POROS™ 20 MC Perfusion Chromatography™ Bulk Media for Metal Chelate 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™ technology 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 you can operate 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/h.

Linear velocity (cm/h) is calculated by dividing volumetric flow rate (cm^3/min) by the column cross-sectional area (cm^2) and multiplying by 60 min/h.

The maximum flow rate is not limited by the media itself (high resolution separations have been achieved at 10,000 cm/h) 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/h.

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/h)
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 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™ or BioCad™ Workstation for Perfusion Chromatography™.

Product description

POROS™ MC media is a polymeric packing designed for immobilized metal affinity chromatography of peptides, proteins, 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 very rapid mass transport.

Particles are surface-coated with a cross-linked polyhydroxylated polymer functionalized with imidodiacetate groups. This functionality allows binding through bidentate ligation of a wide range of transition metals. Binding to proteins is by the formation of coordination complexes between the remaining metal coordination sites and certain surface amino acids, particularly histidine, cysteine, and tryptophan.

Table 2 Product characteristics

Support Matrix	Cross-linked poly(styrene-divinylbenzene)
Surface Functionality	Imidodiacetate ($-CH_2N(CH_2CO_2^-)_2$)
Dynamic Binding Capacity @ 3600 cm/h	Myoglobin, Cu^{2+} form 20 mg/ml
Metal Capacity	65 µmol/ml (Cu^{2+})
Particle Size	20 µm
Recommended maximum flow rate	10,000 cm/h
Maximum pressure drop	170 bar (2,500 psi, 17 MPa)
Permeability	<3 bar at 1,000 cm/h (3 cm bed height)

Table 3 Chemical resistance

pH Range	1-14 (Up to 5.0 M NaOH, 1.0 M HCl)
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	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).
Operating Temperature	5-80 °C

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. Use column bed supports (frits or screens) with a porosity of 10 µm or less.

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: 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 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.
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 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 giving a final pressure about 20 to 50% greater than the maximum anticipated operating pressure.
7. Once 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 loading the metal ion

The metal ion loaded on the imidodiacetate chelating groups on POROS™ MC media has a critical effect on both the binding strength and selectivity for different proteins. Consider the following information as you choose a metal ion:

- The most commonly used ions, in order of greatest to lowest binding strength, are:

  Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} (these last two ions are of about equal binding strength)
- You can also use other metal ions, including Fe^{3+} and Al^{3+}. Fe^{3+} is unusual in that, unlike other ions, elution can often be effected by increasing pH or increasing concentrations of salt.
- Use Cu^{2+} for initial studies when nothing is known about the characteristics of the protein of interest. Cu^{2+} tightly binds to any protein that will work with metal chelate chromatography. Then substitute other metal ions to increase selectivity for the protein of interest or key contaminants, or to reduce binding strength for improved recovery.

Saturating the imidodiacetate sites

Make sure that the imidodiacetate sites on the column are fully saturated with the proper metal. To do this:

1. Before loading the metal, perform a stripping wash with 10 to 20 column bed volumes of 50 mM EDTA in 1 M NaCl.
2. Wash with 5 to 10 column bed volumes of water.
3. Depending on pH levels, do the following:
   - Load metal ions as sulfate or chloride salts in weakly acidic solutions (pH 4.5 to 5) to avoid precipitation of metal hydroxide complexes.
   - Load Fe^{3+} under more acidic conditions (pH 2 to 4) because of solubility difficulties at higher pH.

   The concentration of the metal salt is not critical (0.05 to 0.25 M concentrations are generally used).
4. Load at least 0.25 mM metal/ml column volume (50 ml of 0.1 M for a 1.7 ml column).

   With Cu and Ni, you can observe metal breakthrough visually or with a UV detector.
5. Wash with 5 to 10 column bed volumes of water to remove excess metal.
6. Wash the column with 5 to 10 column bed volumes of 0.1 to 0.5 M NaCl to remove metals that may be bound ionically.
7. Wash with 5 to 10 column bed volumes of starting buffer.

Note: Wash thoroughly with salt solution between metal loading steps. Any condition with free metal ions in solution can lead to precipitation and column plugging. If plugging occurs, wash the column at low flow rate with dilute acid to redissolve the metal.

Stripping and reloading the column

Whether or not you should strip and reload the column with metal between runs depends on the metal used and the elution protocol:

- Reloading is essential with weakly complexing ions such as Zn^{2+} or Co^{2+}.
- Cu^{2+}-loaded columns can sometimes be used for many runs without reloading of metal, unless stripping eluents such as EDTA are used.

In all cases, stripping and reloading after every run gives maximum reproducibility.

Selecting a buffer and an elution method

Because metal chelate chromatography is not as well developed as more traditional chromatography modes such as ion-exchange or reversed-phase, it is virtually impossible to predict retention behavior. As a result, conditions for binding and elution must often be developed by trial and error.

However, very short run times on POROS™ MC columns make this development process much faster than with conventional media.

