POROS® HQ and PI
Perfusion Chromatography®
Columns for Anion Exchange
Chromatography

AB Applied Biosystems

Operating Instructions

NOTE: *These instructions cover the specific operational characteristics of POROS® prepacked columns. POROS media are also available in bulk quantities for direct scale-up of separations developed on prepacked columns. Contact your Applied Biosystems representative for more details.*

Your New POROS Column Is Unique!

Read this section before doing anything!

Applied Biosystems POROS columns are made for Perfusion Chromatography® flow-through particle chromatography—a patented new technology which performs bioseparations 10 to 100 times faster than conventional HPLC or LC without loss in capacity or resolution.

Although POROS columns can be operated on standard HPLC instrumentation, they are substantially different from any columns you have used before. You may have to change the way you run, and, to some extent, the way you think about chromatography. In particular, the higher flow rates made possible by Perfusion Chromatography allow you to perform experiments you might once have considered a luxury, given the constraints of conventional chromatography's longer run times.

Please read the operating instructions carefully to ensure that you run the column to its full capability and take maximum advantage of the benefits that Perfusion Chromatography technology provides.

Increase the Flow Rate

The largest single difference between POROS columns and conventional columns is the flow rates under which high capacity and resolution are achieved.

Although you can operate the column at flow rates typical of conventional chromatography, you can realize the full benefits of Perfusion Chromatography only by increasing the flow rate so that the linear velocity is in the range of at least 1,000 to 5,000 cm/hr. Higher flow rates are possible but may not be practical, depending on the system you are using, the viscosity of your solvents, or the pressure rating on the column.

Linear velocity (cm/hr) is calculated by dividing volumetric flow rate (cm^3/min) by the column cross-sectional area (cm^2) and multiplying by 60 min/hr.

NOTE: *If you are operating with a peristaltic pump, you may need to run at lower flow rates to keep within the pressure rating of the pump.*

The typical volumetric flow rate ranges for various column diameters are listed in the following table.

Table 1   Typical Flow Rates for Perfusion Chromatography

Column Diameter (mm)	Flow Rate Range (ml/min)	Linear Velocity (cm/hr)
2.1	0.5–5	900–8,500
4.6	3–15	1,000–5,300
10	15–75	1,100–5,700
16	30–150	900–4,500

The dramatically higher flow rates of POROS columns and media introduce new considerations into the design and execution of experiments. This is particularly true of adapting a method developed on conventional media.

Be sure to read Section 8, Guidelines for Using Perfusion Chromatography for a full discussion of these considerations. Another excellent reference is The Busy Researcher's Guide to Biomolecule Chromatography, available from your Applied BiosystemsTechnical Representative.

Reoptimize Your Method as Needed

You may need to reoptimize the separation to account for possible differences in selectivity between the POROS column and your old column.

The short run times associated with Perfusion Chromatography make optimization quick and easy, especially if you are using the VISION™ or BioCAD® Workstation for Perfusion Chromatography.

Contents

1   Product Description

POROS HQ and PI columns are polymeric packings designed for anion exchange chromatography of peptides, proteins, polynucleotides and other biomolecules in the Perfusion Chromatography flow-through particle chromatography mode. They consist of crosslinked poly(styrene-divinylbenzene) flow-through particles with a patented bimodal pore size distribution for very rapid mass transport.

POROS HQ media is surface-coated with fully quaternized polyethyleneimine. It is a strong anion exchanger with complete surface ionization over a pH range of 1 to 14.

POROS PI media is surface-coated with polyethyleneimine. It is a weak anion exchanger for which the surface ionization varies continuously over a pH range of approximately 3 to 9. Different selectivities at different pHs can be used to advantage to develop an optimum separation.

POROS HQ and PI column packages include the following items:

- Packed column, with sealing end caps
- Product *Operating Instructions*
- Column Test Certificate
- EZ™ Grip stainless steel fittings

1.1   POROS Column Sizes

POROS anion exchange columns are available in different sizes. Check that the column you have purchased is appropriate for your type of application and is compatible with the pressure limits of the system you are using (see Table 2). See Section 8.1, Account for System Pressure, for a more detailed discussion of pressure considerations for running Perfusion Chromatography columns on conventional systems.

