

# REPLACE

## PRODUCT MANUAL

for the

PROPAC PA1 (4 x 250 mm)  
(P/N 039658)

PROPAC PA1 (4 x 50 mm)  
(P/N 039657)

PROPAC PA1 (9 x 250 mm)  
(P/N 040137)

® Dionex Corporation, 1987-2003

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# TABLE OF CONTENTS

<b>SECTION 1 - INTRODUCTION .....</b>	<b>4</b>
<b>SECTION 2 - INSTALLATION .....</b>	<b>5</b>
2.1 System Requirements .....	5
<b>SECTION 3 - OPERATION .....</b>	<b>6</b>
3.1 Operation Precautions .....	6
3.2 Chemicals Required .....	6
3.3 Method Development .....	6
3.4 Eluent Preparation .....	6
3.5 Sample Preparation .....	7
3.6 Screening For Initial Conditions .....	7
3.7 Bracketing Your Peak .....	7
3.8 Enhancing Detection Sensitivity and Selectivity .....	8
3.9 Enhancing Capacity .....	8
<b>SECTION 4 - TROUBLESHOOTING GUIDE .....</b>	<b>9</b>
4.1 High Backpressure .....	9
4.2 High Background Noise .....	10
4.3 Poor Peak Resolution .....	10
4.4 Spurious Peaks .....	11
<b>APPENDIX A - QUALITY ASSURANCE REPORTS .....</b>	<b>12</b>
A.1 Production Test Chromatogram PA1 (4 x 50) .....	12
A.2 Production Test Chromatogram PA1 (4 x 250) .....	13
A.3 Production Test Chromatogram PA1 (9 x 250) .....	14

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**APPENDIX B - COLUMN CARE ..... 15**

**B.1 Recommended Operation Pressures ..... 15**

**B.2 Column Start-up ..... 15**

**B.3 Column Storage ..... 15**

**B.4 Column Cleanup ..... 15**

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## SECTION 1 - INTRODUCTION

The ProPac PA1 column is a high efficiency column for use in protein analysis and lab-scale purification. This manual, based on work in our laboratories, provides general guidelines for obtaining satisfactory results with the ProPac PA1.

**Table 1**  
**ProPac PA1 Packing Specifications**

Column	Particle Diameter µm	Substrate X-Linking %	Latex Diameter nm	Latex X-Linking %	Functional Group	Hydrophobicity
ProPac PA1 4 x 250	10–11	2	250	5.5	Alkyl quaternary amine	High
ProPac PA1 4 x 50	10–11	2	250	5.5	Alkyl quaternary amine	High
ProPac PA1 9 x 250	10–11	2	250	5.5	Alkyl quaternary amine	High

**Table 2**  
**ProPac PA1 Operating Parameters**

Column	Typical Back Pressure psi (MPa) at 30 °C	Standard Flow Rate mL/min
<b>ProPac PA1 Analytical Column</b> 4 x 250	< 2000 (13.79)	1.5
<b>ProPac PA1 Guard Column</b> 4 x 50	< 600 (4.14)	1.0
<b>ProPac PA1 Analytical Column</b> 9 x 250	< 1500 (10.34)	4.0

Always remember that assistance is available for any problem that may be encountered during the shipment or operation of Dionex instrumentation and columns through the Dionex North America Technical Call Center at 1-800-DIONEX-0 (1-800-346-6390) or through any of the Dionex Offices listed in, "Dionex Worldwide Offices."

## **SECTION 2 - INSTALLATION**

### **2.1 System Requirements**

Optimal performance is obtained when the ProPac PA1 column is used with a metal-free system, like the Dionex BioLC chromatograph. Standard stainless steel systems may leach metal ions that will eventually poison the column. If your column loses resolution with use, regeneration may be required (see Appendix B - Column Care). As with all chromatographic systems, tubing between the injector, column and detector should be kept to a minimum.