Starting buffers

Regardless of the starting buffer and elution method you choose, it is always important to:

1. Use buffers of the highest purity practical.
2. Degas and filter (0.22 or 0.45 µm) all buffers prior to use.
3. Maintain relatively high ionic strength (0.1 to 1.0 M) throughout.

Follow these guidelines when you select a starting/wash buffer:

- Starting buffers can be in the pH range 4 to 8.5. Generally, pH 7 to 8 gives the best results.
- Acetate and phosphate buffers often result in strong binding.
- Buffers containing primary amines (such as Tris) often weaken binding and can strip metals, but may still be used in some cases.
- 0.1 to 1.0 M NaCl is recommended to suppress secondary ionic interactions and protein/protein interactions.
- If the column is run saturated with elution agent (see "Elution method" on page 3), the starting buffer should also contain 0.5 mM of the elution agent.
- Chaotropic agents such as guanidine/HCl or urea may be used if needed.

Elution method

Several methods of elution are possible:

- Reduce the pH to the range of 3.0 to 6.0.

  Acetate and phosphate buffers are widely used since they can be used to form continuous pH gradients in this range.

  However, reducing the pH frequently does not elute tightly binding proteins and often gives poor reproducibility and resolution. In addition, the pH may pass through the isoelectric point of some sample proteins, causing precipitation.
- Use increasing gradients of solutes that compete with the protein for metal binding sites.

  Increasing the gradient gives better results than reducing the pH. In most cases, imidazole works well in relatively low concentrations (usually 1 to 50 mM, although as high as 250 mM may be needed).

  In cases where binding is too weak for imidazole to be useful, use 2-methyl pyridine. Ammonium salts or glycine are sometimes used in relatively high concentrations (0.1 to 1.0 M), but usually give poor resolution.
- Use a chelating agent such as EDTA (50 to 500 mM) to remove both the metal ion and the protein.

  A chelating agent always elutes specifically bound protein, and it is recommended for regeneration of the column. However, it does not work in gradient mode and cannot be used to separate bound proteins from each other.

Improving selectivity and recovery

For many applications, you can improve selectivity and recovery by saturating the column with the displacing elution agent (such as imidazole) before loading the sample. This replaces water on most of the metal coordination sites with the elution agent and results in a favorable reduction in the binding strength. To do this:

1. After the metal is loaded and free metal washed off, wash the column with 5 to 10 column volumes of the highest concentration of the agent used for elution (or until a breakthrough is observed on the UV detector).
2. Wash with 5 to 10 column volumes of starting/wash buffer. The starting/wash buffer should contain a low concentration (0.5 mM) of the elution agent, to keep the column saturated.

Preparing and loading the sample

To ensure efficient binding and prevent column plugging:

1. Dissolve or exchange samples into the starting buffer. It is important to expose the sample to the salt concentration in the starting buffer to remove any components which may precipitate.
2. Centrifuge or filter (0.22 or 0.45 µm) samples before injection.
3. Delipidate samples, if possible. Lipids can cause irreversible column fouling.

Determining sample load

The dynamic binding capacity of POROS™ MC media is in the range of 5 to 20 mg/ml for most peptides and proteins.

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

However, the maximum loading at which a given resolution can be obtained (the loadability) 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™'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 (through increasing either injection volume, 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 reoptimization quick and easy, especially if you use the template features of the VISION™ or BioCad™ Workstation.

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 during blank gradient runs

Note: 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.

Regenerating the column

In any regeneration step, it is vital to first strip the column of metal ions. Otherwise, they may precipitate irreversibly and destroy the column.

1. To strip the column, wash with 1 to 5 column volumes of 0.1 M EDTA in 1 M NaCl.

   Usually, stripping the column with EDTA is enough to restore column performance. If it is not:
2. Wash with 1 to 5 column volumes of 1 M NaOH (preferably including 1 to 2 M salt).
3. Wash with 1 to 5 column volumes of 1.0 M acetic acid, 1.0 M hydrochloric acid, or 1% TFA.
4. Wash with water to remove the acid.
5. Reequilibrate the column with starting/wash buffer.

Removing lipids and lipoproteins

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

- Use a mixture of 50% methanol or acetonitrile with the acid or base.
- Use a mixture of 50% methanol and 50% 3 M guanidine thiocyanate.
- Store the column overnight in 1 mg/ml pepsin, DNAse, or other enzymes

Note: Take care when using thiocyanate with metal systems. Thiocyanate forms complexes with iron that strongly absorb UV light.

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: 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 prepacked column:

- Carefully seal the ends of the column to prevent drying. Drying results in decreased chromatographic efficiency.
- Store the column between 5 and 30 °C.

Short-term storage

Store the column in any appropriate mobile phase.

Long-term storage

- Flush the column with 0.1 M EDTA in1 M NaCl
- Store the column in 0.1 M Na_2SO_4 with any of these solutions:
  - 0.2% sodium azide
  - 30% ethanol
  - 30% methanol

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 in step 1 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 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 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 × 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

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**.

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