POROS™ 20 AL, EP and OH Perfusion Chromatography Bulk Media for Activated Affinity Chromatography

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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™ 20 bulk media. The same media are also available in prepacked columns. Contact your Thermo Fisher Scientific representative for more details.

Read this section before you begin

Your New POROS™ Media Is Unique

Thermo Scientific™ POROS™ 20 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™ 20 media can be operated on standard HPLC and LC 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 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 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 2000 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 maximum flow rate is not limited by the media itself (high resolution separations have been achieved at 10,000 cm/hr) but rather is a function of the system used, the pressure limit on the column selected, and how the column is packed. A typical linear flow rate for POROS™ 20 media is 3,600 cm/hr.

The corresponding volumetric flow rates for various column diameters are listed in the following table.

Table 1 Typical flow rates for Perfusion Chromatography™

Column Diameter (mm)	Volumetric Flow Rate (ml/min)	Linear Velocity (cm/hr)
2.1	2.0	3,600
4.6	10.0	3,600
10	47.5	3,600
16	120.0	3,600

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

Be sure to read "Guidelines for using Perfusion Chromatography™" 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.

Reoptimize Your Method as Needed

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™ BioCad™ Workstation for Perfusion Chromatography™.

Product description

POROS™ AL, 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 aldehyde (POROS™ AL) or 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 2 Product characteristics

Support Matrix	Cross-linked poly(styrene-divinylbenzene)	
Surface Functionality	POROS™ OH	Hydroxyl
	POROS™ AL	Aldehyde
	POROS™ EP	Epoxide
	POROS™ NH	Primary amine
	POROS™ HY	Hydrazide
Shrinkage/Swelling	<1% from 1–100% solvent	
Particle size	20 µm	
Recommended maximum flow rate	10,000 cm/hr	
Maximum pressure drop	170 bar (2,500 psi, 17 MPa)	
Permeability	<3 bar at 1,000 cm/hr (3 cm bed height)	

Table 3 Chemical resistance

pH Range	POROS™ AL, OH POROS™ EP	1–14 (Up to 5.0 M NaOH, 1.0 M HCl) 3–9
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.	
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).	
Operating Temperature	5–80 °C	

General considerations

General considerations regardless of chemistry are given below. Guidelines (and in some cases, suggested protocols) for specific surface chemistries are given in the following sections.

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 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 in other properties, the optimal conditions for immobilization can be determined only experimentally.

However, published papers and textbooks should be consulted, where appropriate.

Surface concentration versus yield and activity

In general, maximum surface concentration of immobilized ligand is achieved by high concentrations of both ligand and support and high ratios of ligand to support during the coupling stage.

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

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:

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.

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™ 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 range 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.
- Do not use a stir bar. It generates fines and may denature proteins.
- After 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.
- Wash the support with starting buffer, and pack the slurry into the desired column.

Packing the column

POROS™ 20 media are mechanically rigid and can therefore be packed effectively both in low-pressure glass columns and in high-pressure PEEK or stainless steel columns. The column bed supports (frits or screens) should have a Porosity of 10 µm or less.

Do not exceed 170 bar (2500 psi) pressure drop across the column during or after packing.

Packing the column

To ensure best results when you pack the column:

- Use a reservoir or adjustable column large enough to hold the entire slurry, so that the bed may be packed all at once.
- Use flow-packing techniques. Gravity settling is time consuming and usually results in poor performance.

Follow these steps:

1. Gently stir the slurry just before adding it to the column.

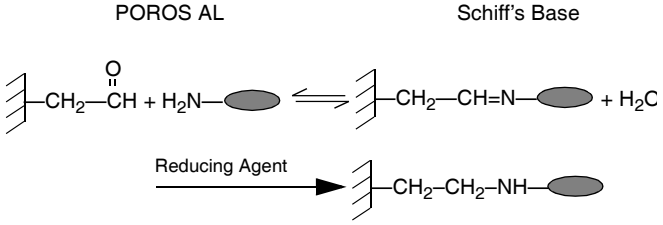
   POROS™ beads have a density similar to water, so rapid settling is not usually a problem.
2. Pour the slurry in gradually to minimize trapping air bubbles.
3. Tap the column gently to remove air bubbles.
4. Top off the column with the slurry solvent.
5. Connect the column to the packing pump.
6. Pack the column at a flow rate that yields a final pressure about 20 to 50% greater than the maximum anticipated operating pressure.
7. After the bed is formed and the final pressure is reached, pump the column with 10 to 20 bed-volumes of slurry solvent to stabilize the bed.

