac SCX-10G Guard Column (4x50 mm) | 079930 |
| ProPac SCX-10 Semi-Preparative Column (9x250 mm) | 063700 |
| ProPac SCX-10 Semi-Preparative Column (22x50 mm) | 088769 |
| Lot select column set – 3 columns from 1 resin lot (4x250 mm) | 088772 |
| Lot select column set – 1 column from each of 3 resin lots (4x250 mm) | 088773 |

## 1.4 ProPac WCX/SCX-10 Operating Conditions

ProPac cation exchange columns are stable between pH 2 and 12, and are compatible with both aqueous mobile phases and those containing solvents, such as acetonitrile. Alcohols should be avoided when operating the ProPac WCX-10 column. The ProPac cation exchange columns can be operated at any flow rate, as long as the backpressure remains below 3,000 psi. When setting up a system for use with this column, check the special precautions listed in Section 3, "Operation". PEEK™ (polyetheretherketone) is used to make the column hardware. PEEK has excellent resistance to most organic solvents and inorganic solutions.

## 1.5 Key Applications of the ProPac WCX/SCX-10

The ProPac cation exchange columns provide excellent peak efficiencies and exceptional resolution of proteins and protein variants including monoclonal antibody variants. Some typical applications for which the columns have been used are listed below.

| COLUMN | APPLICATION |
|---|---|
| ProPac WCX-10 | Monoclonal antibodies; acidic and basic variants |
| ProPac WCX-10 | MAb C-terminal lysine truncation variants |
| ProPac SCX-10 | Hemoglobin sequence variants |
| ProPac WCX-10 | Ribonuclease A and its deamidation variants |

# 2 SYSTEM REQUIREMENT

## 2.1 A METAL FREE SYSTEM IS STRONGLY RECOMMENDED

The ProPac columns were designed to be used with a standard bore HPLC system having a gradient pump module, injection valve, and a UV detector.

**A metal-free system is recommended for halide-salt mobile phases which may otherwise cause corrosion of metallic components. Metal leaching from the system will lead to decreased performance from metal contamination. A metal-free pump is also recommended to avoid denaturation of the protein samples. Use of stainless steel tubing, ferrules, and bolt assemblies is also not recommended..**

| | |
|---|---|
| Typical Flow Rate: | 1 mL/min |
| Injection Volume: | 5–25 µL |
| Autosampler: | AS |
| System Void Volume: | Minimize the lengths of all connecting tubing and remove all unnecessary switching valves and couplers. |
| Pumps: | SP (single pump) or DP (dual pump) |
| Detectors: | VWD (Variable Wavelength Detector) |

## 2.2 SYSTEM VOID VOLUME

Tubing between the injection valve and detector should be < 0.010” ID PEEK tubing. Minimize the length of all liquid lines, but especially the tubing between the column and the detector. The use of larger diameter and/or longer tubing may decrease peak efficiency and peak resolution.

## 2.3 MOBILE PHASE LIMITATIONS

The ProPac cation exchange columns are compatible with typical mobile phases, such as sodium or potassium chloride salts in phosphate, MES or acetate buffers, up to the limit of their solubility. Use of organic solvents in the mobile phase is usually unnecessary. If you choose to use one, test the solubility limit of the mobile phase in the presence of the chosen organic solvent. Some combinations of salts and organic solvents are not miscible.

**WARNING** ***Do not use methanol or other alcohols as additives to the buffer when using the ProPac WCX-10, because alcohols will alter the anionic properties of this column. The formation of esters with alcohols will significantly reduce the capacity of the column. This ester formation is reversible but the regeneration process is rather time consuming.***

**WARNING** ***Do not operate the ProPac WCX-10 in the absence of a minimal concentration of salt. If the ionic strength is too low, the conformation of the stationary phase will be affected, causing a significant increase in backpressure. This effect can be reversed by pumping 500 mM NaCl through the column at low flowrate (0.1-0.2mL/min) until the backpressure is reduced, but it is recommended that salt be present in the mobile phase at all times.***

**WARNING** ***Cationic detergents (i.e. SDS) will irreversibly bind to the ProPac cation exchange columns and their use should be avoided.***

## 2.4 CHEMICAL PURITY REQUIREMENTS

Reliable, reproducible results require mobile phases that are free from impurities and prepared consistently.

### 2.4.1 Deionized Water

The deionized water used to prepare your mobile phase should be Type I reagent grade water with a specific resistance of 18 megohm-cm. The water should be free from ionized impurities, organics, microorganisms and particulate matter. UV treatment in the water purification unit is recommended. Follow the manufacturer's instructions regarding the replacement of ion exchange and adsorbent cartridges. All filters used for water purification must be free from UV-absorbing components. Contaminated water in the mobile phase causes high background signals, gradient artifacts, and even sample degradation.

# 3 OPERATION

## 3.1 MOBILE PHASE SELECTION

The mobile phase for the ProPac cation exchange columns consists of a buffer component and a salt component. The buffer selected depends upon the pI of the proteins to be separated, and should provide minimal UV interference at the wavelength to be monitored. Although phosphate buffers are widely used for various applications, usage of MES containing buffers is becoming increasingly popular for MAb separations between the pH range of 5 - 6.5. The advantage of using MES buffer is the buffering of the stationary phase of the column effectively with improved resolution.