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## SECTION 3 - OPERATION

### 3.1 Operation Precautions

#### NOTE

The polymeric packing used in the ProPac PA1 column is damaged by organic solvents. The use of acetonitrile and/or methanol at concentrations greater than 2%, and the use of more hydrophobic solvents than these, will void the ProPac PA1 column warranty. Because anionic detergents can mask the ion exchange sites, these are also contraindicated. Cationic detergents can also mask some of the ion change sites. Some cationic detergents can be used without incurring permanent damage. Consult your DIONEX Regional Office (see Dionex Worldwide Offices) for further instruction if you feel that you must use cationic detergents.

### 3.2 Chemicals Required

The compounds separated in the Column Quality Control Test are commercially available in acceptable purity from Sigma Chemical Co., St. Louis, MO. The elution order is: Angiotensin 11 (Sigma #A-9525), MET-Enkephalin (Sigma M-6683), and the oxidized A-chain of insulin (Sigma 1-1633). Dissolve these compounds in deionized water having a specific resistance of 18.2 megohm-cm and inject approximately 1.9 nmol of Ang.-II, 2.6 nmol of Met-Enk., and 3.2 nmol of the insulin A-chain. Refer to test chromatograms shipped with column. If the column performance is unsatisfactory, contact the nearest Dionex Regional Office (see Dionex Worldwide Offices).

#### NOTE

Many pH electrodes do not accurately measure the pH of Tris solutions. The pH of the test eluent (pH 9.9) was measured with a ROSS™ electrode. This eluent, when measured with a standard gel-filled electrode, gave a pH of 9.6.

### 3.3 Method Development

The ProPac PA1 column is intended for use in the analysis and purification of hydrophilic anionic proteins and peptides. This enormous class of compounds cannot be resolved under a given set of conditions by any single column. However, careful choice of sample preparation and gradient elution conditions can resolve many of these proteins.

Proteins and peptides that are very hydrophobic (e.g. integral membrane proteins), or very cationic proteins (i.e. those with very high isoelectric points), may be resolved on this column, but some exhibit substantial binding to the ProPac PA1. Examples of proteins with high pIs include cytochrome C (pI 9.4), and Lysozyme (pI 11). Cytochrome C elutes normally from the ProPac PA1 while lysozyme exhibits substantial binding, at pH 8–9.

As you work with a particular protein, you will find the conditions (pH, temperature, presence of cofactors, substrates, or reducing agents, and other salts) that best stabilize it. Whenever possible, it is advisable that those conditions be kept constant during chromatography.

Determining the best conditions for resolution of proteins can be described as a three-step process. These steps are: eluent and sample preparation, screening for initial conditions, and finally ‘bracketing’ your peak.

### 3.4 Eluent Preparation

The buffer employed will depend on the isoelectric point (pH at which the protein’s net charge is zero) of the protein to be examined. While much of the development and testing of the ProPac PA1 employed Tris buffers, any buffer that neither contributes contaminant peaks, nor interferes with detection, will be acceptable. Among those commonly used are phosphate, MOPS, borate and bicarbonate.

In addition to the buffer, a salt is used to provide the anion that competes with the protein’s charges for ion exchange sites on the column. This salt is chosen to present a minimum signal at the detector, while still being an effective competitor with the protein’s ionic domains. In practical terms, the salt should contribute a maximum ionic strength of 1–2 molar (M). When chloride is used,

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few proteins are retained on the ProPac PA1 column at salt concentrations above 1 molar. However, the ProPac PA1 can tolerate ionic strengths up to 4 molar.

Occasionally the buffer and salt can be the same. When a salt-free protein preparation is needed, volatile salts like  $\text{NH}_4\text{HCO}_3$  are used to both elute the protein and buffer the system. Repeated lyophilization of fractions collected in this buffer/salt will yield an essentially salt-free preparation. Alternatively, desalting can usually be accomplished with a small size-exclusion column (e.g. PIERCE GF-5 columns).

### 3.5 Sample Preparation

This step is of variable importance depending on the matrix, or chemical composition, of your sample. If the sample contains many smaller compounds, you should consider dialysis or desalting. Two alternative techniques for the latter process are filtration through low molecular weight pore size membranes and size exclusion chromatography.

Samples with many proteins may require a 'salting-out' step. This is usually accomplished by treatment with high concentrations of ammonium sulfate, centrifugation to collect the precipitate, and resuspension followed by desalting. When the salting-out step is impractical, extra care must be devoted to the screening and bracketing steps.

