POROS® 50 R1 and R2 Perfusion Chromatography® Bulk Media for Reversed-Phase Chromatography

Applied Biosystems

Operating Instructions

Note: *These instructions cover the specific operational characteristics of POROS® 50 bulk media. The same surface chemistries are available as bulk media with 20-µm particle size (POROS 20), as well as in prepacked columns. Contact your Applied Biosystems representative for more details.*

Your New POROS 50 Media Is Unique

Read this section before doing anything!

Applied Biosystems POROS 50 bulk media 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 columns packed with POROS 50 media can be operated with standard low-pressure and high-pressure systems, they are substantially different from any columns you have used before. You may have to change the way you run, and, to a large 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.

Using Perfusion Chromatography, you now have the capability to perform true methods development by systematically investigating a wide range of chromatographic variables in a short time frame. In addition, the higher flow rates translate into significant gains in throughput and productivity when scaling up to production.

Please read the operating instructions carefully to ensure that you take maximum advantage of the benefits that Perfusion Chromatography technology provides.

Increase the Flow Rate

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

Although the media can be operated without problems at flow rates typical of classical chromatography, increasing the flow rate to the range of 500 to 1,000 cm/hr allows maximum productivity without the loss of performance.

The maximum recommended flow rate for POROS 50 media is 2,000 cm/hr.

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 you adapt 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 Biosystems Technical Representative.

Reoptimize Your Method as Needed

When you transfer a method from an existing column, you may need to reoptimize the separation to account for possible differences in selectivity between POROS media and your old media.

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.

1 Product Description

POROS 50 R1 and R2 media are polymeric packings designed for reversed-phase chromatography of peptides, proteins, polynucleotides and other biomolecules in the Perfusion Chromatography mode.

POROS R2 media is designed for general reversed-phase separations of proteins, peptides, and nucleic acids.

POROS R1 media is designed for very hydrophobic proteins and peptides.

Contents

Both media columns consist of cross-linked poly(styrene-divinylbenzene) flow-through particles with a patented bimodal pore-size distribution for rapid mass transport.

Table 1 Product Characteristics

Support Matrix	Cross-linked poly(styrene-divinylbenzene)
Surface Functionality	None (native poly(styrene-divinylbenzene))
Dynamic Binding Capacity @ 1,000 cm/hr	Lysozyme 1% acetonitrile
POROS 50 R1	6 mg/ml
POROS 50 R2	9 mg/ml
Shrinkage/Swelling	<1% from 1–100% solvent
Particle Size	50 µm
Recommended maximum flow rate	2,000 cm/hr
Maximum pressure drop	100 bar (1,500 psi, 10 MPa)
Permeability	<3 bar at 1,000 cm/hr (10-cm bed height)

Table 2 Chemical Resistance

pH Range	1–14
Buffer Additives	All common agents, including tetrahydrofuran, 8 M urea, 6 M guanidine hydrochloride, ethylene glycol, and detergents. **⚠ WARNING** ***CHEMICAL HAZARD***. ***Tetrahydrofuran*** *is a flammable liquid and vapor. It may be harmful if swallowed. Exposure may cause eye and respiratory tract irritation, central nervous system depression, and liver and kidney damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.* **⚠ WARNING** ***CHEMICAL HAZARD***. ***Guanidine hydrochloride*** *may be harmful if swallowed or absorbed through the skin. Exposure may cause eye, skin, and respiratory tract irritation and adverse effects on the central nervous system and bone marrow. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*
Solvents	Water, 0–100% alcohols, acetonitrile, other common organic solvents. **⚠ WARNING** ***CHEMICAL HAZARD***. ***Acetonitrile (ACN)*** *is a flammable liquid and vapor. Exposure may cause eye, skin, and respiratory tract irritation, central nervous system depression, and damage to the heart, blood system, liver, and kidneys. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.* ***Warning:*** *Do not expose solvents to strong oxidizers (such as hypochlorite), oxidizing acids (such as nitric), or strong reducing agents (such as sulfite).*
Operating Temperature	5–80 °C

2 Packing the Column

POROS 50 media are mechanically rigid, and therefore, can be packed effectively both in low-pressure glass columns and in high-pressure PEEK or stainless steel columns. Use column bed supports (frits or screens) with a porosity of 20 µm or less.