Proteins are eluted using a gradient of increasing ionic strength. Optimum performance is obtained if a minimum salt concentration is maintained in buffer A at all times. Dionex recommends a minimum concentration of 2mM NaCl or equivalent in buffer A. Failure to maintain a minimum ionic strength in buffer A will result in alteration of the stationary phase conformation resulting in an increase in the column backpressure beyond the maximum recommended value. If this occurs, remove the column from the system, flush the buffer from the system and replenish with buffer B containing your high salt concentration. Replace the column and pump buffer B through the column at low flow rate (0.1-0.2 mL/min), until the backpressure falls back to normal.

| MOBILE PHASE CONSTITUENT | RECOMMENDATIONS |
|---|---|
| Buffer | Sodium phophate, MES, Tris |
| Salt | Potassium or sodium salts of chloride, acetate |
| pH Modifier | Phosphoric acid, HCl |
| Column Cleaning / Pretreatment | Sodium hydroxide |
| Solvent | Up to 80% acetonitrile |
| Detergent | Non-ionic, anionic, or zwitterionic detergents |
| Anti-Microbial | 0.1% sodium azide |

**WARNING** ***Do not operate the ProPac WCX-10 in the absence of a minimal concentration of salt. If the ionic strength is too low, the structure of the stationary phase will be affected, causing a significant increase in backpressure. This effect can be reversed by pumping 500 mM NaCl through the column at a low flowrate (0.1-0.2mL/min) until the backpressure is reduced, but it is recommended that salt be present in the mobile phase at all times.***

## 3.2 MOBILE PHASE PREPARATION

### 3.2.1 Adjusting the pH of the Mobile Phase

The mobile phase should contain all the electrolytes before adjusting the pH. To make sure that the pH reading is correct, the pH meter should be calibrated at least once a day. Stirring and temperature correction should be employed. (Note that pH measurements of buffers containing Tris should not be performed with a Ross electrode as this electrode produces erroneous results with amine containing solutions.)

### 3.2.2 Filtering the Mobile Phase

To extend the lifetime of your column as well as your HPLC pump, the high salt concentration buffers must be filtered using a 0.2 mm membrane filter to remove insoluble contaminants from the eluents.

### 3.2.3 Degassing the Mobile Phase

Before using them, the buffers must be degassed. The degassing can be done either using the Dionex pump degas function as described in the manual, or by using a vacuum pump. Vacuum degas the solvent by placing the mobile phase reservoir in a sonicator and drawing a vacuum on the filled reservoir with a vacuum pump for 5-10 minutes while sonicating.

## 3.3 VALIDATING COLUMN PERFORMANCE

Dionex recommends that you perform an efficiency test on your ProPac cation exchange column before you use it. The purpose of column performance validation is to make sure that no damage has been done to the column during shipping. Test the column using the conditions described on the Quality Assurance Report enclosed in the column box, and also included in the appendix of this manual. Repeat the test periodically to track the column performance over time. Note that slight variations may be obtained on two different HPLC systems due to system electronic, plumbing, operating environment, reagent quality, column conditioning, and operator technique.

Please see the example Quality Assurance Report in **Error! Reference source not found.**.

### 3.3.1 Procedure for Validating Column Performance

1. Connect the column to the LC system.
2. Purge the column with the mobile phase listed on the QA report for 20 to 40 column volumes.
3. Inject the test mix shown in the QA report and collect the data.
4. Compare your result with the QA report provided in the column box.
5. If the chromatograms look similar, you can use the column for your application work.

## 3.4 EQUILIBRATING THE COLUMN

Equilibrate the column after installing it for the first time. Always re-equilibrate the column prior to use following periods of storage.

Purge the column of shipping or storage solvent until the baseline is stable. Equilibrate the column with at least 15 column volumes of mobile phase A, or until a stable baseline is achieved.

## 3.5 CARING FOR THE COLUMN

To ensure the high performance of the ProPac cation exchange columns, the following guidelines should be followed.

1. Protect the column from contamination using a ProPac WCX/SCX guard column.
2. Make sure that solvents are miscible when changing mobile phases.
3. Always degas and filter mobile phases through a 0.22-mm membrane filter.
4. When switching to a new mobile phase, the column should be equilibrated with at least 30 column volumes before injecting the sample.
5. The recommended pH range is from pH 2 to 12. However, it is preferred that the column be used between pH 3 and pH 11 to achieve longer lifetime.
6. The column can be stored in mobile phase for short term storage (e.g. overnight). However, it is highly recommended that the column be stored in 20 mM $Na_2HPO_4/H_3PO$, pH 6.5 wth 0.1% sodium azide (more than 2 days).
7. The recommended operating maximum temperature is below 50 °C. In most cases, temperature control between ambient and 30 °C gives good results.
8. The recommended maximum backpressure is 4000 psi.

# 4 APPLICATIONS

## 4.1 SEPARATION OF ACIDIC AND BASIC VARIANTS IN MONOCLONAL ANTIBODIES USING PROPAC WCX COLUMN

Monoclonal antibodies (MAbs) are currently developed by pharmaceutical and biotechnology companies for various therapeutic applications. MAbs undergo several post-translational modifications including oxidations, deamidations, truncations as well as glycan modifications. Manufacturing of MAbs and subsequent stability testing procedures involve routine analysis and monitoring of the impurities resulting from asparagines deamidation, aspartic isomerization, disulfide interchange, peptide bond cleavage and oxidation. ProPac WCX is widely used to characterize MAb heterogeneity. One of the example applications is shown below (Figure 1).

