POROS™ 20 Micron HP2 Perfusion Chromatography™ Bulk Media for Hydrophobic Interaction Chromatography

Pub. No. 8-0140-40-1095 **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™ 20 micron 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 micron bulk media are made for Perfusion Chromatography™ flow-through particle chromatography—a patented new technology which performs bioseparations 10 to 100 times faster than conventional HPLC or LC without loss in capacity or resolution.

Although columns packed with POROS™ 20 micron 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.

While 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 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 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 micron media is 3,600 cm/hr.

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

Table 1 Typical flow rates for Perfusion Chromatography™

Column Diameter (mm)	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 true of adapting 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 this quick and easy, especially if you are using the VISION™ or BioCad™ Workstation for Perfusion Chromatography™.

Product description

POROS™ 20 micron HP2 media is a polymeric packing designed for hydrophobic interaction chromatography (HIC) of peptides, proteins, and other biomolecules 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 very rapid mass transport. The particles are surface coated with a

cross-linked polyhydroxylated polymer. This coating is further functionalized with phenyl groups.

Table 2 Product characteristics

Support Matrix	Crosslinked poly(styrene-divinylbenzene)
Surface Functionality	Phenyl (High ligand density)
Dynamic Binding Capacity @ 3,600 cm/hr	Bovine serum albumin, pH 7.0 10 mg/ml
Shrinkage/Swelling	<1% from 0–100% 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	1–14 (Up to 5.0 M NaOH, 1.0 N HCl)
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. **Note:** Do not expose to strong oxidizers (such as hypochlorite), oxidizing acids (such as nitric) or strong reducing agents (such as sulfite).
Solvents	Water, 0–100% alcohols, acetonitrile, other common organic solvents
Operating Temperature	5–80°C

Packing the column

POROS™ 20 micron media are mechanically rigid and can 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 for POROS™ packings.

Precautions

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

Do not exceed 170 bar (2,500 psi) pressure drop across the column during or after packing.

Preparing the slurry

Follow these steps to form the slurry:

Note: Avoid magnetic stirring that may abrade the particles and cause fines to form.

1. 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.

   Note: POROS™ HP2 media is very hydrophobic. Wet with 100% organic such as acetone to form a paste before adding the desired aqueous slurry solvent.
2. Add buffer or dilute saline solutions, such as 1 to 3% w/v NaCl for the slurry solvent.

   The volume to add depends on the equipment you are using. In general, the final slurry volume should be a minimum of 2 to 3 times the final packed bed volume.
3. Mix the slurry gently.

Packing the column

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 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 the trapping of air bubbles.
3. Tap the column gently to remove air bubbles.
4. Top the column off with the slurry solvent.
5. Connect the column to the packing pump.
6. Pack the column at a flow rate giving a final pressure about 20 to 50% greater than the maximum anticipated operating pressure.
7. Once the bed is formed and final pressure is reached, pump the column with 10 to 20 bed volumes of buffer to stabilize the bed.

Selecting and preparing the starting buffer

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

- Use buffers of the highest purity practical. Some crude grades of ammonium sulfate may contain UV-absorbing materials which can cause baseline shifts.
- Degas and filter (0.22 or 0.45 µm) all buffers prior to use. This is especially critical with high salt concentration solutions.

Most applications of HIC require that the column be equilibrated with a relatively high concentration of an anti-chaotropic ("salting out") salt to promote hydrophobic binding of the proteins with the packing surface.

- Ammonium sulfate is by far the most commonly used salt for equilibration.
- Other salts, such as sodium, potassium or magnesium acetate, phosphate, sulfate, or chloride are also sometimes used.

Choosing the starting salt concentration

The concentration of salt is the most important factor in determining binding and elution characteristics. To select a concentration of salt, consider:

- The hydrophobicity of the proteins in the sample and the surface functionality used.

  Proteins rarely require more than 3 M ammonium sulfate for binding to any HIC media.

  In some cases, proteins or peptides may be so hydrophobic that they bind to one or more of the POROS™ HIC media with no salt present. This can be a highly desirable situation, since the column will be extremely selective.

