POROS™ 50 HE Perfusion Chromatography™ Columns for Affinity Chromatography

Pub. No. 4470890 **Rev.** B

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

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.*

Read this section before you begin

Your New POROS™ Column Is Unique

Thermo Scientific™ POROS™ columns are made for Perfusion Chromatography™ flow-through particle chromatography—a patented new technology that 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.

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™ media and classical media is the flow rates at which experiments are run.

Although POROS™ media can be operated without problems at flow rates typical of classical chromatography, increasing the flow rate to between 500 and 1000 cm/hr allows maximum productivity without the loss of performance.

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.

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

Be sure to read "Guidelines for Using Perfusion Chromatography™ Media" on page 4 for a full discussion of these considerations. Another excellent reference is *The Busy Researcher's Guide to Biomolecule Chromatography* , available from your Thermo Fisher Scientific Technical Representative.

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	0.1–2.0	100–2000
4.6	0.3–6.0	100–2000
10	1.0–30	100–2000
16	3.0–60	1000–2000

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 itself.

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 reoptimization quick and easy, especially if you are using the BioCad™ Workstation for Perfusion Chromatography™.

The following chemicals are used in these operating instructions. Read the MSDS for each chemical, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

DANGER! CHEMICAL HAZARD. Guanidine thiocyanate causes eye burns and can cause skin and respiratory tract irritation. It is harmful if absorbed through the skin or swallowed. Contact with acids and bleach liberates toxic gases. DO NOT ADD acids or bleach to any liquid wastes containing guanidine thiocyanate.

CAUTION! CHEMICAL HAZARD. May irritate eyes, skin, and respiratory tract:

- Heparin – May be harmful if swallowed and may cause blood damage
- Tris (hydroxymethyl)aminomethane (TRIS)
- Urea

WARNING! CHEMICAL HAZARD. Flammable liquid and vapor. Exposure may irritate eyes, skin, and upper respiratory tract and depress the central nervous system:

- Acetone – Prolonged or repeated contact may dry the skin. Keep away from heat, sparks, and flame.
- Acetonitrile (ACN) – Exposure may damage the heart, blood system, liver, and kidneys.
- Ethanol – Prolonged or repeated contact may dry the skin. Exposure may damage the liver. Keep away from heat, sparks, and flame.
- Methanol – Exposure may cause blindness.

WARNING! CHEMICAL HAZARD. Exposure may irritate eyes, skin, and respiratory tract and depress or adversely affect the central nervous system. May be harmful if swallowed:

- Ethylene glycol – Exposure may damage the kidneys.
- Guanidine hydrochloride – May be harmful if absorbed through the skin. Exposure may adversely affect bone marrow.

Product Description

POROS™ HE columns are designed for analytical and preparative affinity purification of coagulation proteins, lipoproteins, restriction endonucleases, and nucleic acid polymerases using Perfusion Chromatography™ technology. The packings consist of

cross-linked poly(styrene-divinylbenzene) flow-through particles with a patented bimodal pore size distribution for very rapid mass transport. The particle surface is coated with a cross-linked polyhydroxylated polymer. This coating is further derivatized with heparin functional groups.

POROS™ HE column packages include the following items:

- Packed column, with sealing end caps
- *Product Information Sheet*
- Column Test Certificate
- Fitting Adaptor Kit
- EZ™ Grip stainless steel fittings

POROS™ Columns Series and Sizes

POROS™ HE (Heparin) columns are available in various performance series and 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). Refer to "Account for System Pressure" on page 4 for a detailed discussion of pressure considerations for running Perfusion Chromatography™ columns on conventional systems.

Table 2 Product characteristics

Support Matrix	Cross-linked poly(styrene-divinylbenzene)
Immobilized Ligand	Heparin
Dynamic Binding Capacity @ 3,600 cm/hr	Lysozyme, pH 6–7 15 mg/mL
Particle Size	50 µm
Shipping Solvent	0.1 M phosphate pH 7.0, 20% ethanol
Shrinkage/Swelling	<1% from 1–100% solvent
Recommended maximum flow rate	2000
Maximum pressure drop	103 bar (1500 psi, 10 MPa)
Permeability	<3 bar at 1000 cm/hr (10 cm bed height)

Table 3 Chemical resistance

pH Range	5–10
Ionic Strength Range	0–5 M, all common salts
Buffer Additives	All common agents, including 8 M urea, 6 M guanidine HCl, ethylene glycol, and detergents. Agents that may degrade the ligand not recommended.
Solvents	0–100% water, alcohols, acetonitrile, other common organic solvents. **Note:** Do not expose to strong oxidizers (such as hypochlorite), oxidizing acids (such as nitric), or strong reducing agents (such as sulfite).
Operating Temperature	5–40 °C

Connecting and Preparing the Column

The column fitting is Parker 10-32 female.