Table 2   Classification of POROS Anion Exchange Columns

Particle Size	Maximum Pressure Drop	Type of Separation	Chromatography System
POROS 10 micron	170 bar (2,500 psi, 17 MPa)	Analytical	BioCAD Workstation, or conventional HPLC instrumentation
POROS 20 micron	170 bar (2,500 psi, 17 MPa)	Preparative	BioCAD Workstation, or conventional HPLC instrumentation

Table 3   Product Characteristics

Support Matrix	Crosslinked poly(styrene-divinylbenzene)

Surface Functionality
    POROS HQ
    POROS PI
	Quaternized polyethyleneimine
	Polyethyleneimine

Dynamic Binding Capacity @ 3,600 cm/hr
    POROS HQ
    POROS PI
	Bovine serum albumin, pH 8.0
	
	55 mg/ml
	45 mg/ml

Counterion as Supplied	SO_4^{2-}
Shipping Solvent	0.1 M Na_2SO_4, 30% methanol
Packing Density	0.35 g/ml
Shrinkage/Swelling	<1% from 1–100% solvent

Table 4   Chemical Resistance

pH Range	1–14 (Up to 5 M NaOH, 1 M HCl)
Ionic Strength	0–5 M, all common salts
Buffer Additives	All common agents suitable, including 8 M urea, 6 M guanidine hydrochloride, ethylene glycol, and detergents. Anionic detergents not recommended, as they can bind. **NOTE:** *Do not expose to strong oxidizers (such as hypochlorite), oxidizing acids (such as nitric), or strong reducing agents (such as sulfite).*
Solvents	Water, 0–100% alcohols, acetonitrile, other common organic solvents.
Operating Temperature	5–80 °C

2   Connecting and Preparing the Column

This section describes how to connect your column to your liquid chromatography system and prepare the column for use.

WARNING: *Always wear eye protection when working with liquid chromatography systems.*

The column fitting is an Upchurch Scientific 10-32 female fitting.

POROS columns come with EZ Grip stainless steel fittings that are designed to be tightened by hand.

NOTE: *For PEEK columns, do not use standard steel fittings that require tightening with a wrench. Overtightening can strip the threads of the column.*

Extra EZ Grip fittings are available from Applied Biosystems (see Section 10, Accessories, Spare Parts and Ordering Information).

Connecting the Column

Columns can be connected to M-6 metric fitting systems (such as the Pharmacia FPLC® system) by using the adaptor kit (see Table 9 on page 7).

1. Connect the short tubing section to the column using the red Fingertight fittings.

2. Slip the black metric nuts over the other end of the tubing, followed by a blue ferrule, with the conical end *pointing toward the nut.*

3. Connect the nut to a female M-6 fitting.

The columns can also be connected to 1/4 -28 fitting systems using the green 1/4-28 male nut in place of the black M-6 nut.

Preparing the Column

POROS anion exchange columns are shipped in 0.1 M Na_2SO_4, 30% methanol. Before you use the column for the first time, pump the column with 5 to 10 column volumes of water to remove the methanol.

To prepare the column for a routine injection:

1. Pump 5 to 10 column volumes of high-salt eluent buffer.

2. Equilibrate with 10 to 15 column volumes of starting buffer.

Unlike the lower flow rates of conventional chromatography, the high flow rates possible with Perfusion Chromatography allow a thorough column equilibration cycle in a matter of minutes.

3 Selecting and Preparing the Starting Buffer

Regardless of the buffer system you choose, it is always important to:

1. Use buffers of the highest purity practical.

2. Degas and filter (0.22 or 0.45 µm) all buffers prior to use.

3.1 Buffer pH

The buffer pH is the most critical variable in ion exchange chromatography.

- Because POROS PI media is a weak anion exchanger, the positive surface charge of the packing increases with decreasing pH while the charge of the protein becomes more positive. These two effects must be balanced for good binding.

- With the strong anion-exchange packing (POROS HQ), the charge of the packing itself is essentially unchanged from pH 1 to 14. This allows operation at high pH for binding of proteins with a very high isoelectric point or surface pKa.

Examine the effect of pH carefully in a systematic screening or mapping experiment. For anion exchangers, examine a pH range of 6 to 9.

The very high flow rates possible with Perfusion Chromatography mean that such an experiment can be completed quickly. The VISION or BioCAD Workstation has a template feature that automatically performs an entire pH mapping study in less than one hour.