When the sample is not limited, but the salt and protein concentrations are low, a preconcentration step may be useful. The 4 x 50 mm ProPac PA1 (P/N 039657) can be used as a concentrator column for this purpose. After concentration of the sample, replace the injection loop with the loaded concentrator column so that the eluent flows in the opposite direction of the concentration flow.

### 3.6 Screening For Initial Conditions

In order to elute anionic proteins from anion exchange columns, ions that compete with the protein for binding sites on the column must be present. The two most commonly employed variables used to control this competition are concentration of salt and pH of solution. As mentioned above, any salt that does not impair detection is acceptable, although chloride is most commonly used.

When starting to determine the best conditions for your application, use a pH that is 1.5 to 2 pH units higher than the pI of your protein. If the pI is not known, begin with the highest pH that permits protein or enzyme stability. Then reduce the pH until the protein elutes in a sharp peak when the salt gradient is applied.

Since proteins vary widely in their ability to bind to ion exchange resins, salt gradients are used to elute them. Good efficiencies are obtained with salt concentration changes as high as 50 mM per mL and with flow rates up to 2 mL/min, using the ProPac PA1. Therefore, determining the right salt concentration gradient to best resolve your protein on the ProPac PA1 can be accomplished quickly. For example, a screening gradient of 0–1 M NaCl would take less than 15 min, including pre-equilibration (12 mL) and a 6 mL isocratic hold at 1 M NaCl.

When your protein elutes in the first half of the rapid gradient, repeat the run using from 0 to half of the concentration of salt initially used. Similarly, if your protein elutes in the last half of the gradient, repeat the run starting at from 30 to 40% of the final concentration of salt initially used. After 2–3 steps of this screening process, reduce the flow rate to 0.9 to 1.2 mL/min and lower the salt concentration slope to about 4–15 mM/mL.

### 3.7 Bracketing Your Peak

At this point, you must consider what your goal is. If you wish to analyze for the presence of a protein in a sample, you will probably want to maximize throughput and detection sensitivity. However, if you wish to purify the protein from a mixture of similar proteins, maximizing resolution and capacity will be more important.

Both throughput and resolution can be enhanced by running a program that elutes the protein(s) of interest in a shallow gradient segment between two steep gradient segments. This allows fast removal of proteins that do not elute near, and high resolution analysis of the proteins that do elute near, the protein you are assaying.

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### 3.8 Enhancing Detection Sensitivity and Selectivity

The most general mode of detection that confers some selectivity is absorption of UV light at 280 nm. For proteins that contain Tyr, Phe, and/or Trp residues, this detection mode will allow detection of just under 1 pg of protein. If your protein does not contain any of these residues or if you require greater sensitivity, absorbance at 235 nm can be used with Tris (Sigma Cat. #T- 1503) buffer, and NaCl as salt on the ProPac PA1 column. Use of other salts or buffers may result in 'system' peaks due to the presence of contaminating ions. This problem becomes more pronounced as the detection wavelength decreases. Detection sensitivity is best at 210 nm, but few eluents are available in adequate purity to elute proteins without substantial baseline disturbances. Sodium phosphate will buffer at  $\text{pH } 6.8 \pm 0.7$ , and contributes little or no absorbance, but may harbor contaminants that produce system peaks. However, please experiment, for we have not exhaustively examined commercially available buffers with the ProPac PA1.

Sometimes it is useful to add a buffered reaction mixture directly to the column eluent. Such a solution, containing substrate and cofactors, mixed with the eluent allows the formation of detectable product only in the presence of enzyme activity. Since this is usually temperature, time and concentration dependent, a temperature controlled reaction coil may be required. The products generated from enzyme-substrate reactions may be fluorescent, cause a pH change, or absorb light at wavelengths where few proteins absorb. This increases the options for sensitive and selective detection of your proteins. Post-column reagent delivery systems that require only modest air pressure to operate (P/N 037460), and appropriate reactions coils (P/N 039349) are available from Dionex.

### 3.9 Enhancing Capacity

If your goal is to purify a protein for subsequent analysis, the most effective way to enhance the effective loading capacity of the ProPac PA1 column is to minimize the presence of similar unwanted protein.