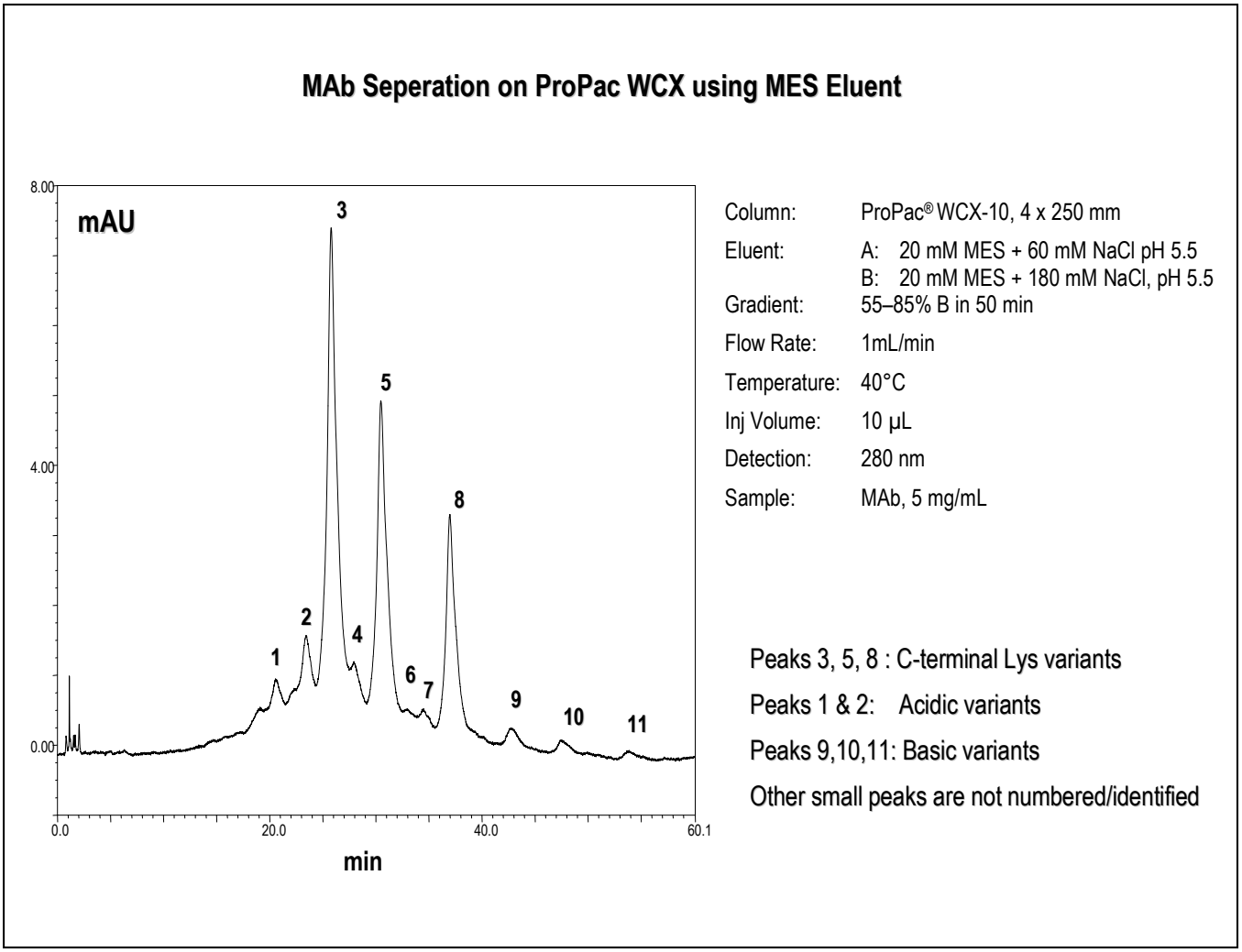


**Figure 1**
**Separation of Acidic and basic variants of MAbs on ProPac WCX column**

## 4.2 MONITORING PROCESSING OF C-TERMINAL LYSINE AND ARGININE RESIDUES OF PROTEINS ISOLATED FROM MAMMALIAN CELL CULTURE BY CATION EXCHANGE CHROMATOGRAPHY

Processing of C-terminal lysine and arginine residues of proteins isolated from mammalian cell culture has been described [1]. As a result of processing techniques, the presence of C-terminal Lys or Arg residues, which could be expected based on gene sequence information, are often absent in proteins isolated from mammalian cell culture. This discrepancy, which is common in plasma derived proteins, may result from the activity of one or more basic carboxypeptidases. Charge heterogeneity can result if the processing is incomplete. The resulting charge heterogeneity of the variant forms can be identified by cation exchange chromatography. C terminal processing of lysine residues from heavy chains of monoclonal antibodies from a variety of sources has been reported [2-6].

In this example the ProPac WCX-10 cation exchange columns were used to separate variants of a humanized IgG, suspected of having lysine residue variation at the C-terminal of the heavy chains. As shown in Fig. 2, a shallow NaCl gradient (40-150 mM NaCl in 30 min), at neutral pH, resolves three variant forms differing by the presence of lysine at the C-terminal of the heavy chains (with either 0, 1, or 2 lysine residues).

To verify that the reason for the different retention times of the three peaks was the different content of heavy chain C terminal lysine, the IgG preparation was treated with carboxypeptidase B, an exopeptidase that specifically cleaves C terminal lysine residues. This treatment of the IgG preparation resulted in the quantitative disappearance of peaks 2 and 3 (containing 1 and 2 terminal lysine residues, respectively, on their heavy chains). The decreased peak areas in peaks 1 and 2 were accompanied by a corresponding quantitative increase in peak area 1 (variant with no terminal lysine, Fig. 2), confirming that peaks 2 and 3 differed from peak 1 in that they contained IgG with 1 and 2 terminal heavy chain lysine residues, respectively.