3.2 Buffer Ions

Keep this information in mind as you choose your buffer system:

- Buffer ions should be cationic or at least zwitterionic.
- Avoid anionic buffers such as phosphate and borate, because they can bind to the functional groups and cause local changes in pH.
- Keep buffer ion concentration in the range of 20 to 50 mM for POROS HQ columns.
- Use higher buffer concentrations (50 to 100 mM) for POROS PI columns because of the buffering capacity of the column itself.
- After equilibrating the column, check that the pH of the effluent buffer is at the desired value, especially with POROS PI columns.

Table 5   Recommended Cationic Buffers

pH Range	Buffer Ion	pKa
4.5–5.0	N-methylpiperazine	4.75
5.0–6.0	Piperazine	5.68
5.8–6.4	Bis-Tris	6.50
6.4–7.3	Bis-Tris propane	6.80
7.3–7.7	Triethanolamine	7.77
7.5–8.0	Tris	8.16
8.0–8.5	N-methyldiethanolamine	8.54
8.4–8.8	Diethanolamine	8.88
8.5–9.0	1,3-diaminopropane	8.64
9.0–9.5	Ethanolamine	9.50
9.5–9.8	Piperazine	9.73
9.8–10.3	1,3-diaminopropane	10.47

3.3 Additives

You can use POROS HQ and PI columns with most additives:

- Urea, ethylene glycol and nonionic or cationic detergents. Avoid anionic detergents, because they bind tightly to the packing.
- Adding up to 30% alcohol or acetonitrile can be a very useful technique for peptides or small proteins which may not be fully soluble or which bind by mixed ionic/hydrophobic interactions in 100% aqueous eluents.

4 Preparing and Loading the Sample

To ensure efficient binding and prevent column plugging, it is important to:

1. Dissolve or exchange samples in the starting buffer, if possible.

2. Centrifuge or filter samples (0.22 or 0.45 µm) prior to injection.

3. Delipidate samples, if possible. Lipids can cause irreversible fouling.

4.1 Determining the Sample Load

The dynamic binding capacities of POROS HQ and PI are listed in Table 3.

In general, high-resolution separations are generally achieved at 20% of the total binding capacity or less. However, the maximum loading at which a given resolution can be obtained (the loadability) is dependent on a number of factors, including sample solubility, column selectivity, and so on.

The long run times associated with conventional chromatography usually prohibit a systematic determination of loadability. Your POROS column's shorter run times make it easy to perform this determination.

Perform a loadability experiment as follows:

1. Determine the most effective elution conditions (eluent, gradient, and flow) at low loading.

2. Gradually increase the sample load (either through increasing injection volume or sample concentration or both) until the required resolution no longer occurs.

3. If you are using a VISION or BioCAD Workstation, take advantage of the loading study template designed specifically for this purpose.

NOTE: As the loading is increased, the peaks may elute earlier on the gradient, possibly necessitating reoptimization of the gradient.

Once again, the short run times made possible by Perfusion Chromatography make this quick and easy, especially if you use the template features of the VISION or BioCAD Workstation.

5   Eluting the Sample

To elute, increase the ionic strength of the starting buffer.

- NaCl or KCl are the most commonly used salts for elution, although sulfate, formate, or acetate salts can also be used.
- Up to 1.0 M ionic strength elutes most proteins.
- Use up to 2.0 M ionic strength to remove very tightly bound proteins or for column cleanup (see below).
- Ionic strength changes may be either by step or continuous gradients.
- Gradient volumes of 10 to 20 column volumes normally provide a good compromise between resolution and peak dilution.

Note that with the very high flow rates possible with Perfusion Chromatography, increased gradient volumes (with decreased slope and therefore increased resolution) are possible without the excessive times normally experienced with conventional media.

6   Cleaning Up and Regenerating the Column

In some applications, sample molecules may not fully elute or may precipitate on the column. Regenerate the column if these symptoms appear:

- Loss of resolution
- Loss of binding capacity
- Loss of recovery
- Increased pressure drop
- "Ghost" peaks during blank gradient runs

NOTE: In any cleanup method, reversing the flow direction is recommended to help flush out particulates and to prevent contamination of the lower part of the bed. Also, slow the flow rate to give several minutes' exposure time to the regeneration solution at each step of the cleaning protocol.

Simple Regeneration

Wash with 1 to 5 column volumes of 1 to 2 M of the salt used for elution.

If simple regeneration does not restore column performance, a more complex regeneration is required.