To accomplish this, wet chemistry and preliminary chromatography on media that does not employ ion exchange, are recommended. Examples of wet chemistry procedures are ammonium sulfate fractionation and acid precipitation. Non-ion exchange chromatographic methods include size-exclusion, reversed phase and hydrophobic interaction chromatography. Chromatofocusing may also be useful. These techniques can remove a very large fraction of the sample protein, enriching the protein of interest.

When the sample to be purified is loaded onto the column, it should be washed with a buffer having an ionic strength just below that which elutes your protein. This will elute proteins that might otherwise contaminate your purified preparation. After this wash, a shallow gradient is applied. If chloride is the gradient anion, we recommend a chloride concentration slope of about 2 mM/mL. This slope has resulted in separation of proteins differing only by the presence of bound iron, or sialic acid containing glycans.

Proteins thus purified can be concentrated by a variety of methods, so the dilution that occurs during purification is probably unimportant. However, if conditions dictate that dilution is of major importance, steeper salt gradient slopes or lower flow rates can certainly be used.

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## SECTION 4 - TROUBLESHOOTING GUIDE

The purpose of the Troubleshooting Guide is to help you solve operating problems that may arise while using the ProPac PA1 column. For more information on problems that originate with the pumps or detectors refer to the troubleshooting manuals provided with those devices. If you cannot solve the problem on your own, call the Dionex Regional Office nearest you (see Dionex Worldwide Offices).

### 4.1 High Backpressure

System pressure depends on the column format (4 x 50, 4 x 250, or 9 x 250), flow rate, and eluent viscosity. Typically, the 4 x 50 gives a back pressure less than 600 psi at 1 mL/min, the 4 x 250 exhibits less than 2000 psi at 1.5 mL/min, and the 9 x 250 exhibits less than 1500 psi at 4 mL/min. If the pressure is much higher than this, it is advisable to find out what is causing the high pressure.

- A. Make sure that the pump is set to the appropriate flow rate. Higher flow rates will cause higher pressure. Measure the pump flow rate with a graduate cylinder and a stopwatch if necessary.
- B. Find out what part of the system is causing the high pressure. Check for plugged or collapsed tubing, plugged injection valve ports or tubing-to-connector unions, plugged column bed supports or restricted detector flow cells.

To find out which part of the 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 be more than 50 psi. Reconnect the injection valve only and double check the new pressure. Continue adding components (injection valve, guard column, analytical column and detector) to the flow path until the cause of the high pressure is found. The pressure should increase to 1500–2000 psi when the 4 x 250 column is attached at 1.5 mL/min. No other system component should add more than 200 psi to the backpressure. Refer to the appropriate manual for repair of the problem component.

- C. If the column is the cause of high backpressure, its inlet bed support may be contaminated. To change the bed support, follow the instructions below using one of the two spare bed support assemblies (P/N 042310 for the 4-mm column, 042297 for the 9-mm column) included in the ShipKit.
  1. Disconnect the column from the system.
  2. Using two open-end wrenches (3/4, 5/16 and 1/2 inch are used with ProPac PA1 column hardware), carefully unscrew the inlet (top) column fitting.
  3. Turn the end fitting over and tap it against a benchtop or other hard, flat surface to remove the bed support and seal assembly. Alternatively, when the end fitting is removed, the bed support may be displaced with air or gas pressure applied to the column inlet. Discard the old assembly.
  4. Place a new bed support assembly (P/N 042310 for the 4-mm column, P/N 042297 for the 9-mm column) into the end fitting. Use the end of the column to carefully push the bed support assembly into the end fitting.
  5. Screw the end fitting back onto the column. Tighten it fingertight, then an additional 1/4 turn (25 in./lb.). Tighten further only if leaks are observed.