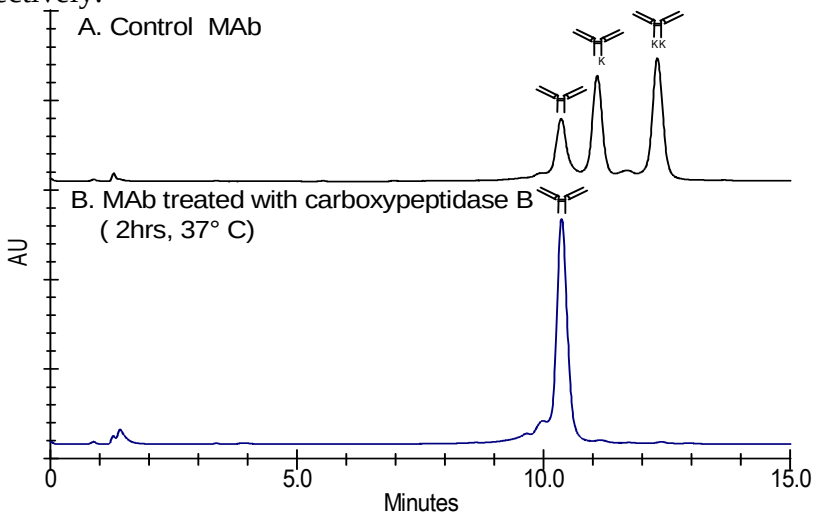


**Figure 2**
**Chromatograms Obtained for MAb Sample Before and After Treatment with Carboxypeptidase B (Boeringer Mannheim) for 2 hrs at 37º C**

## 4.3 MONITORING FORCED ASPARGINE DEAMIDATION GLYCOPROTEINS BY CATION EXCHANGE CHROMATOGRAPHY

Deamidation of Asn residues or the isomerization of Asp residues occurs in a variety of protein-based pharmaceuticals including human growth hormone [7], tissue plasminogen activator [8], hirudin [9], monoclonal antibodies [10], acidic fibroblast growth factor [11], and interleukin 1 [12], with varying effects on the activity or stability of the therapeutic protein. Hence, monitoring the deamidation of Asn residues in proteins is of interest to analytical and protein chemists in quality control and process departments at biotechnology and pharmaceutical companies [13].

As described by A. D. Donato et al. [14], separation of the Asn67 deamidation products of ribonuclease A required cation exchange on Mono S followed by hydrophobic interaction chromatography to resolve the two deamidation variants (Asp and isoAsp at residue 67). In contrast, using only a ProPac WCX-10 Column, deamidation variant forms having Asp or isoAsp at Asn67 were baseline-resolved from each other and from native ribonuclease A in a single chromatographic analysis (Figure ).

The chromatograms in this example show the separation of ribonuclease A and its two deamidation products at several time points during the course of the forced deamidation. The baseline separation made it possible to quantify the change in amounts of each form within the mixture as a function of time. Based on the increase in the amount of Asn67 deamidated forms of ribonuclease A as a function of time, it was observed that the kinetics of deamidation appear to be first order with a t1/2 of 159 hours. See Figure 4.

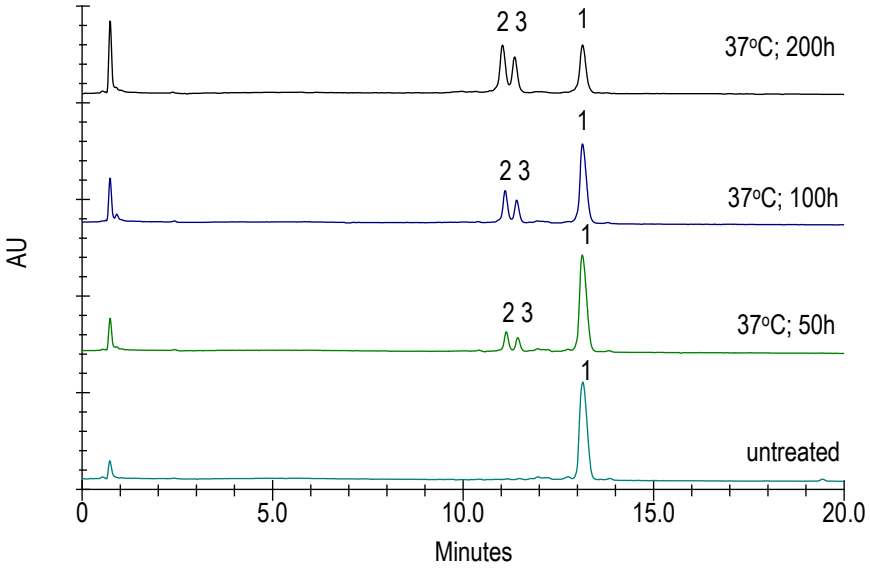


**Figure 3**
**Monitoring Deamidation of Glycoprotein**

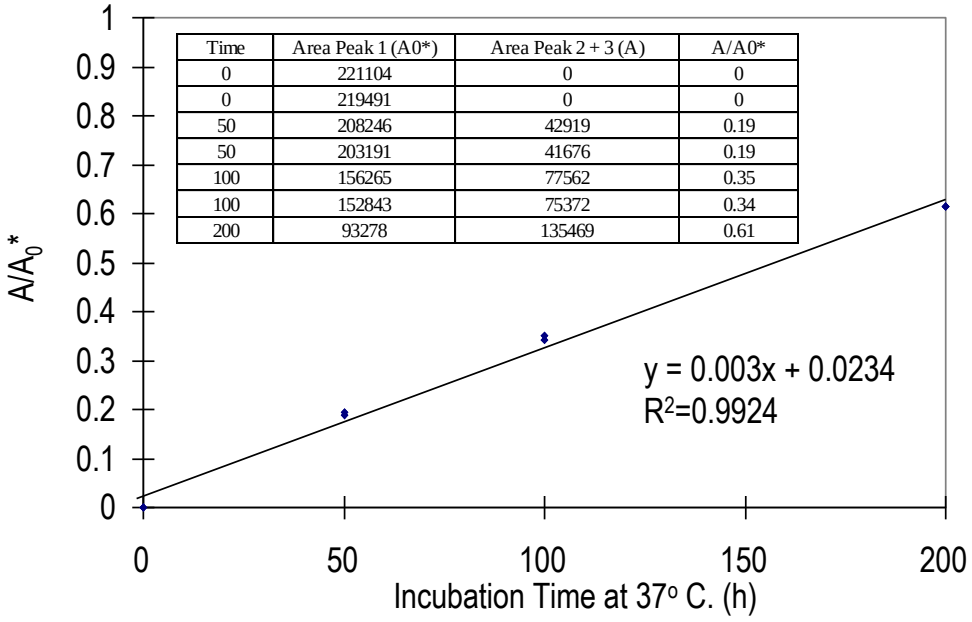


**Figure 4**
**Fractional Amount of Deamidation Products Formed as a Function of Time when Ribonuclease A (3 mg/mL) was Incubated in 1% Ammonium Carbonate Buffer, pH 8.2, at 37 ºC**