2.1 Precautions

Warning: *POROS media is provided as a dry powder, which may form a light dust. Read the Material Safety Data Sheet and follow the handling instructions. Use one of the following when handling dry POROS media:*

- *NIOSH*/MSHA**-approved respirator with dust cartridge*
- *Fume hood*

** National Institute for Occupational Safety and Health*
*** Mine Safety and Health Administration*

Warning: *Always wear eye protection when working with solvents.*

Keep the media container closed when it is not in use.

Do not exceed 100 bar (1,500 psi) pressure drop across the column during or after packing.

2.2 Preparing the Slurry

To prepare the slurry:

1. Calculate the amount of dry powder needed to form the final bed volume of your column. Use the ratio of dry powder to packed bed volume listed on the product label.

   Example: If the label indicates that 8.3 g of powder forms 25 ml of packed bed, to pack a 10-ml column, weigh out 3.3 g of powder.

   The packed bed volume specified on the label is based on a packing pressure of 7 bar.

2. Add a volume of organic solvent equivalent to at least 2 to 3 times the final bed volume. Isopropanol is recommended.

⚠ WARNING ***CHEMICAL HAZARD. Isopropanol*** *is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry skin and cause irritation. Exposure may cause central nervous system effects such as drowsiness, dizziness, and headache. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

3. Suspend the POROS 50 packing by stirring with a glass rod.

Important: *Do not use a magnetic stirrer. It may abrade the particles and cause fines to form.*

4. Make sure that the POROS 50 packing is fully wetted by dispersing any floating material.

2.3 Packing the Column

To ensure best results when you pack the column:

- Use a reservoir or adjustable column large enought to hold the entire slurry, so that the bed can be packed all at once.
- Use flow packing techniques.

To pack a column:

1. Gently stir the slurry just before adding it to the column.
2. Pour the slurry into the column gradually to minimize the trapping of air bubbles.
3. Tap the column gently to remove air bubbles.
4. Top off the column with the slurry solvent.
5. Connect the column to the packing pump.
6. Start the flow slowly, until a clear space between the top column plunger and the slurry forms.
7. Increase the flow rate to the maximum flow rate and pressure obtainable with the equipment used.

   At a minimum, the final packing pressure should be at least 20% greater than the maximum anticipated operating pressure. POROS 50 media are mechanically stable up to backpressures of 1,500 psi.
8. After the bed is formed and the final pressure is reached, lower the plunger until it touches the top of the bed.

   POROS 50 media does not shrink or swell, so an open “head space” is not recommended.
9. Pump the column for 10 to 20 bed volumes to stabilize the bed.

   Use “equilibration run” conditions (no sample applied), including the minimum and maximum ionic strengths used in actual operation along with any anticipated cleaning cycles.

   You can now reequilibrate the column and operate it.

3 Selecting and Preparing the Mobile Phase

Regardless of the mobile phase you choose, always:

- Use eluents of the highest purity practical.
- Degas all eluents prior to use.

3.1 Solvents

⚠ WARNING ***CHEMICAL HAZARD. Acetonitrile (ACN)*** *is a flammable liquid and vapor. Exposure may cause eye, skin, and respiratory tract irritation, central nervous system depression, and damage to the heart, blood system, liver, and kidneys. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

Keep this information in mind as you select the solvent for the mobile phase:

- Acetonitrile is recommended as the solvent for mobile phases.
- For the A buffer in a water/acetonitrile gradient system, use a solution with a minimum concentration of acetonitrile (1%).
- Alcohols such as methanol or isopropanol may give poor peak symmetry or efficiency. Adding 10% THF or acetonitrile to alcohol-based mobile phases can improve chromatographic performance.

3.2 Mobile Phase

The polymeric nature of POROS R1 and R2 media enables it to withstand prolonged exposure to high-pH conditions.

Switching to high-pH mobile phases may improve selectivity.

This opens up new possibilities that are not available with conventional silica media.