  If any proteins bind this way, be careful in designing the elution and regeneration scheme to recover all the protein and restore the full capacity of the column. See "Eluting the sample" on page 3.
- The solubility of the proteins in the sample mix.

  Ammonium sulfate or other salts at sufficient concentration cause proteins to precipitate. If possible, keep the starting salt concentration low enough to avoid precipitation. If precipitation does occur in the starting sample, clarify the sample prior to injection on the column.

Buffer ions, pH, and additives

Keep this information in mind when you select buffer ions, pH, and additives:

- Most buffer ions can be used with POROS™ HIC media without problem, because the buffer pH affects the conformation of the proteins and not the actual binding process itself.
- pH can have a strong effect on selectivity. Investigate this variable.

- Most common additives can be used. Avoid additives in the starting buffer that may affect the hydrophobic binding such as chaotropes (urea, thiocyanate, guanidine/HCl), detergents or solvents. These additives may be used for elution. See "Eluting the sample" on page 3.
- Note that the viscosity of typical loading buffers (for example, 3M $(NH_4)_2SO_4$) in hydrophobic interaction chromatography (HIC) is high. This may significantly alter the flow rates recommended (Table 1) to stay within the back-pressure recommendations (Table 2).

Preparing and loading the sample

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

1. Dissolve or exchange large samples (> 10% of the column bed volume) into the starting/wash buffer (high ammonium sulfate concentration). Small samples can be loaded without dissolving or exchanging on the column, since buffer exchange will occur on the top of the column bed.
2. Centrifuge or filter samples (0.22 or 0.45 µm) prior to injection.
3. Delipidate samples, if possible. Lipids can cause irreversible fouling.

Determining sample load

The binding capacity of HIC media is very difficult to measure because conditions that make proteins bind tightly also tend to reduce solubility.

A typical range for working capacity of proteins under normal operating conditions is 5 to 10 mg/ml column bed volume.

In general, the maximum loading at which a given resolution can be obtained (the loadability) is dependent upon a number of factors, including sample solubility, column selectivity, and so on.

The long run times associated with conventional chromatography usually prohibit a systematic determination of loadability. Perfusion Chromatography™ technology's short run times make it easy to perform this determination.

Perform a loadability experiment as follows:

1. Determine the most effective elution conditions (eluent, gradient, and flow) at low loading.
2. Gradually increase the sample load (either through increasing injection volume or sample concentration or both) until the required resolution no longer occurs.
3. If you are using a VISION™ or BioCad™ Workstation, take advantage of the loading study template designed specifically for this purpose.

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

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

Eluting the sample

Follow these guidelines to elute the sample:

- Use either linear gradient or stepwise reductions in the salt concentration. Most proteins should elute with a wash in straight buffer (no added salt).
- If they do not, use chaotropic or polarity-reducing agents such as ethylene or propylene glycol (up to 80%), ethanol or isopropanol (up to 50%), either in steps or increasing gradients.
- Chaotropic agents such as urea (4 to 8 M) or guanidine HCl (2 to 8 M) can also be used, but tend to disrupt protein structure.
- Ionic or non-ionic detergents can also be used, although these sometimes can cause variability in the behavior of the column.
- Temperature can have a complex but often significant effect on HIC. Because of these effects, it is important to carry out method development at the final operating temperature.

  In general, lower temperatures weaken binding strength. Because of the very short run times possible with Perfusion Chromatography™, high recovery can often be obtained at room temperature, even for labile proteins.
- Be careful with concentrated eluents (especially at low temperature). At the high flow rates normal with Perfusion Chromatography™, high viscosity of concentrated eluents may generate a pressure that approaches or exceeds the maximum pressure drop of the column.
- Watch the pressure drop on the column carefully and adjust conditions appropriately.

Cleaning up and regenerating the media

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

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

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

1. Wash with 5 to 10 column volumes of the low salt eluent.
2. Wash with 5 to 10 column volumes of 50% organic solvent (ethanol, isopropanol or ethylene glycol).