## 4.4 ANALYSIS OF HEMOGLOBIN VARIANTS

Clinical laboratories frequently separate and quantify the levels of different hemoglobin variants. Two very important hemoglobin variant separations are, respectively, the resolution of glycosylated hemoglobins in Figure 5 and hemoglobin sequence variants in Figure 6. For the physician, the determination of glycated hemoglobin levels in the blood of a diabetic patient serves as an excellent indication of the average glucose level in the patient's blood during the preceding 1–2 months. Separating and identifying hemoglobins associated with serious hemopathies, including sickle cell, hemoglobin C, and Barts disease are also extremely important in the diagnosis, treatment, and counseling of afflicted children.

Typically, isoelectric focusing gel electrophoresis (IEF) is used for the analysis of hemoglobin sequences, including Hb S, C, F, A, and A2. However, two IEF steps, using cellulose acetate electrophoresis with alkaline pH, followed by confirmation using citrate agar electrophoresis at acidic pH are necessary. The ProPac SCX-10 column successfully resolves these hemoglobin species in a single run within 20 minutes.

| | |
|---|---|
| Sample Volume Injected: | 10 µL |
| Column: | ProPac SCX-10 |
| Eluent: | A: 50 mM sodium phosphate and 2 mM potassium cyanide adjusted to 6.0 pH with H3PO4<br>B: 50 mM sodium phosphate, 2 mM potassium cyanide and 0.5 mM sodium chloride adjusted to 6.0 pH with H3PO4 |
| Gradient: | See tables. |
| Eluent Flow Rate: | Any PEEK HPLC pump capable of delivering gradients |
| Detection | UV, 220 nm |

**Table 1**

| Time (min.) | %A | %B |
|---|---|---|
| Initial | 97 | 3 |
| 0.0 | 97 | 3 |
| 20.0 | 88 | 12 |
| 22.0 | 88 | 12 |
| 30.0 | 60 | 40 |
| 30.1 | 97 | 3 |
| 40.1 | 97 | 3 |

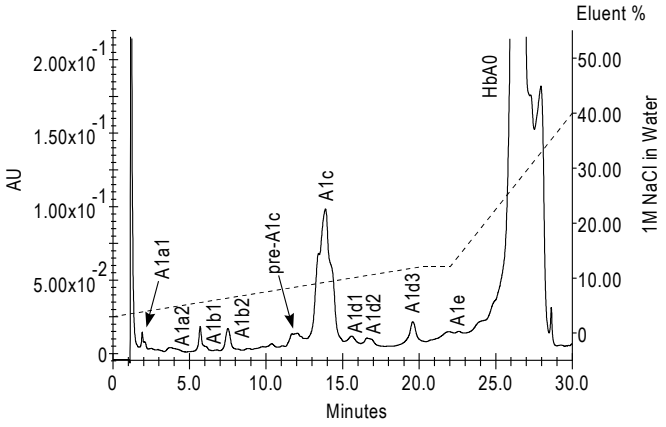


**Figure 5**
**Resolution of Glycosylated Hemoglobins**

**Table 2**

| Time (min.) | %A | %B |
|---|---|---|
| Initial | 100 | 0 |
| 0.0 | 100 | 0 |
| 30.0 | 50 | 50 |
| 30.1 | 100 | 0 |
| 40.0 | 100 | 0 |

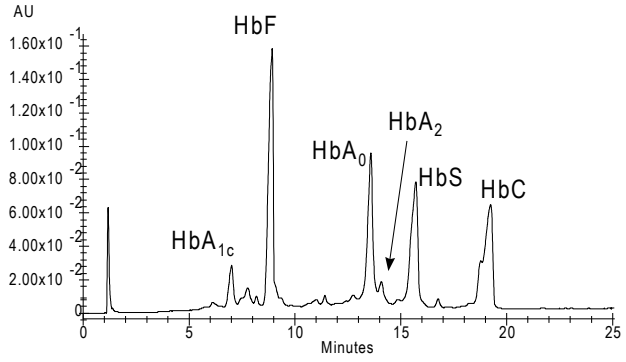


**Figure 6**
**Hemoglobin Sequence Variants**

**NOTE**

***Dispose of the waste eluent containing potassium cyanide using basic conditions.***

## TROUBLESHOOTING GUIDE

### 4.5 FINDING THE SOURCE OF HIGH SYSTEM BACKPRESSURE

**WARNING** ***NEVER WASH THE PROPAC WCX/SCX COLUMN WITH $H_2O$. Always maintain minimum ionic strength (20 mM Sodium phosphate, or equivalent) in the eluents.***

a. If you observe high back pressure, wash the column with an eluent containing high salt (Buffer containing 1M NaCl) at a lower flow rate (0.1 to 0.5 mL/ min) until the pressure becomes normal.
b. A significant increase in the system backpressure may be caused by a plugged inlet frit (bed support).
c. Before replacing the inlet bed support assembly of the column, make sure that the column is the cause of the excessive backpressure.
d. Check for pinched tubing or obstructed fittings from the pump outlet, throughout the eluent flow path to the detector cell outlet. To do this, disconnect the eluent line at the pump outlet and observe the backpressure at the usual flow rate. It should not exceed 50 psi (0.3 MPa). Continue adding components (injection valve, column, detector) one by one while monitoring the system backpressure. The 4 x 250 mm ProPac WCX-10 and SCX-10 should add no more than 1,800 psi backpressure at 1 mL/min. The 4 x 50 mm ProPac WCX-10 and SCX-10 columns should add no more than 400 psi (2.6 MPa) back pressure at 1 mL/min. No other component should add more than 100 psi (0.7 psi) to the system backpressure.
e. If the high backpressure is due to the column, first try cleaning the column. If the high backpressure persists, replace the column bed support at the inlet of the column.