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 **thermofisher.com**.

Connecting the Column

Columns can be connected to M-6 metric fitting systems (such as the Pharmacia FPLC™ system) by using the fitting adaptor kit provided.

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™ HE columns are shipped in 0.1 M phosphate pH 7.0, 1 M NaCl, 20% ethanol. Before you use the column for the first time, pump the column with 5 to 10 column-volumes of high-strength eluent buffer.

To prepare the column for a routine injection, pump the column with 10 to 15 column-volumes of starting/wash 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.

Selecting and Preparing the Starting Wash Buffer

Regardless of the starting/wash buffer system you choose, always:

- Use buffers of the highest purity practical.
- Degas and filter (0.22 or 0.45 µm or less) all buffers before use.

Follow these guidelines when you select and prepare the starting/wash buffer:

- In most cases, a simple buffer like 10 to 25 mM phosphate or Tris can be used.
- The starting/wash buffer pH can range from 6.0 to 9.0, although binding is usually strongest near neutral pH.
- Heparin is negatively charged and acts as a cation exchanger. Add salt (0.1 to 0.2 M NaCl or KCl) to prevent non-specific adsorption due to heparin/protein interactions.

Preparing and Loading the Sample

To ensure efficient binding and prevent column plugging:

- Dissolve or exchange samples into the starting/wash buffer, particularly for large samples (>25% of the column volume).
- Centrifuge or filter samples (0.22 or 0.45 μm or less) before injection.
- Heat-treat serum samples (56 °C for 30 minutes) to remove residual fibrinogen that can clog the column on multiple runs.
- Delipidate samples, if possible. Lipids can cause irreversible fouling.

Determining the Sample Load

The dynamic binding capacity of POROS™ HE media listed in Table 2 for the test protein lysozyme approximates the binding capacities expected under experimental conditions for most proteins.

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.

To perform a loadability experiment:

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.

If you use a BioCad™ Workstation, take advantage of the loading study template designed specifically for this purpose.

Note that 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 BioCad™ Workstation.

Concentrating Dilute Samples

The binding of protein to POROS™ HE columns and the resulting elution peak depend on the total mass, not the concentration, in the sample. This is because of the very high binding constants inherent in the protein-ligand interaction. Therefore, the column can concentrate very dilute samples such as cell culture supernatants.

Because of the high flow rates possible with Perfusion Chromatography™, concentration of a dilute sample does not take much time. On analytical 2.1 mmD/30 mML columns, sample sizes can be as large as 2 to 3 mL (20 to 30 column-volumes) or more and still give good results in 2 to 5 minutes or less.

Wash and Elution Protocols

Elution is usually carried out with an increasing concentration of salt.

- NaCl or KCl are the most commonly used salts for elution, although sulfate salts can also be used.
- Although most proteins elute with less than 1.5 M of salt, you may use up to 4 M or 5 M, if necessary.
- You can also elute by adding heparin (1 to 5 mg/mL) in the starting/wash buffer.

After loading the sample, follow these steps:

1. Wash the column with the starting/wash buffer.

   Generally a 5 to 15 column-volume wash is sufficient to remove all unbound proteins from the column. Samples with high contaminant concentrations, however, may require a longer wash to return to a stable baseline.
2. Use 5 to 15 column-volume steps to elute with the chosen eluent and to reequilibrate with the starting/wash buffer.

   You can also use gradient elution.

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 you observe:

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

Note: In the cleanup method, reverse the flow direction to help flush out particulates and to prevent contamination of the lower part of the bed. Also, slow the flow rate to expose the column to the regeneration solution for several minutes at each step of the cleaning protocol.

To regenerate the column, wash with 5 to 10 column-volumes of 2 to 3 M salt buffer to remove any remaining proteins.

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

- Use a mixture of 50% methanol or acetonitrile with 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.
- Use 8 M urea, or 6 M guanidine hydrochloride.

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 does not solve the problem, replace the inlet frit.

Storing the Column

When you store the column, always be sure to:

- Store columns in the refrigerator, but DO NOT FREEZE THEM!
- Store the column with the endcaps in place, carefully sealed to prevent drying. Drying results in decreased chromatographic efficiency.

Short-Term Storage

Store columns in any appropriate starting/wash buffer.

Long-Term Storage

Store the column in 0.05% sodium azide as a preservative.

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

Guidelines for Using Perfusion Chromatography™ Media

There are a few important guidelines to keep in mind when you make the transition to Perfusion Chromatography™. The guidelines are categorized as:

The chromatography system

- Account for system pressure
- Check the gradient system
- Adjust the data collection system
- Maintain your column and system

Experimental design

- Think in terms of column volumes
- Adjust the sample load
- Measure recovery properly

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, you need to:

1. Determine the system pressure by connecting a union in place of the column, then 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 in Step 1 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 prevent 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 drop of a POROS™ column is listed in Table 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 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.