#### NOTE

**If any of the column packing becomes lodged between the end of the column and the bed support washer assembly, no amount of tightening will seal the column. Make sure that the washer and the end of the column are clean before screwing the end fitting back onto the column.**

6. Reconnect the column to the system and resume operation.

**Replace the outlet end fitting ONLY if high pressure persists after replacement of the inlet fitting.**

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## 4.2 High Background Noise

Typically, high noise problems can be traced to the detector, other chromatograph components or eluent problems. Dirty flow cells, excessive sensitivity to refractive index, optical alignment problems and contaminated eluents that contribute to background absorbance are all fairly common problems. Similarly, contaminated tubing, gradient mixers and guard columns can cause extra noise.

If the excessive noise can be traced to the column, replace the end fittings as described in Section 4.1. If this does not help, see Column Care.

## 4.3 Poor Peak Resolution

Poor resolution may be due to:

### A. Loss of column efficiency.

1. Headspace may have developed in the column (e.g. due to improper use of the column, such as use with organic solvents, or submitting it to very high pressures). Inspect the inlet bed of the column by removing the inlet end fitting (section 4.1). If the resin does not fill the column body all the way to the top, the resin bed has collapsed creating a headspace. The column will need to be replaced
2. Extra column effects could result in sample band dispersion, making the peaks look less efficient. Make sure that you are using 0.010 inch or narrower i.d. tubing and that the tubing lengths are minimized.

### B. Column overloading.

1. Poor resolution is also indicative of sample overload, suboptimal pH or gradient composition and improper sample preparation. Overload can be caused by application of samples high in salt concentration or by sample preparation that employs anion exchange methods.

Preparation by anion exchange chromatography will generate a sample containing proteins that have similar anion exchange properties. Because that is the primary mode of separation on the ProPac PA1, the proteins thus obtained will probably elute near to one another, or may form a broad band of peaks on the ProPac PA1.

2. Elution of milligram quantities of protein in one peak will cause band broadening. This may, or may not, be important. If you need to resolve peaks that elute near to one another, apply less protein. If your purpose is to purify one of a pair of closely spaced proteins, reduce the gradient salt concentration slope and/or flow rate or try changing the pH.

### C. Shorter retention times where peaks elute too fast, compromising resolution.

1. Excessive flow rates. Measure the flow rate at the column outlet with a graduated cylinder and a stopwatch.
  2. Incorrect eluent concentration or pH. Gradient salt slopes may not reflect what you programmed into the system. Salt concentrations can often be measured with handheld refractometers. Double check your gradient salt concentration and buffer pH. If your system is proportioning the eluent components from 3 or 4 reservoirs, the resulting eluent composition might not be accurate. This is more likely when one of the components is proportioning at less than 10%. Try reducing the concentration of buffer or salt in the offending eluent reservoir and increasing the range of percentage proportioned to produce the same salt or buffer concentrations. Alternatively, try proportioning between two eluents.
  3. Column contamination can lead to a loss of column capacity (e.g. when ion exchange sites are consumed by strongly bound anions). The contaminants usually come from chemical impurities in the buffers and salts used in the eluents. Use deionized water with a specific resistance of 18.2 megohm-cm and high purity reagents. The
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ProPac PA1 column can be regenerated as described in Column Care. After regeneration, install the column in the system and equilibrate it with your gradient program. The original column capacity should be restored by the column cleanup treatment.

#### 4.4 Spurious Peaks

Spurious peaks may be due to:

A. Eluent contamination.

1. Since proteins are eluted from the ProPac PA1 by gradients, ionic eluent contaminants can bind to the column at low salt concentration, and elute as the salt concentration increases. This problem is very common and becomes more pronounced as the detection wavelength is decreased below 250 nm.