### 4.6 COLUMN PERFORMANCE IS DETERIORATED

#### 4.6.1 Loss of Resolution and efficiency for Monoclonal Antibody (MAb) separations

For certain applications involving the characterization of monoclonal antibodies, optimum resolution of the analyte separation on the ProPac WCX-10 column is desired. If your column fails to exhibit the expected efficiency and resolution, perform the following treatments as suggested in the following steps and then try the separation again using your own optimized conditions.

**STEP 1: 20 mM NaOH column wash procedure**
Eluent 1: 20 mM NaOH
Eluent 2: 20 mM MES (pH 6.5)

**Procedure:**

1. Prepare 20 mM MES (pH 6.5) by adding 3.9 gm of MES (Sigma M-8250) to 950 mL of 18-megohm Milli Q water or equivalent.

2. Adjust the pH with NaOH to pH 6.5 and bring to volume (1 liter) with 18-megohm water. Filter and degas the solution before use.
3. Treat the ProPac WCX-10 column by pumping 20 mM NaOH through it at 0.5 mL/min for 15 minutes at room temperature (25 ºC to 30 ºC).
4. Following the sodium hydroxide treatment, wash the column with 20 mM MES pH 6.5, 1.0 mL/min for 1hr.

The column should now be ready for your analysis using routine conditions. If you do not see any improvement with your column performance proceed to Step 2 (See below)

**STEP 2: 20 mM MES/ 50°C treatment procedure**

Prepare MES as described in Step 1 and treat the WCX-10 Column with 20 mM MES (pH 6.5) at 50°C for 48hr at a flow rate of 0.2 to 0.5mL/min.

(Alternatively, WCX-10 column may be treated with 20 mM MES (pH 6.5) for 8 hr at a flow rate of 0.5 mL/min at 80°C.)

**ANALYSIS**: Use your routine conditions for the analysis of the analyte. A few examples are shown below.

Example 1: Effect of MES/50°C treatment on the separation of MAbs: As you can see in Fig 7, the column treated with 20 MES (pH 6.5) at 50°C for 48hrs showed improved peak shapes and efficiencies as compared to the untreated controls. The chromatography conditions are given in the figure.

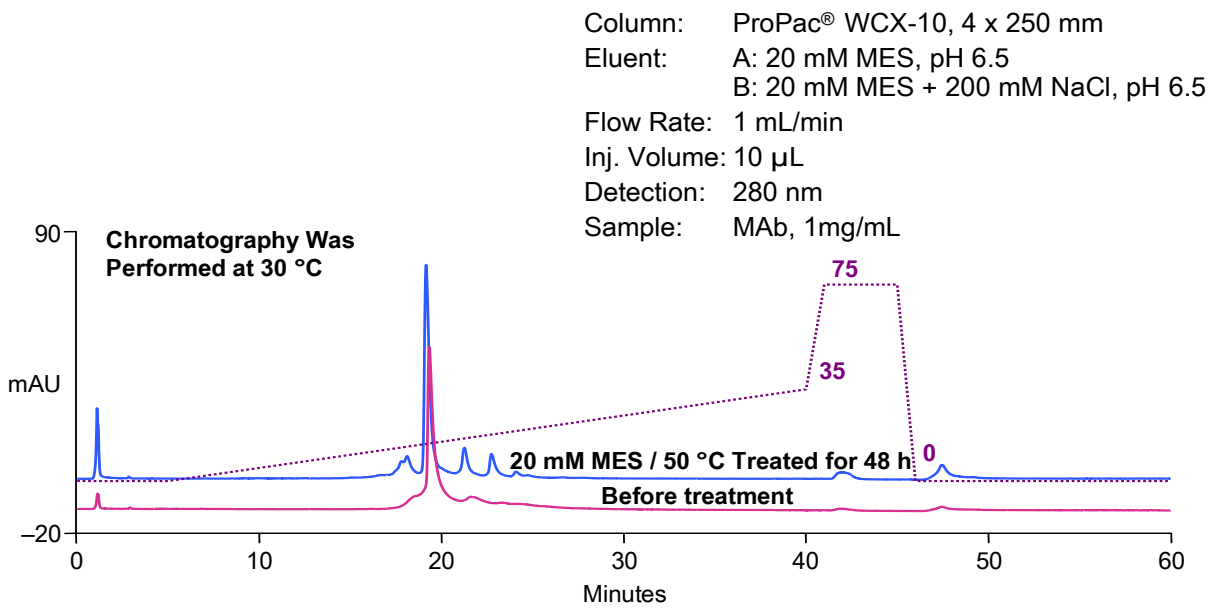


**Figure 7**
**Effect of MES/50 ºC Treatment on WCMAb Separation**

Exmple 2: Time Course of MES/50 °C Treatment on WCX-MAb Separation: This experiment shows (Figure 8) treatment of ProPac WCX column with 20 mM MES at 50°C for various intervals followed by separation of MAb on the treated column. The chromatography is compared at 30°C vs 50°C to document differences in chromatography at different temperatures. The maximum benefit of treatment was observed between 41 hrs and 61 hrs of treatment. Therefore 48 hrs was chosen and recommended for the MES/heat treatment experiments to improve efficiency of MAb separations and performance.