Note the following when selecting a mobile phase:

- 10 mM tribasic phosphate solution has a pH of around 11.5 and is convenient for many applications.
- You can also use triethylamine (TEA) for high-pH mobile phases.
- Examine the effect of pH on selectivity by doing a systematic pH screening or mapping experiment at pH 2, 7, and 11.

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.

3.3 Additives

⚠ DANGER ***CHEMICAL HAZARD. Trifluoroacetic acid (TFA)*** *causes eye, skin, and respiratory tract burns. It is harmful if inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

⚠ DANGER ***CHEMICAL HAZARD. Formic acid*** *causes burns to the eye, skin, and respiratory tract. It is harmful if swallowed. Prolonged or repeated exposure may cause allergic reactions. It is a combustible liquid and vapor. Exposure may cause damage to the kidneys and liver. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

⚠ WARNING ***CHEMICAL HAZARD. Isopropanol*** *is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry skin and cause irritation. Exposure may cause central nervous system effects such as drowsiness, dizziness, and headache. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

The completely nonionic nature of POROS R1 and R2 media and the resistance to high pH allow great flexibility in the use of mobile phase additives:

- Additives such as TFA may no longer be necessary for separation performance, although they may still be needed for solubilization of the sample.
- Less toxic additives such as phosphoric, formic, or acetic acids may often be just as effective as TFA. However, adding organic acids in concentrations greater than 5 to 10% (v/v) can significantly reduce binding strength.
- Inorganic phosphate is not recommended as an additive because of poor solubility in acetonitrile.
- When sample selectivity is partially based on charge differences, adding appropriate hydrophobic additives (ion pairing agents) to the mobile phase can mimic the selectivity of silica-based media.

  Select the pH and additive so that the additive has an ionic charge opposite that of the ionic groups on the solute.
- For differences in negative charge (such as an oxidized sulfhydryl group), use agents such as 1 mM trimethyloctadecylammonium chloride at neutral or high pH.
- For differences in positive charge, use agents such as 5 mM pentane sulfonic acid or 0.1% hexafluorobutyric acid (HFBA), in place of TFA at low pH.

The effects of mobile phase solvent, pH, and additives can be determined only by experimentation. However, the very high flow rates in Perfusion Chromatography shorten experimentation time.

4 Preparing and Loading the Sample

To ensure efficient binding and prevent column plugging:

1. Dissolve or exchange samples for POROS R1 and R2 columns into the starting mobile phase.
2. Centrifuge or filter (0.22 or 0.45 µm) samples before injection to prevent column plugging.

Important: *If the sample contains more than 10 mM phosphate, other salts, or other components that may not be soluble in acetonitrile, you must inject the sample at 0% of the organic solvent. Failure to do this may irreversibly foul the column.*

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

Determining the Sample Load

Many peptides and proteins have limited solubility in mobile phases that also allow binding to the column. Limited solubility may be exaggerated on the column, because the column concentrates the sample as it is injected. If the solubility limit is exceeded on the column, poor resolution or even column plugging can occur.

Because of limited solubility, the dynamic binding capacities of POROS R1 and R2 media are in the range of 0.1 to 5 mg/ml for most proteins.

In general, high-resolution separations are achieved at 20% or less of the total binding capacity .

However, the maximum loading at which a given resolution can be obtained (the loadability) normally depends on a number of factors, including sample solubility, column selectivity, pH, molecular weight, and buffer ionic strength.

To perform a loadability experiment:

1. Determine the most effective elution conditions (eluent, gradient, and flow) at low sample 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 use 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 requiring reoptimization of the gradient.*

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

5 Eluting the Sample

You can elute the sample using isocratic or gradient conditions.

- Many peptides are optimally eluted isocratically, but because of the high sensitivity of peptides to elution strength, very shallow gradients (<5% elution strength range) are usually more practical.
- Gradient volumes of 10 to 20 column volumes normally provide a good compromise between resolution and peak dilution.
- With the very high flow rates possible with Perfusion Chromatography, increased gradient volumes are possible without the excessive times typically required when using conventional reversed-phase media.

