

POROS® 50 EP and OH Perfusion Chromatography® Bulk Media for Activated Affinity Chromatography



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- μ m 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 based on 50- μ m flow-through particles. POROS 50 media enable you to employ Perfusion Chromatography® technology under low operating pressures. Chromatographic separations of biomolecules can be performed 10 to 100 times faster than classical liquid chromatography separations, while maintaining high resolution and high binding capacity.

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 classical 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 processing capacity 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 classical media is the flow rates at which experiments are run.

While 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 starting flow rate range for POROS 50 EP and OH media is 1,000 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 of adapting a method developed on diffusion limited media.

Be sure to read Section 10, 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.

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1 Product Description

POROS EP and OH media are polymeric packings designed for affinity chromatography of peptides, proteins, polynucleotides, and other biomolecules with user-immobilized ligands in the Perfusion Chromatography mode. The packings consist of cross-linked poly(styrene-divinylbenzene) flow-through particles with a patented bimodal pore size distribution for rapid mass transport. This base matrix is coated with a cross-linked polyhydroxylated polymer (POROS OH), which is then activated with epoxide (POROS EP) functional groups.

POROS OH media is designed to be activated by the user, using any of the conventional chemistries like glutaraldehyde, tresyl, divinylsulfone, CNBr, and so on.

Table 1 Product Characteristics

Support Matrix	Cross-linked poly(styrene-divinylbenzene)
Surface Functionality POROS OH POROS EP	Hydroxyl Epoxide
Shrinkage/Swelling	<1% from 1–100% solvent
Particle size	50 μm
Recommended maximum flow rate in 10 cm bed length	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 POROS OH POROS EP	1–14 (up to 5.0 M NaOH, 1.0 M HCl) 3–12*
Ionic Strength Range	0–5 M. All common salts.
Buffer Additives	All common agents, including 8 M urea, 6 M guanidine hydrochloride, ethylene glycol, and detergents. Reactive amine-containing buffers (for example, Tris) can react with EP.
Solvents	Water, 0–100% 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). Reactive amine-containing solvents (for example, ethanol amine) can react with epoxide functional groups.

*Stability depends on storage time at a given pH. Epoxide functionality hydrolyzes at pH extremes. For long-term storage, milder conditions are recommended (for example, pH 5–9).

2 General Considerations

General considerations regardless of chemistry are given below. Guidelines (and in some cases, suggested protocols) for specific surface chemistries are given in Section 5, POROS EP Media, and Section 6, POROS OH Media.

In activated affinity chromatography, coupling yield (ratio of molecule offered to molecule immobilized), surface concentration (mass of the immobilized molecule per unit support column bed volume), and specific activity (biological activity per unit mass of immobilized molecule), are determined by these key variables:

- Binding buffer (pH)
- Concentration of molecule in binding solution
- Solubility of the molecule in binding solution
- Ratio of molecule mass to support mass or bed volume
- Concentration of support in binding solution
- Reaction time and temperature
- Reaction quenching
- Mode of reaction (batch or column)

Because molecules for immobilization differ substantially in reactivity and other properties, the optimal conditions for immobilization can only be determined experimentally. However, published papers and textbooks should be consulted, where appropriate.

Surface Concentration Versus Yield and Activity

In general, maximum surface concentration is achieved by high concentrations of both molecule and support and high ratios of molecule to support.

However, high surface concentration is often obtained at the expense of low coupling yield and specific activity of the immobilized molecule.

Note that the surface concentration of reactive groups on POROS activated supports has been carefully optimized to reduce overcoupling, which causes low specific activity.

Maximum utilization of rare or expensive molecules is usually achieved at a lower ratio of molecule to support, and thus lower surface concentration.

Use of Salting-Out Conditions

In general, with macromolecules such as proteins, coupling under salting-out conditions can achieve both high coupling yield and high surface concentration. To achieve this, add a saturated solution of sodium sulfate or other anti-chaotropic salt to the coupling solution until the solution just starts to become cloudy (just before the concentration reaches the precipitation point). Avoid ammonium sulfate because primary amines in the buffer interfere with all the reaction chemistries.

Although the support surface is quite hydrophilic, it is less hydrophilic than the solution. When exposed in high salt concentration, the protein partially precipitates on the support surface. This dramatically raises the local concentration at the surface, driving the coupling reaction to a much higher level.

Preparation of Concentrated Sodium Sulfate

At the high concentrations required in a salting-out procedure (2 M, for example), sodium sulfate can precipitate during preparation. To prevent precipitation, follow these guidelines:

1. Dissolve the appropriate amount of solid sodium sulfate in hot (>80°C) water in a volume close to the final buffer volume.
2. Allow the solution to slowly cool at room temperature.
3. When the temperature of the solution reaches approximately 30°C, titrate to the final pH using as small a volume as possible so that the buffer does not cool too rapidly.
4. Allow the buffer to cool to 25 to 30°C.
5. Proceed with the immobilization.