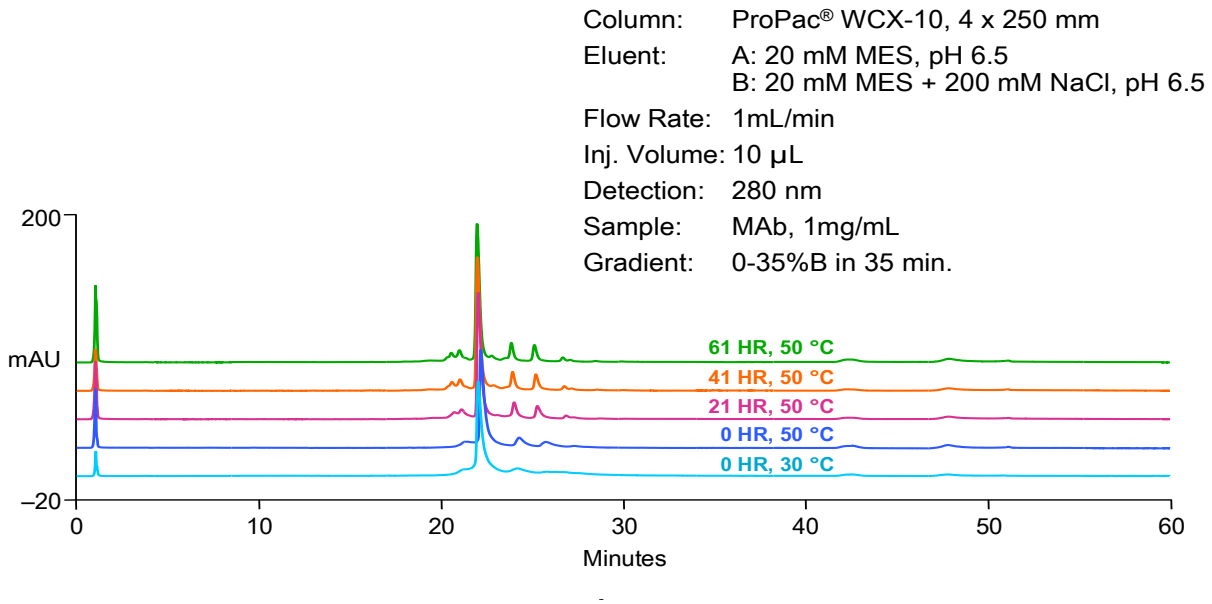


**Figure 8**
**Time Course of MES/50 °C Treatment on WCX-MAb Separation**

Example 3: Time Course of MES/80 °C Treatment on WCX-MAb Separation. Figure 9 shows time course effect of MES/ 80°C treatment on ProPac WCX MAb separations. At 80°C it takes only about 8hrs to get the improved efficiency as compared to 48hrs treatment at 50°C. It should be noted that the chromatography was done at 30°C.

**Column:** ProPac WCX-10 (4x250mm)
**Eluents:** A: 20 mM MES (pH6.5) B: 20 mM MES + 200 mM NaCl (pH6.5)
**Flow:** 1.0 mL / min
**WVL:** 280nm
**Sample:** MAb (1mg / mL)
**Inj volume:** 10 µl
**Gradient:** 0-35%B in 35 min.

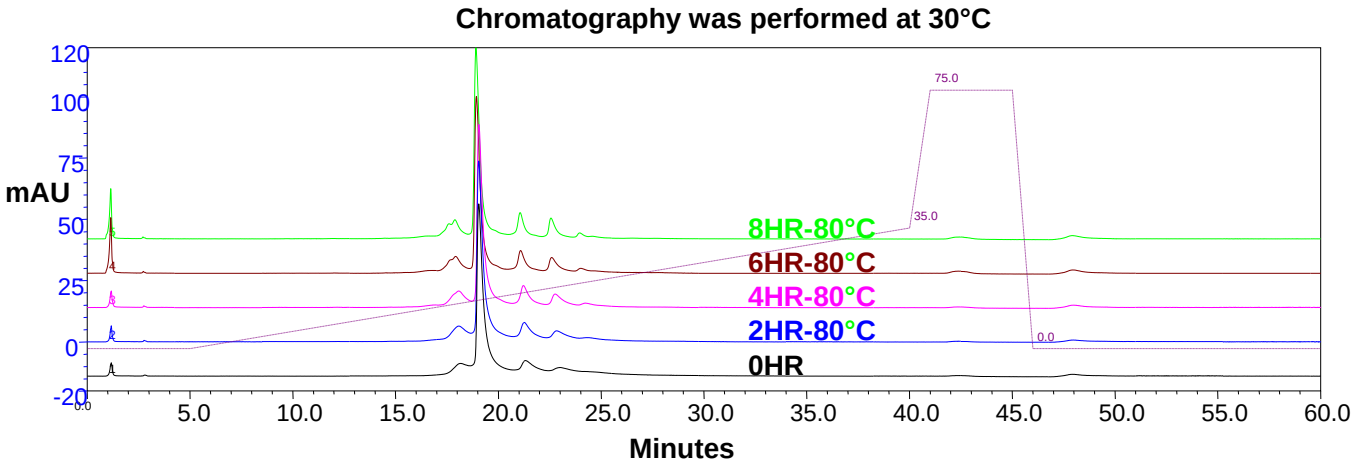


**Figure 9**
**Time course of MES/80C treatment on WCX-MAb separation**

### 4.6.2 Peak efficiency and resolution is decreasing; loss of efficiency.

**WARNING** **One of the sources of decreased performance could be metal leaching from the system. To avoid denaturation of the protein samples and corrosion of components with halide-salt mobile phases we strongly recommend a metal-free system, including pump, tubing, ferrules, and bolt assemblies.**

a. If changes to the system plumbing have been made, check for excess lengths of tubing, tubing diameters larger than 0.010 ID in., larger than normal tubing diameter, and for leaks.
b. Check the flow rate and the gradient profile to make sure your gradient pump is working correctly.
c. The column may be fouled. Clean the column using the recommended cleaning conditions.

d. If there seems to be a permanent loss of efficiency, check to see if headspace has developed in the column. This is usually due to improper use of the column such as submitting it to high backpressure. If the resin doesn't fill the column body all the way to the top, the resin bed has collapsed, creating a headspace. The column must be replaced.
e. If the peak shape looks good, but the efficiency number is low, check and optimize the integration parameters. If necessary, correct the integration manually, so the start-, maximum-, and end of the peak are correctly identified.