Complex Regeneration

To regenerate more completely:

1. Wash with 1 to 5 column volumes of 1.0 M NaCl, 1.0 M NaOH.

2. Wash with water to remove base.

3. Wash with 1 to 5 column volumes of 1.0 M acetic or hydrochloric acid (or 1% TFA).

4. Wash with water to remove the acid.

5. Reequilibrate the column with starting buffer, 1 M NaCl.

6. Reequilibrate with starting buffer.

To remove fouling lipids or lipoproteins, try one of these approaches:

- Use a mixture of 50% methanol or acetonitrile with the acid or base.
- Use a mixture of 50% methanol and 50% 3 M guanidine thiocyanate.

  NOTE: Take care when using thiocyanate with metal systems. Thiocyanate forms complexes with iron that strongly absorb UV light.

- Store the column overnight in 1 mg/ml pepsin, DNAse, or other enzymes.

Multiple Injections

It is possible to use multiple injections of regeneration solutions instead of pumping them directly. This method is recommended for very aggressive or very viscous solvents.

To clean by injections:

- Make the injection volume as large as possible.
- Use a low flow rate that allows at least several minutes exposure time to the regeneration solution.

NOTE: Backpressure increase is sometimes caused by a plugged inlet frit. If backflushing the column fails to solve the problem, replace the inlet frit. See Section 10, "Accessories, Spare Parts and Ordering Information," for details.

7   Storing the Column

When you store the column, always be sure to:

- Store the column between 5 and 30 °C.
- Store the column with the end plugs in place, carefully sealed to prevent drying. Drying results in decreased chromatographic efficiency.

Short-Term Storage

Store the column for short periods in any appropriate buffer.

Long-Term Storage

Flush the column with 1 M NaCl, followed by water with either 0.02% sodium azide or 30% alcohol.

WARNING: Sodium azide is toxic. Follow precautions and decontamination procedures recommended by the National Institute for Occupational Safety and Health.

8   Guidelines for Using Perfusion Chromatography

There are a few simple but important things to keep in mind when you make the transition to Perfusion Chromatography. They can be grouped into two general categories:

Related to the Chromatography System:

- Account for System Pressure
- Check the Gradient System
- Adjust the Data Collection System
- Maintain Your Column and System

Related to Experimental Design:

- Think in Terms of Column Volumes
- Adjust the Sample Load
- Measure Recovery Properly

8.1   Account for System Pressure

The high flow rates used with Perfusion Chromatography cause a higher-than-usual system pressure (resulting from the chromatography hardware itself). In some cases, this system pressure can be equal to or even greater than the column pressure.

Therefore, when you use your POROS column, you cannot simply set the upper pressure limit of the system at the pressure rating of the column. Instead:

1. Determine the system pressure by:
   - Connecting a union in place of the column
   - Pumping the highest salt concentration to be used at the planned flow rate

2. Set the upper pressure limit by adding the system pressure observed above to the column pressure rating.

If the system pressure is too high:

1. Check carefully for plugged or crimped tubing or other restrictions in your plumbing.

2. Use larger ID or shorter tubing.

3. Use a larger detector flow cell.

In some systems, excessive system pressure can preclude the high flow rates required to take full advantage of Perfusion Chromatography technology.

It is important to isolate the relative contribution of the column and instrument when pressures approach the maximum column pressure. Take the column out of line to determine those contributions (measured pressure = column pressure drop + system pressure). The maximum allowable pressure drops of POROS columns are listed in Table 2.

Typical pressure drops of POROS columns (at 2,000 cm/hr with water as the mobile phase) are shown in Table 6. Use this table to verify column performance or to help you decide if column regeneration is required.

Table 6   Typical Column Pressure Drops
Pressure: Bar (psi) (1 bar = 0.1 MPa)

Column	30 mmL	50 mmL	100 mmL
POROS 10	40 (600)	60 (900)	80 (1,200)
POROS 20	20 (300)	30 (450)	40 (600)

8.2   Check the Gradient System

High flow rates and short run times can expose both operational and design problems in gradient blending systems. Gradient system problems can affect step changes as well as linear gradients. Most problems come from one of two sources:

- Excessive delay (dwell) or mixing volume can cause both delay in the start of the gradient at the column and rounding or distortion of the edges of the gradient. Mixing or delay volume can be reduced by using a smaller mixer and shortening the tubing between the mixer and sample injector.

- Poor gradient proportioning can cause either short-term fluctuations or long-term inaccuracies. Adding a mixer can sometimes help.

