POROS™ 20 R1 and R2 Perfusion Chromatography™ Bulk Media for Reversed-Phase 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**.

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

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.

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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 3600 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 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.

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 use the BioCad™ Workstation for Perfusion Chromatography™.

Product description

POROS™ R1 and R2 media are polymeric packings designed for reversed-phase chromatography of peptides, proteins, polynucleotides, and other biomolecules in the Perfusion Chromatography™ mode. The packing consists of cross-linked poly(styrene-divinylbenzene) flow-through particles with a patented bimodal pore size distribution for rapid mass transport.

POROS™ R1 and R2 media have somewhat different selectivity from conventional silica-based, reversed-phase media. Binding strength for proteins and peptides is similar to low-carbon-loading C4 supports for POROS™ R1 media and C8 or C18 supports for POROS™ R2 media. POROS™ R1 and R2 media show stronger binding toward some highly aromatic species. In addition, the complete lack of residual silanol activity modifies the binding characteristics of ionic species.

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

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

Table 2 Product characteristics

Support Matrix	Cross-linked poly(styrene-divinylbenzene)	
Surface Functionality	None [native poly(styrene-divinylbenzene)]	
Dynamic Binding Capacity @ 3,600 cm/hr	Lysozyme, 1% acetonitrile **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.	
	POROS™ R1	5 mg/ml
	POROS™ R2	10 mg/ml
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 M HCl)
Buffer Additives	All common agents, including THF (tetrahydrofuran), 8 M urea, 6 M guanidine hydrochloride, ethylene glycol, and detergents. **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	0–100% water, alcohols, acetonitrile, other common organic solvents **Note:** Do not expose to strong oxidizers (such as hypochlorite), oxidizing acids (such as nitric), or strong reducing agents (such as sulfite). **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.
Operating Temperature	5–80 °C

Packing the column

POROS™ 20 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 10 µm or less.

Precautions

CAUTION! POROS™ 20 bulk media are supplied as a dry powder, which may form a light dust. Read the MSDS and follow handling instructions. Avoid inhaling the dust: some surface chemistries are irritants. Keep the media container closed when it is not in use.

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

Preparing the slurry

To form the slurry:

IMPORTANT! Do not use a magnetic stirrer. It 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 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 170 bar.
2. Add the desired mobile phase for packing. Isopropanol is recommended.

   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 the best results when you pack the column:

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

To pack a column:

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 off the column 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. 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.

Selecting and preparing the mobile phase

Regardless of the mobile phase you choose:

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

Solvents

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

- Acetonitrile is the preferred solvent for reversed-phase chromatography.
- 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. However, adding 10% THF or acetonitrile to alcohol-based mobile phases can improve chromatographic performance.

Mobile phase

The polymeric nature of POROS™ R1 and R2 media means that they can 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 the mobile phase:

- 10 mM tribasic phosphate solution gives 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 a pH mapping experiment can be completed quickly.

Additives

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 (trifluoroacetic acid) may no longer be necessary for separation performance, although they may still be needed for solubilization of the sample.
- Other additives such as hydrochloric, 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 upon 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.

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, it is necessary to do the sample injection at 5% of the organic. 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 capacity of POROS™ R1 and R2 media is in the range of 0.1 to 5 mg/ml for most proteins.

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

However, the maximum loading at which a given resolution can be obtained (the loadability) depends on a number of factors, including sample solubility, column selectivity, and so on.

To perform a loadability experiment:

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

IMPORTANT! 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 reoptimization quick and easy, especially if you use the template features of the BioCad™ Workstation.

Eluting the sample

You can elute the sample using isocratic or gradient conditions.

- Many peptides are optimally eluted isocratically, but because of the extremely high sensitivity to elution strength, it is usually more practical to use very shallow gradients(<5% elution strength range).
- 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.

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 you observe:

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

IMPORTANT! 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), and 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 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, and extends the practical life of your column.

Multiple injections

You can make multiple injections of regeneration solutions instead of pumping them directly. This method is recommended when using 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.

IMPORTANT! 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.

To store a packed column:

- Carefully seal the ends of the column to prevent drying. Drying results in decreased chromatographic efficiency.
- Store the column in any appropriate mobile phase.
- 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.

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 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 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.

If there is any question about gradient performance, 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 any solutions you make up from solid components.

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 × 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 BioCad™ Workstation allows 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 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 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 the standard test protocols for POROS™ 20 R1 and R2 media to:

- Confirm that the media 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 Reversed-Phase Protein Test Standard available from **thermofisher.com**.

Run the separation with a linear gradient in acetonitrile. Run conditions are described below.

To perform the protein separation test:

1. Dissolve the lyophilized sample mixture in 1 ml of Eluent A (5 mg/ ml soybean trypsin inhibitor, (5 mg/ml bovine heart cytochrome C).
2. Filter the sample after thorough mixing.
3. Store the reconstituted test mix frozen.
4. Run the sample.

Run conditions

Table 4 Protocol

Parameter	Specification
Eluent A	0.1% TFA in water
Eluent B	0.085%-0.1 % TFA in acetonitrile
Flow rate	2,000 cm/hr
Sample	1–2% of column bed volume
Gradient	15–45% B in 5 minutes
Detection	220 nm

Results

The standard chromatogram shown in Figure 1 on page 5 is for a 4.6mm × 100mm column packed with POROS™ 20 R1. Although the retention times and bandspreading may vary with different column sizes and systems, the general profile should be similar.

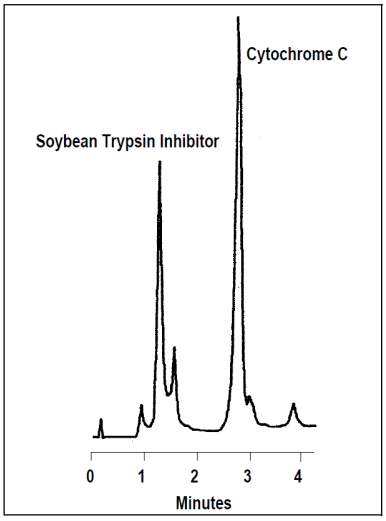

Fig. 1 POROS™ 20 R1 chromatogram

Ordering information

A reversed-phase test standard is available from **thermofisher.com**.

Table 5 Test standard ordering information

Description	Quantity	Cat. No.
Reversed-Phase Protein Test Standards	Package of 5 vials	1-9001-05

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

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

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