### 4.6.3 Unidentified peaks appear as well as the expected analyte peaks.

a. The sample may be degrading. Proteins tend to degrade faster in solutions; therefore, store your protein samples in the freezer in dry form, and prepare only a small amount of solution/mixture for analysis.
b. The eluent may be contaminated. Prepare fresh, filtered eluent.
c. Run a blank gradient to determine if the column is contaminated. If ghost peaks appear, clean the column.

### 4.6.4 Peak efficiency and resolution is poor.

a. Try to use different eluents (buffer, pH, concentration etc.), to make sure you are using the optimum conditions for your separation problem.
b. The column may be overloaded. Dilute the sample and/or inject smaller volumes.

### 4.6.5 5.2.4 Peak retention time varies from run-to-run.

The column may not be adequately equilibrated or washed.

a. Make sure that the equilibration time is adequate and remains constant after every gradient run. Re-equilibration should be part of the method.
b. Column washing is usually not necessary between every run, unless your sample is extremely "dirty." If you need to use a wash, a consistent and adequate method for washing and equilibrating should be part of the method.

## 4.7 NO PEAKS, SMALL PEAKS, NOISY BASELINE

### 4.7.1 Detection Problem

Make sure that you are using the correct wavelength for your sample/buffer system. Adjust the selected detector range (AU) according to your injected sample amount. Check your lamp: aged UV lamps tends to give noisier response. Replace the lamp if necessary.

### 4.7.2 Chromatographic Problem

Make sure that your sample can be eluted with the buffers and conditions you are using. Before trying a gradient separation, try isocratic elution with 100% B (high salt) buffer: the sample should elute at, or near to, t0 (void). If not, try a higher salt concentration or different pH.

## APPENDIX A. REFERENCES

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# APPENDIX B. PROPAC WCX-10 AND SCX-10 COLUMN CARE

## B.1. NEW COLUMN EQUILIBRATION

The columns are shipped in 10 mM Na2HPO4/H3PO4 pH=6.5 buffer containing 0.1% sodium azide. Before use, wash the column with approximately 20 mL of the starting eluent (20 min at 1 mL/min).

## B.2. COLUMN CLEAN UP

**NOTE** ***When cleaning an analytical and guard column in series, move the guard column after the analytical column in the eluent flow path. Otherwise contaminants that have accumulated on the guard column will be eluted onto the analytical column.***

1. For minor contamination, use a mild cleaning protocol by running consecutive gradient runs, using a high (1-2 M) salt concentration at the end of the gradient.

2. For more severe contamination, inject 100-500 µL (or more as needed) of 0.1-1 M NaOH consecutively.

3. If necessary, the column can be washed with strong acid and/or base such as 1.0 M HCl and/or 0.1 - 0.5 M NaOH. Usually 5 - 30 min at 1 mL/min is sufficient. Do not exceed 20x the column volume of 0.5 M NaOH (60 mL). The use of high concentrations of base and/or larger volumes of base are not recommended. The WCX-10 columns can be washed at temperatures up to 60 °C. For the SCX-10 columns, the above mentioned strong acid or base cleaning solutions should be used at room temperature (<30 °C). After the wash, rinse the column with at least 20 mL of the starting buffer solution. Note: Do not store the column in strong acid or base solution.

## B.3. COLUMN STORAGE

Short Term Storage: For short term storage, use the low salt concentration eluent (pH = 3 - 10) as the column storage solution.

Long Term Storage: For long term storage, use 20 mM $Na_2HPO_4$/H3PO4 pH=6.5 eluent (or other low salt concentration eluent with pH=6.0-7.5) with 0.1% sodium azide added to avoid bacteria growth on the column.

Flush the column with at least 10 mL of the storage eluent. Cap both ends, securely, using the plugs supplied with the column.

## B.4 REPLACING COLUMN BED SUPPORT ASSEMBLIES

**NOTE** ***Replace the inlet bed support ONLY if the column is determined to be the cause of high system backpressure, AND cleaning of the column does not solve the problem.***

1. Carefully unsrew the inlet (top) column fitting. Use two open end wrenches.
2. Remove the bed support. Tap the end fitting against a hard, flat surface to remove the bed support and seal assembly. Do not scratch the wall or threads of the end fitting. Discard the old bed support assembly.
3. Removal of the bed support may permit a small amount of resin to extrude from the column. Carefully remove this with a flat surface such as a razor blade. Make sure the end of the column is clean and free of any particulate matter. Any resin on the end of the column tube will prevent a proper seal. Insert a new bed support assembly into the end fitting and carefully thread the end fitting and bed support assembly onto the supported column.
4. Tighten the end fitting fingertight, then an additional ¼ turn (25 in x lb.). Tighten further only if leaks are observed.

**WARNING** ***If the end of the column tube is not clean when inserted into the end fitting, particulate matter may prevent a proper seal between the end of the column tube end the bed support assembly. If this is the case, additional tightening may not seal the column but instead damage the column tube or break the end fitting.***