POROS™ AL media

Chemistry

The figure below shows the reaction chemistry of POROS™ AL media:

- Aldehyde groups on the surface of the support react selectively with primary amines on the molecule, ultimately to form a Schiff's base.
- The Schiff's base is subject to easy hydrolysis. Therefore, a highly stable secondary amine linkage is formed by reacting the Schiff's base with a mild reducing agent.



Coupling buffer

- Do not use any buffer that contains primary amines (such as Tris).
- The optimum coupling pH is usually between 4 and 8.
- pH 6 to 7 usually produces good results.
- The use of salting out conditions is highly recommended (see "Use of salting out conditions" on page 2 for details).

Reaction time and temperature

The required reaction time depends on the ligand and the reaction temperature.

Reaction times vary:

- At room temperature, allow at least 8 hours.
- At cold-room temperature, allow up to several days.

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

When in doubt, perform an overnight reaction at room temperature for good results, especially with proteins.

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

Reduction step

After the initial reaction and Schiff's base formation, you must react the support with a reducing agent to form a stable bond.

Consider the following when selecting a reducing agent:

- Sodium cyanoborohydride (NaCNBH3) is the most widely used reducing agent. It is mild enough to prevent reduction of disulfide bonds on proteins, but it is toxic and may present disposal problems when used on a large scale.
- Sodium borohydride (NaBH4) should not be used in prepacked columns. Although it reduces aldehyde groups, eliminating the need for a quenching reaction, it may produce gas bubbles.

To reduce the support chemically:

1. Make up your reducing agent as a concentrated solution. A suggested concentration is 5 mg of reducing agent per ml of column-bed volume.
2. Start the reduction reaction according to the reducing agent you are using. If you use:
   - Cyanoborohydride: Begin the reduction reaction simultaneously with the coupling reaction by mixing the protein solution, coupling solution, and cyanoborohydride
   - Other reducing agents: Add the reducing agent after the coupling reaction is complete.
3. Allow the reduction reaction to proceed for approximately 2 hours at room temperature or 8 hours in the cold room.
4. After the reduction reaction is complete, wash the support with coupling buffer.

Quenching

Residual aldehyde functionality is likely to remain after the coupling reaction. To quench these aldehydes:

1. React the support with a low-molecular-weight primary amine, such as:
   - 0.2 M Tris buffer
   - 1 M ethanolamine in phosphate buffer
2. Include reducing agent in the quenching reaction mixture.

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

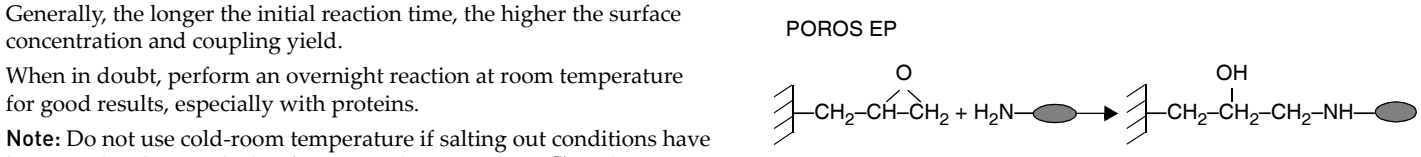
POROS™ EP media

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.
- Hydroxyls require high pH (for example, pH 10-12), resulting in an α-hydroxy ether linkage. However, hydroxyls are less reactive than amines.
- Epoxides also react with sulfhydryl groups. The pH of the coupling buffer can be adjusted (for example, pH 6-8) to make the sulfhydryl reaction selective in the presence of amines.
- At the elevated pH used for coupling amines and hydroxyls, there is a slow competing hydrolysis of the epoxide groups to form diols.



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 9–11 for amines and 6–8 for sulfhydryls.
- 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 media has a limited shelf life after the 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 on the:

- 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 the following 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.

POROS™ OH media

POROS™ OH media offers a surface that 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 matrixes, including:

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

Consult standard textbooks for specific chemistries and protocols.

Affinity chromatography

- The binding and elution buffers used for affinity chromatography depend entirely on the nature of the ligand interaction.
- Although the POROS™ support itself and the coupling linkage are highly stable chemically, do not 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).

Cleaning up and regenerating the media

POROS™ activated affinity media are stable to most agents typically used in a regeneration procedure. Refer to “Product description“ on page 1 for details.

The regeneration protocol to use 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 of the column 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 media

Store the dry powder at room temperature, except for POROS™ EP, which must be stored 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

Add 0.02% sodium azide or 30% methanol to the column.

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

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

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:
   a. Connecting a union in place of the column
   b. 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 prevent 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).

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.

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™ 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 specified 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 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 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.

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.

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.

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

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