Make sure all other buffers are at ambient temperature.

If prepared carefully, 2 M sodium sulfate is stable at room temperature.

3 Reacting the Support

It is desirable to react the support in a batch reaction before packing it into a column. The concentrations of ligand and support can readily be varied independently to give more flexible immobilization development.

Warning: POROS EP media is provided as a dry powder, which may form a light dust. 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

Follow these guidelines to react the support:

- Form the binding solution by dissolving or exchanging the ligand protein into the desired coupling buffer. For proteins, the concentration should typically be in the order of 10 to 20 mg/ml. Lower concentrations give higher coupling yield; higher concentrations give higher surface concentration.
- Calculate the amount of dry powder needed to give 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 gives 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 170 bar.
- Add the desired amount of dry support to the binding solution. Keep in mind that a concentration of 0.15 g/ml or less is typically used as a slurry concentration during the reaction itself.
- Alternatively, the gel slurry can be prepared in advance in sample buffer and washed extensively (at least 10 column volumes) with the coupling buffer using a Buchner funnel. The binding solution can then be added to the slurry in the desired concentration.
- When reacting the support, it is necessary to gently agitate the reaction tubes or vessels to prevent settling of the beads. Shaking or tumbling are preferred techniques.
- Once immobilization and any quenching reactions are complete, wash the support extensively first with coupling buffer, then with a high salt (1 M NaCl) buffer to eliminate any ligand that may be bound to the support through protein/protein interactions.

- Excess epoxide functionality with POROS EP can be blocked by reacting the beads as described in Section 5, POROS EP Media.
- Wash the support with starting buffer, and pack the slurry into the desired column.

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

4 Packing the Column

This section discusses these topics:

- Packing buffers
- Preparing the slurry
- Packing procedures

POROS 50 media are mechanically rigid, and therefore, can be packed effectively both in low-pressure glass columns and in high-pressure stainless steel columns. The screen size must be less than 15 µm.

4.1 Packing Buffers

Use these buffers:

- Slurry buffer: 0.5 M NaCl
- Packing buffer: 0.1 M NaCl

4.2 Preparing the Slurry

POROS 50 EP media is a dry powder. POROS 50 OH media is a slurry containing 20% ethanol as a bacteriostat and gives the final packed bed volume indicated on the label. Calculate the total volume of slurry needed to pack your own column by keeping in mind that the ratio of slurry volume to packed bed volume is 1.8:1.

Example: 18 ml of slurry contains enough media for a 10 ml packed bed volume. The packed bed volume specified on the label is based on a packing pressure of 3 bar.

To prepare the slurry for packing:

1. Allow the media to settle for 3 hours.
2. Pour off the supernatant.
3. Resuspend the media in 0.5 M NaCl.

The volume of 0.5 M NaCl to add depends on the column equipment you use. In general, the final slurry volume should be 2 to 3 times the final packed bed volume.

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

4.3 Packing Procedures

To ensure best results when you pack the column:

- Use a large enough reservoir or adjustable column to contain the entire slurry, so that the bed may be packed all at once.
- Use flow packing or pressure packing techniques.

Flow Packing

1. Gently stir the slurry just before adding it to the column.
POROS 50 beads have a density similar to the density of water, so rapid settling is not a problem.
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 the column off with the slurry buffer.
5. Prime the packing pump and top adjuster with packing buffer. Remove all air bubbles.
6. Connect the top adjuster to the column.
7. Start the flow slowly, until a clear space between the column top adjuster and the slurry forms.
8. Increase the flow rate to the maximum flow rate and pressure obtainable with the equipment used.
The final packing flow rate should be at least 20% greater than the maximum anticipated operating flow rate.
9. Once the bed is formed and the final flow rate is reached, bring the column top adjuster into contact with the top of the bed.
10. Restart the flow for three bed volumes to stabilize the bed.
POROS 50 media does not shrink or swell, so an open "head space" is not recommended.
11. Pump the column for 5 to 10 bed volumes.
Use "equilibration run" conditions (no sample applied), including the minimum and maximum ionic strengths used in actual operation along with any anticipated cleaning cycles.
The column is ready for operation.

Note: You may observe some fine material in the eluent as packing begins. This will clear as packing proceeds and 2 to 3 bed volumes of packing buffer pass through the column.

Pressure Packing

1. Gently stir the slurry just before adding it to the column.
POROS 50 beads have a density similar to the density of water, so rapid settling is not a problem.
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 the column off with the slurry buffer.
5. Prime the top adjuster with packing buffer.
6. Connect the top adjuster.

7. Expel trapped air from the column by applying 5 psi to the system.
8. Select the packing pressure on the pressure gauge.
9. Pack the column.
10. Apply flow for 3 bed volumes to stabilize column bed. The flow rate should generate no more than 80% of the packing pressure.
11. Pump the column for 5 to 10 bed volumes.