   Running rapid "sawtooth" gradients from 0 to 50% solvent may work better to elute proteins that have solubility limits in high solvent concentration.
3. If steps 1 and 2 do not restore column performance, mix the solvent in 1 M NaOH or 50% acetic or phosphoric acid instead of water.

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

- Use a mixture of 50% methanol and 50% 3 M guanidine thiocyanate.

  Note: Take care when using thiocyanate with metal systems. Thiocyanate forms complexes with iron that strongly absorb UV light.
- Store the column overnight in 1 mg/ml pepsin, DNAse or other enzymes.

Multiple injections

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

- Make the injection volume as large as possible.
- Use a low flow rate that exposes the column to the regeneration solution for several minutes.

Note: Back pressure 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.

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 mobile phase.

Long-term storage
Flush the column with a storage solution of 0.1 M Na_2SO_4 with either 0.02% sodium azide or 30% ethanol.

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™
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

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

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

1. Determine the system pressure by:
   - Connecting a union in place of the column
   - Pumping the highest salt concentration to be used at the planned flow rate
2. Set the upper pressure limit by adding the system pressure observed above to the column pressure rating.

If the system pressure is too high:

1. Check carefully for plugged or crimped tubing or other restrictions in your plumbing.
2. Use larger ID or shorter tubing.
3. Use a larger detector flow cell.

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

It is important to isolate the relative contribution of 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 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/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 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 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/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.

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 HP2 media to:

- Confirm that your column and hardware are functioning properly in the Perfusion Chromatography™ mode
- Obtain working experience with Perfusion Chromatography™ before running a real sample
- Troubleshoot

Protein separation

For the sample, use the HIC Protein Test Standard available from **thermofisher.com**. Run the separation with a gradient from 3 to 0 M ammonium sulfate. Run conditions are given below.

To prepare the protein test standard:

1. Mix 0.5 ml of Eluent A with 0.5 ml of Eluent B.
2. Dissolve the test standard in the Eluent A/B mixture (concentration—4 mg/ml bovine pancreas ribonuclease A, 1 mg/ml chicken egg lysozyme).
3. Filter the test standard using a 0.22 µ filter.
4. Store unused reconstituted test mix frozen.

Conditions

Table 4 Protocol

These conditions are common to all column sizes:	
Eluent A	20 mM phosphate pH 7.0/3.0 M ammonium sulfate
Eluent B	20 mM phosphate pH 7.0
Flow rate	2,000 cm/hr
Sample size	1–2% of column bed volume
Gradient	0–100% B in 5 minutes
Detection	280 nm

Results

The standard chromatogram shown in Figure 1 on page 5 is for a 4.6mmD/100mmL POROS™ 20 micron HP2 column. The retention times and bandspreading may be somewhat different with different column sizes and gradient systems. The other surfaces will show less retention. The general profile should be similar, however.

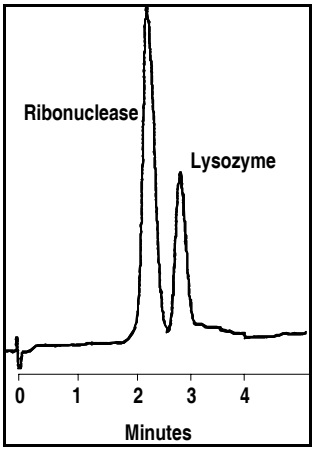

Fig. 1 POROS™ 20 micron HP2 chromatogram

Ordering information

Test standards are available from **thermofisher.com**.

Table 5 Test standard ordering information

Description	Quantity	Part Number
Hydrophobic Interaction Test Standards	Package of 5 vials	1-9005-05

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. 8-0140-40-1095

Revision	Date	Description
B	27 March 2017	Baseline for this revision history.

Important Licensing Information: These products may be covered by one or more Limited Use Label Licenses. By use of these products, you accept the terms and conditions of all applicable Limited Use Label Licenses.

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