B. Baseline upsets.

1. When a leaky injection valve or column switching valve is activated, a baseline disturbance can appear. This baseline upset appears as a peak of varying size and shape. It happens when the valve needs to be cleaned or adjusted. If cleaning and torquing of the valve fittings does not help, replace or rebuild the valve.
  2. Baseline disturbances at the end of gradients are common, and frequently caused by elution of contaminants and by sudden changes in salt concentration. These sudden changes may cause refractive index anomalies that most optical detectors cannot correct. Because these rarely occur during the part of the gradient where sample components elute, they can usually be ignored.
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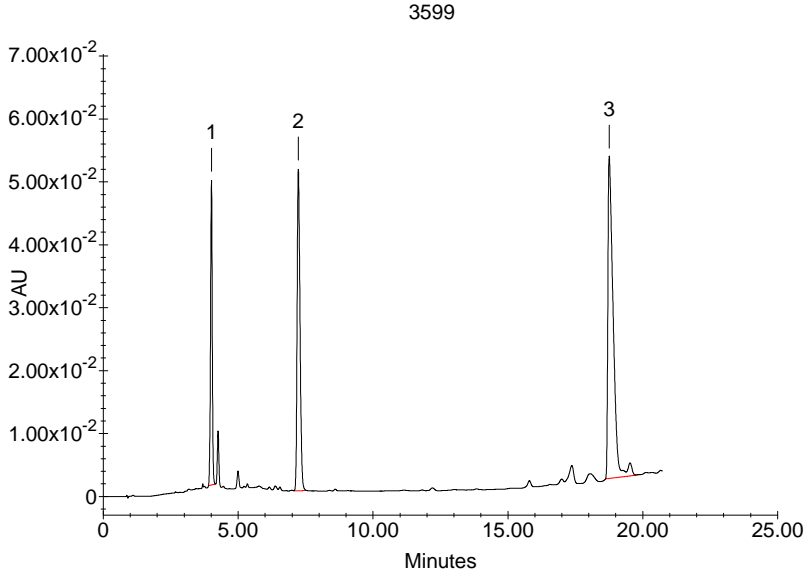


**ProPac™ PA1**  
**Analytical (4 x 250 mm)**  
**Product No. 39658**

Serial No. : 3599

Pressure (PSI) : 1410

Date : 7/17/00 9:25:20 AM



**Eluent:** E1: Deionized Water  
 E2: 100 mM TRIZMA BASE  
 E3: 1,000 mM NaCl

**Gradient:**

Time	%E1	%E2	%E3	V5	V6	Comment
0	79	20	1	on	off	Start
5	0	20	80	on	off	Wash #1
10	79	20	1	on	off	
15	0	20	80	on	off	Wash #2
20	79	20	1	on	off	
25	0	20	80	on	off	Wash#3
30	79	20	1	on	off	Init. Conditions&Equilibrate
37.6	79	20	1	off	off	Load Sample
38	79	20	1	on	off	Inject
58	0	20	80	on	off	Ramp in 20 min.
60	0	20	80	on	off	Hold 2 min.
60.1	79	20	1	on	off	Leave each column in low salt
68.0	79	20	1	on	on	

**Flow Rate:** 1.5 mL/Min.

**Standard:** 3.0 nMol Angiotensin II;  
 5.6 nMol Met-Enkephalin  
 3.2 nMol Oxidized A-chain of Insulin  
 in 50 µL H<sub>2</sub>O

**Detection:** Absorbance 280 nm

**Storage Solution:** E2

Peak Information : Found Components

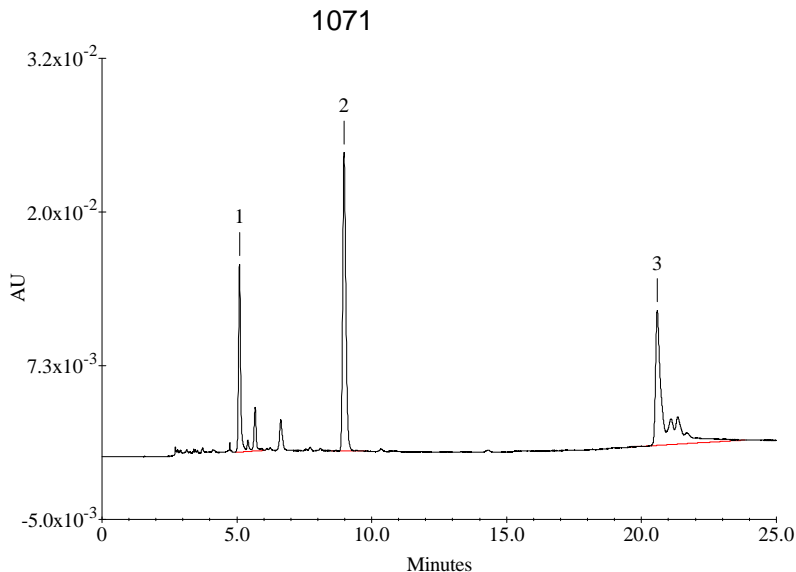
Peak No.	Retention Time	Name	(nmol)	Efficiency	Asymmetry (10%)	Resolution
1	4.01	Angiotensin	3.0	18180	0.9	20.00
2	7.23	Met Enkephalin	5.6	20111	1.6	41.61
3	18.75	Insulin A-Chain	3.2	45601	3.7	n/a