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.

Maintain Your Column and System

Perfusion Chromatography™ allows 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.

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 4 provides bed volumes of POROS™ columns to help you make the necessary calculations.

Table 4 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, because 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™ 4.6 mmD/100 mmL 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 BioCad™ Workstation allows you to program directly in column volumes.

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. Refer to Table 4 for POROS™ column bed volumes to help you make the necessary calculations.

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. If you use this approach, take care 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.

Run the Test Standard

Run a standard to verify that your system and column are running properly. Refer to "Standard Test Protocols" on page 5 for details.

Standard Test Protocols

Use this performance-test protocol for POROS™ HE columns to:

- Confirm that the column and hardware are functioning properly in the Perfusion Chromatography™ mode
- Obtain working experience with the column
- Troubleshoot

Chromatographic Efficiency

The Column Test Certificate lists the chromatographic efficiency test method and initial test chromatogram for the column. The test involves running a small, non-retained molecule at low flow rate to obtain the most sensitive measurement of the packed column bed. Plate count is determined by the half-height method.

Protein Separation

You must create the test standard to use for this test (directions are given below). The separation is run using gradient elution.

To prepare the protein test standard:

1. Dissolve 5 mg bovine serum albumin and 5 mg lysozyme in 1 mL of buffer.
2. Filter the test standard using a 0.22-µm filter.
3. Store the unused reconstituted test mix in a freezer.

Table 5 Protocol

Conditions Common to All Column Sizes	
Eluent A	20 mM MES, pH 5.5
Eluent B	1 M NaCl in A
Flow rate	5 mL/min for FPLC™ system 10 mL/min for BioCad™ Workstation
Sample	20 µL
Gradient	0–100% B in 10 column-volumes
Detection	280 nm

Results

The standard chromatogram shown in "Results" on page 5 is for a 4.6 mmD/100 mmL POROS™ HE column. The retention times and bandspreading may vary with different HPLC systems. The general profile should be similar, however.

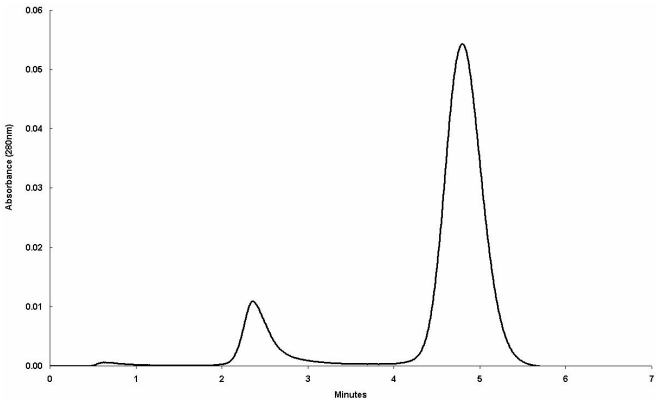

Fig. 1 A POROS™ 50 HE chromatogram

Support

For service and technical support, go to **thermofisher.com/poros** or call toll-free in US: 1.800.831.6844.

For the latest service and support information at all locations, or to obtain Certificates of Analysis or Safety Data Sheets (SDSs; also known as MSDSs), go to **thermofisher.com/support**, or contact you local Thermo Fisher Scientific representative.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

The information in this guide is subject to change without notice.

DISCLAIMER: TO THE EXTENT ALLOWED BY LAW, LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Revision history: Pub. No. 4470890

Revision	Date	Description
B	10 January 2017	Baseline for this revision history.

Limited Use Label License No. 495: Internal Research, Quality Control, and Bioproduction Use for Columns and Resins: Notice to Purchaser: The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product, under intellectual property rights that are owned and/or controlled by Life Technologies and relate specifically to the product, to perform (a) internal research for the sole benefit of the purchaser; (b) quality control testing conducted by the purchaser on a fee for service or contract basis for or on behalf of third parties; and (c) performance of research or manufacturing services conducted by the purchaser on a fee for service or contract basis for or on behalf of third parties. The purchase of this product does not grant the purchaser any additional rights, including, without limitation, the right to transfer or resell the product in any form, the right to use the product as a therapeutic agent or diagnostics test component, or to use the product to perform tests other than what is indicated in this Limited Use Label License on a contract or fee per test basis for or on behalf of third parties. For information on obtaining additional rights, please contact **outlicensing@thermofisher.com** or Out Licensing, Life Technologies Corporation (part of Thermo Fisher Scientific), 5823 Newton Drive, Carlsbad, California 92008.

Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288