On the VISION or BioCAD Workstation, gradient performance is tracked on each run with the internal conductivity detector. If there is any question about gradient performance on other systems, the gradient can be visualized as follows:

1. Connect a union in place of the column.

2. Form a gradient with water as the A solvent and 0.5% acetone in water as the B solvent with detection at 280 nm.

   The UV absorbance is directly proportional to the concentration of B solvent and can be compared to the programmed gradient.

Consult your system vendor for serious gradient problems.

8.3   Adjust the Data Collection System

Because Perfusion Chromatography runs are much shorter than conventional chromatography runs, you may need to adjust your data collection system as follows:

- To obtain high-definition chromatograms, use a shorter total run time and higher data collection rate (or lower average peak width parameter). A typical data collection rate is 10 points/second.

- If you use a chart recorder, increase the chart speed in proportion to the flow rate increase.

8.4   Maintain Your Column and System

Perfusion Chromatography enables you to perform runs more quickly than other chromatography technologies. For this reason, perform maintenance tasks such as replacing filters or regenerating columns after a certain number of runs, rather than after a set period of time. You can reduce the frequency of such maintenance by always filtering the sample and eluent.

8.5   Think in Terms of Column Volumes

In any chromatographic separation, as flow rate increases, gradient time must decrease to maintain constant gradient volume. At the flow rates used for Perfusion Chromatography, the gradient times are dramatically shorter than those you are accustomed to working with. To convert a method to Perfusion Chromatography, keep the gradient volume constant in terms of column volumes and adjust the time of the gradient according to the new flow rate. Table 7 on page 6 provides bed volumes of POROS columns to help you make the necessary calculations.

Table 7   POROS Prepacked Columns

Diameter (mmD)	Length (mmL)	Column Bed Volume (ml)
2.1	30	0.10
2.1	100	0.35
4.6	50	0.8
4.6	100	1.7
10	100	7.9
16	100	20.1

When you work routinely with Perfusion Chromatography, always think of gradients in terms of column volumes, since a slight change in gradient time may result in a dramatic difference in gradient volume and column performance.

For example, on a conventional 4.6 × 250 mm analytical column (volume 4.25 ml), a 45-minute run at 1 ml/min represents a 10.6 column volume gradient.

On a POROS 4.6mmD/100mmL column (volume 1.7 ml), a 5 ml/min flow rate translates into 3 column volumes/min (5/1.7). Therefore, a 10.6 column volume gradient would be completed in 3.5 minutes.

The VISION and BioCAD Workstations allow you to program directly in column volumes.

8.6   Adjust the Sample Load

If the volume of your POROS column is different from the column you are currently using, adjust the sample volume or mass proportionally to keep the same load per unit volume of column. See Table 7 for POROS column bed volumes to help you make the necessary calculations.

8.7   Measure Recovery Properly

Quantitation (recovery) measurements using peak integration are comparable run-to-run only if the conditions are kept nearly constant.

Flow rate affects the value of the integrated peak area for a given mass recovered, because the amount of material recovered in a peak is equal to the concentration (absorbance) times the volume. However, an integrator (or integration software) quantitates peaks by summing absorbance measurements over *time*. A change in flow rate is a change in the amount of volume over time.

Therefore, time integration does not allow comparison of recovery at different flow rates. Direct comparison of your POROS results with previous results on conventional chromatography may lead to the incorrect conclusion that recovery is lower on POROS columns.

There are several ways to compensate for limitations in time-based integration:

- Multiply the peak area by the flow rate. Unfortunately, because integrators often vary the data rate with expected peak width, this approach can give invalid results.
- Use peak height for a general comparison, but bandspreading differences due to different efficiencies or gradient slopes will cause errors.
- Use peak integration at the same flow rate, but *doing this may not show important effects such as a recovery increase due to shorter time on the column with Perfusion Chromatography.*

- Collect the peaks carefully and analyze using spectrophotometry or other methods. Make sure to develop blanks, especially when UV-absorbing components are present in the eluent.
- If peak collection is not feasible, generate standard curves by injecting increasing amounts of calibration standards of known concentration. If the measured peak area increases linearly with load, and the standard curve passes through the origin, it is very likely that recovery is high.

8.8   Run the Test Standard

Run a standard to verify that your system and column are running properly. See Section 9, Standard Test Protocols, for details.