**ProPac™ PA1**  
**Semi Prep (9 x 250 mm)**  
**Product No. 40137**

Serial No. : 1071

Pressure (PSI) : 1280

Date : 8/17/99 8:41:57 AM



**Eluent:** E1: Deionized Water  
 E2: 100 mM TRIZMA BASE  
 E3: 1,000 mM NaCl

**Gradient:**

Time	%E1	%E2	%E3	V5	V6	Comment
0	79	20	1	on	off	Start
5	0	20	80	on	off	Wash #1
10	79	20	1	on	off	
15	0	20	80	on	off	Wash #2
20	79	20	1	on	off	
25	0	20	80	on	off	Wash#3
30	79	20	1	on	off	Init. Conditions&Equilibrate
37.6	79	20	1	off	off	Load Sample
38	79	20	1	on	off	Inject
58	0	20	80	on	off	Ramp in 20 min.
60.0	0	20	80	on	off	Hold 2 min.
60.1	79	20	1	on	off	Leave each column in low salt
68.0	79	20	1	on	on	

**Flow Rate:** 4.0 mL/Min.

**Standard:** 15 nMol Angiotensin II;  
 28 nMol Met-Enkephalin  
 16 nMol Oxidized A-chain of Insulin  
 in 50 µL H<sub>2</sub>O

**Detection:** Absorbance 280 nm

**Storage Solution:** E2

Peak Information : Found Components

Peak No.	Retention Time	Name	(nmol)	Efficiency	Asymmetry (10%)	Resolution
1	5.10	Angiostein II	15.0	22515	1.2	21.75
2	8.97	Met Enkephalin	28.0	26376	1.6	44.19
3	20.58	Insulin A-Chain	16.0	72419	2.7	n/a

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## APPENDIX B - COLUMN CARE

### B.1 Recommended Operation Pressures

Operating above its recommended pressure limit can cause irreversible loss of column performance. The maximum recommended operating pressure for the ProPac PA1 is 4000 psi (-270 atm).

#### NOTE

**The polymeric packing used in the ProPac PA1 column is damaged by organic solvents. The use of acetonitrile and/or methanol at concentrations greater than 2%, and the use of more hydrophobic solvents than these, will void the ProPac PA1 column warranty. Because anionic detergents can also mask the Ion exchange sites, these are also contraindicated. Cationic detergents can also mask some of the ion change sites. Some cationic detergents can be used without incurring permanent damage. Consult the nearest Dionex Regional Office (see “Dionex Worldwide Offices”) for further instruction if you feel that you must use cationic detergents.**

### B.2 Column Start-up

The column is shipped in eluent (20 mM Tris pH 9.6) as storage solution. This eluent is the same one shown on the test chromatogram. Prepare the eluent listed on the test chromatogram, install the column in the system and test the column performance under the conditions described therein. Let the column complete one gradient cycle before applying the sample. Consecutive injections of the standard should give reproducible results. The column can be used with other eluents as discussed in Section 3 of this manual. Remember to equilibrate the column with the new eluent.

### B.3 Column Storage

The column's storage solution should be the same as the eluent used for the test chromatogram (20 mM Tris pH 9.6). If the column will not be used for more than one week, prepare it for long term storage. Flush the column with the test chromatogram eluent (the initial condition, before gradient initiation). Cap both ends securely, using the plugs supplied with the column.

### B.4 Column Cleanup

The ProPac PA1 Column can be cleaned with successive 25 column volume washes of 1 M HCl, deionized water, 1 M NaOH, and deionized water. The deionized water should have a specific resistance of 18.2 megohm-cm. Then, equilibrate the column by running two or more salt gradient programs without sample application.

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