6 Column Regeneration and Cleaning-in-Place (CIP)

⚠ WARNING ***CHEMICAL HAZARD. Acetic acid*** *is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract burns. It may be harmful if inhaled, absorbed through the skin, or swallowed. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

⚠ WARNING ***CHEMICAL HAZARD. Sodium hydroxide (NaOH)*** *causes severe eye, skin, and respiratory tract burns. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves*

⚠ WARNING ***CHEMICAL HAZARD. Isopropanol*** *is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry skin and cause irritation. Exposure may cause central nervous system effects such as drowsiness, dizziness, and headache. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

In some applications, sample molecules may not fully elute or may precipitate on the column and cause fouling. Regenerate the column if you observe:

- Increased bandspreading
- Loss of binding capacity
- Loss of recovery
- Increased pressure drop
- Trace or “ghost” peaks occurring during blank gradient runs

IIMPORTANT: *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.*

In reversed-phase chromatography, the bound species may have very limited solubility in the organic solvent required to remove them from the surface. Therefore, regeneration solutions must be both strong solubilizing agents and strong eluents. These qualities are often mutually exclusive. To manage this situation:

1. Run rapid “sawtooth” gradients from 100% of a very strong solubilizer (such as 50% acetic or phosphoric acid or 0.5 M NaOH, 1 to 3 M guanidine) to 100% of a strong eluent, (such as acetonitrile or isopropanol), then back to the solubilizer.

   Running a gradient helps achieve the correct blend of the two agents needed to remove the bound contaminant.

2. Use a solubilizer that is miscible with the organic solvent selected. Isopropanol is a better choice with base or guanidine.

The stability of POROS R1 and R2 media to high pH allows you to use harsh eluents such as 2 M NaOH for column cleaning. This increases the range of regeneration options available, allowing efficient media sanitization and extending the practical life of your column.

7 Storing the Media

Store the dry POROS powder at room temperature.

To store a packed column:

- For short-term or long-term storage, use a 20% to 80% organic mobile phase such as acetonitrile, methanol, or ethanol.
- Carefully seal the ends of the column to prevent drying. Drying results in decreased chromatographic efficiency.
- Avoid long-term storage of stainless steel columns with halide (Cl) salts, because frit corrosion may result.
- Store the column between 5 and 30 °C.

8 Guidelines for Using Perfusion Chromatography

When you make the transition to Perfusion Chromatography, consider factors related to:

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

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 a POROS column, you cannot simply set the upper pressure limit of the system to the pressure rating of the column. Instead, you need to:

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 column and instrument to the total system pressure when pressures approach the maximum column pressure. Take the column out of line to determine those contributions (measured pressure = column pressure drop + system pressure).

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 you have any question about gradient performance on other systems, you can visualize the gradient 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.

- 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 allows you to perform runs more quickly than other chromatography techniques. For this reason, perform maintenance tasks such as replacing filters or regenerating columns after a certain number of runs, rather than after a specified 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 what 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.

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 4.6mmD/100mmL column (volume 1.7 ml), a 5 ml/min flow rate translates into three column volumes/min (5/1.7). Therefore, a 10.6 column-volume gradient is completed in 3.5 minutes.

The VISION and BioCAD Workstations allow you to program by 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.

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 less on columns packed with POROS media.

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.
- Using peak height can give a general comparison, but bandspreading differences due to different efficiencies or gradient slopes cause errors.
- Recovery may be compared accurately using peak integration at the same flow rate. *However, 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.

9 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 R1 and R2 columns, Perfusion Chromatography, or other products, please contact Applied Biosystems. Refer to the back page of this document for contact information.

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POROS products and perfusive chromatography are covered by U.S. patents 5,030,352; 5,552,041; 5,605,623; and 5,833,861; foreign patents pending. Chromatography materials of certain pore geometries, including certain wide-pore supports, can permit perfusive chromatography, that is, separations in which the rate of intraparticle convection exceeds the rate of intraparticle diffusion. Use of any such chromatography media at sufficiently high linear velocity, without license, may constitute patent infringement. A limited license to use the patented perfusive chromatography process is granted with the purchase of POROS products from Applied Biosystems. The license terminates upon expiration of the useful life of the product.

Subtractive Assay technology, enabled by the use of ImmunoDetection (ID) Sensor Cartridges and the INTEGRAL Micro-Analytical Workstation, is covered by U.S. patent 5,234,586. Other patents pending.

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