9   Standard Test Protocols

Applied Biosystems has designed standard test protocols for POROS HQ and PI columns. Use this test to troubleshoot column performance, whenever in doubt.

9.1   Chromatographic Efficiency

The Column Test Certificate provided with the column lists the chromatographic efficiency, asymmetry, and permeability for the column. The test uses a small, nonretained molecule run at low flow rate, which gives the most sensitive measurement of the packed column bed. Plate count is determined by the half-height method. For detailed test information, contact Applied Biosystems Technical Support.

9.2   Protein Separation

For the sample, use the Anion Exchange Protein Test Standard available from Applied Biosystems. Run the separation with a linear gradient from 0 to 0.5 M NaCl. Run conditions are given below, including flow rate and sample load for various column sizes.

The test consists of these steps:

1. Dissolve the test standard in 1 ml of Eluent A (final concentration 5 mg/ml bovine serum albumin, 5 mg/ml ovalbumin).
2. Filter the test standard using a 0.22 µ filter.
3. Store unused reconstituted test mix frozen.
4. Run the sample.

### 9.3 Conditions

Table 8   Protocol

These conditions are common to all column sizes:	
Eluent A	50 mM Tris hydrochloride pH 8.5
Eluent B	50 mM Tris hydrochloride pH 8.5, 0.5 M NaCl
Gradient	0–100% B in 5 minutes
Detection	280 nm

Flow rate and sample size depend on column diameter and are listed below:				
Column (mmD)	2.1	4.6	10	16
Flow (ml/min)	1	5	25	50
Sample (µl)	5	20	100	200

9.4 Results

The standard chromatogram shown in Figure 1 was generated on a 4.6 mmD/100 mmL POROS 20 micron HQ column. The retention times and bandspreading may be somewhat different with different packing, particle size, column size, and gradient systems. The general profile should be similar, however.
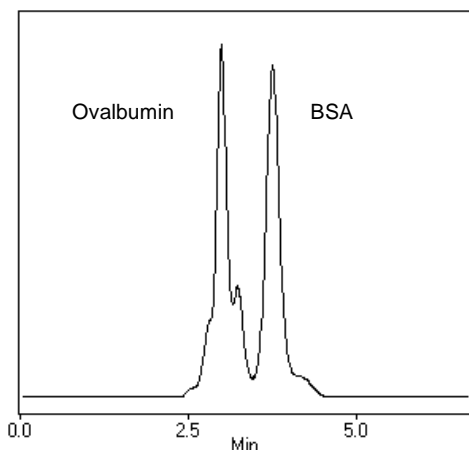
Figure 1 POROS 20 Micron HQ Chromatogram

10 Accessories, Spare Parts and Ordering Information

These accessories are available for your POROS HQ and PI columns:

Table 9   POROS HQ and PI Column Accessories

Description	Quantity	Part Number
Anion Exchange Protein Test Standards	Package of 5 vials	1-9002-05
Frits, PEEK		
2.1mmD	Package of 5	1-9124-05
4.6mmD	Package of 5	1-9125-05
10mmD	Package of 1	1-9127-01
16mmD	Package of 1	1-9128-01
EZ Grip Fittings (SS)	Package of 5	P5-1011-05
Fitting Adaptor Kit		1-9532-00
The Fitting Adaptor Kit lets you connect POROS columns to M-6 (FPLC) and 1/4-28 low-pressure fitting systems. The kit includes two 10-32 fittings, two low-pressure ferrules, two M-6 nuts, two 1/4-28 nuts and 1/16–inch OD PEEK tubing.		

Column parts are available from Applied Biosystems on special order. Please inquire.

11 Technical Support

Applied Biosystems is dedicated to helping you use Perfusion Chromatography and POROS media to the fullest extent possible. Our biochromatographers, bioprocess engineers, and applications development laboratories are available for support ranging from telephone consultation to full-scale method development.

Applied Biosystems also offers a full line of other POROS media for Perfusion Chromatography in the reversed-phase, ion-exchange, affinity, and other chromatographic modes. Please contact your Applied Biosystems representative for technical and ordering information.

Applied Biosystems publishes a continuing series of Application and Technical Notes highlighting specific purification problems and technical aspects of Perfusion Chromatography. Please contact Applied Biosystems directly for a publication list.

For further details or for answers to questions on POROS HQ and PI columns, Perfusion Chromatography, or other products, please contact Applied Biosystems. See the back page of this document for contact information.

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