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

The column is ready for operation.

Note: You may observe some fine material in the eluent as packing begins. This will clear as packing proceeds and 2 to 3 bed volumes of packing buffer pass through the column.

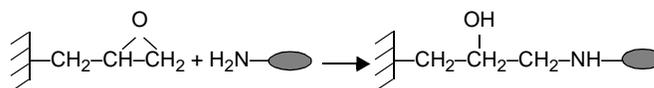
5 POROS EP Media

Chemistry

The figure below shows the reaction chemistry of POROS EP media. Note that:

- The oxirane (epoxide) rings on the support react with primary amines to form secondary amine linkages.
- The epoxide groups also react with sulfhydryl and hydroxyl groups, but at a much lower rate.
- At the elevated pH used for coupling, there is a slow competing hydrolysis of the epoxide groups to form diols.

POROS EP Media



Coupling Buffer

Follow these guidelines when you select a coupling buffer:

- Do not use any buffer that contains primary amines (such as Tris). Recommended buffers are carbonate, borate, and phosphate.
- The optimum pH for coupling is usually in the range of 9 to 11, although it varies for different materials.
- The use of salting-out conditions is highly recommended (see "Use of Salting-Out Conditions" on page 2 for details).
- Organic solvents can be used if you add an appropriate organic soluble base.
- Because of the hydrolysis reaction, POROS EP has a limited shelf life once coupling buffer is added.
- If the packing is to be stored in coupling buffer for any significant length of time, keep the packing cold, but DO NOT FREEZE IT.

Reaction Time and Temperature

The required reaction time depends upon:

- Molecule being coupled
- pH
- Reaction temperature

The reaction is faster at higher pH and temperature and with small molecule ligands. However, the competing hydrolysis reaction is also faster. Reaction times vary:

- At room temperature with proteins, the reaction can take place overnight.
- At cold room temperature, several days are generally required.

Generally, the longer the initial reaction time, the higher the surface concentration and coupling yield.

Note: Do not use cold room temperature if salting-out conditions have been used. Salt crystals that form may damage POROS media.

Quenching

Some residual epoxide functionality is likely to remain after the coupling reaction.

To quench these epoxides, use any of these low molecular weight reactants:

- 0.2 M Tris buffer
- 1 M ethanolamine in phosphate buffer
- 0.1 M mercaptoethanol in phosphate buffer

Quenching reactions are generally complete after 2 hours at any temperature.

6 POROS OH Media

POROS OH media offers a surface which is chemically similar to base cellulose or agarose supports, containing a high concentration of hydroxyl functionality. Activate POROS OH media with any of the coupling chemistries used for agarose or cellulose matrices, including:

- Cyanogen bromide
- Tresyl
- Glutaraldehyde
- Divinylsulfone
- Carbonyldiimidazole
- Periodate
- Triazine
- Diazonium

Consult standard textbooks for specific chemistries and protocols.

7 Affinity Chromatography

- The binding and elution buffers used for affinity chromatography depend entirely upon the nature of the ligand interaction.
- Although the POROS support itself and the coupling linkage are highly stable chemically, be careful not to expose the column to conditions that cause denaturation of the immobilized molecule itself.
- Because Perfusion Chromatography media have such a high rate of mass transport, the volumes required for complete washing and elution of the column are generally quite low (<5 column volumes).

8 Cleaning Up and Regenerating the Media

POROS activated affinity media are stable to most agents typically used in a regeneration procedure. Refer to Table 2 for details.

The regeneration protocol used depends on the stability of the ligand chosen and the nature of the sample containing the target molecule.

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 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.

9 Storing the Media

To store the slurry:

- Store POROS OH media at room temperature.
- Store POROS EP media at 4 to 10°C.

To store a packed column:

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

Short-term Storage

Store the column in any appropriate buffer.

Long-term Storage

For POROS OH media: Add 0.02% sodium azide or 30% methanol to the column.

For POROS EP media: Add 100% ethanol to the column.

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

10 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

10.1 Account for System Pressure

The high flow rates used with Perfusion Chromatography media 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 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).

10.2 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 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 for Perfusion Chromatography, 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/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.

10.3 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.

10.4 Maintain Your Column and System

Perfusion Chromatography enables 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 set period of time. You can reduce the frequency of such maintenance by always filtering the sample and eluent.

10.5 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, 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 x 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/100mL 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 by column volumes.

10.6 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.

10.7 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 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.
- Using peak height can give a general comparison, but bandspreading differences due to different efficiencies or gradient slopes will cause errors.
- Recovery may be compared accurately using 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.

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. Contact Applied Biosystems directly for a publication list.

For further details or for answers to questions on POROS 50 EP and OH media, Perfusion Chromatography, or other products, 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 5,552,041; 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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