Bio-Sil and Bio-Silect SEC HPLC Columns

Instruction Manual

For Technical Service
Call Your Local Bio-Rad Office or
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BIO-RAD

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Section 1
Introduction

Bio-Sil and Bio-Silect HPLC columns line provide excellent column efficiency and reproducibility. Packed with Bio-Rad's proprietary Bio-Sil SEC 125, 250, and 400 packing materials, both Bio-Sil and Bio-Silect SEC HPLC columns are ideal for protein, peptide, and nucleic acid separations in the 5,000 to 1,000,000 Daltons molecular weight range.

Bio-Sil and Bio-Silect SEC columns separate compounds by gel filtration chromatography. Also called size exclusion chromatography, the technique is based on diffusion in and around highly porous spherical silica beads. The degree of retention depends on the size and shape of the solute molecule and the pore size of the packing. Large molecules are sterically excluded and pass around the beads, eluting from the column before smaller molecules, which totally or partially permeate the material and are eluted later. The working range of the gel is the volume between the void volume, those solutes which elute first, and the totally included volume, those solutes which elute last.

Section 2
Column Set-up

2.1 Unpacking

While unpacking the HPLC column, check it carefully for evidence of shipping damage, rough handling, or solvent leakage. Save the shipping container to store the column. If there is evidence of damage, immediately call our Technical Services Hotline at 1-800-4BIORAD or contact your local Bio-Rad representative.

2.2 Column Installation

Bio-Sil columns include a pair of reverse nuts and ferrules for easy installation. All Bio-Sil stainless steel 80 x 7.8 mm guards and 300 x 7.8-mm columns use Parker end-fittings. All Bio-Sil stainless steel 600 x 7.5 min and 600 x 21.5 mm columns use TSK-type end fittings. All Bio-Silect PEEK 50 x 7.8 mm and 300 x 7.8 mm columns include a pair of universal finger tightenable fittings.

If your system tubing is not outfitted with Parker or plastic finger tight fittings, the system can be made Parker compatible by cutting off

the incompatible fittings from the system tubing and replacing the incompatible fittings with either the Parker nuts and ferrules supplied with the column or with universal finger tightenable fittings.

To keep air out of the solvent delivery system, start the mobile phase flowing slowly (0.2 ml/min) through the inlet tubing prior to connecting the column. (If using a guard column, install the guard column before installing the analytical or preparative column, following the column installation instructions below).

1. Remove the protective nut (Bio-Sil stainless steel columns) or end plug (Bio-Silect PEEK columns) from the inlet end of the column (refer to the flow direction arrow printed on the column tag).
2. With a flow rate of 0.2 ml/min, connect the inlet tubing to the column.

   If using a Bio-Sil stainless steel column, push the tubing in until it bottoms firmly. Using a 1/4" wrench, tighten 1/4 turn. The fitting only needs to be tight enough to seal; its lifetime will be diminished by over-tightening.

   If using a Bio-Silect PEEK column, finger tighten the fitting only until the connection does not leak.
3. Immediately remove the end nut or plug from the outlet end of the column.
4. When the solvent is flowing freely from the outlet end of the column, connect the column to the detector. Make sure air does not get into the column. If there is reason to believe that it has, disconnect the tubing from the outlet end of the column and monitor the solvent flow (< 0.3 ml/min for 7.5 mm and 7.8 mm ID columns; 1.5 ml/min for 21.5 mm ID columns) from the column outlet until the air is eliminated and solvent is flowing freely. Then reconnect the column to the detector inlet tubing.
5. The column is delivered in 0.05% sodium azide in water and should be equilibrated with at least 5 bed volumes of mobile phase prior to sample injection.

Section 3
Operating Parameters

Table 1. Operating Parameters

Column Dimensions (mm)	Operating Flow Rate (ml/min)	Maximum Flow Rate (ml/min)l	Operating Pressure (psi)[2]	Maximum Pressure (psi)[2]	Maximum Temperature (°C)
Bio-Sil and Bio-Silect 50 mm, 80 mm, and 300 mm long, 5 µm particle size columns:					
50 x 7.8	0.5-1.0	1.5	<300	1,500	45
80 x 7.8	0.5-1.0	1.5	<500	1,500	45
300 x 7.8	0.5-1.0	1.5	<1000	1,500	45
Bio-Sil 600 mm long, 10 µm particle size columns:					
600 x 7.5	0.5-1.0	1.2	<500	700 (SEC 250)	45
600 x 21.5	3.0-6.0	8.0	<300	400 (SEC 250)	45

1 Flow rates given pertain to low viscosity solvents; use lower flow rates with water-alcohol mixtures, guanidine hydrochloride, or urea aqueous solutions.

2 Pressure ratings are dependent upon system variations, flow rates, and solvent viscosities. Operating and maximum pressure ratings reflect the pressure drop across the column, not the total system pressure. To determine the column back pressure, read the total system operating pressure, disconnect the tubing between the guard column and the analytical column, and note the pressure drop. (Note: remember to reduce the flow rate of the pump before reconnecting the column, then slowly return to the full flow rate over a 2-5 minute period).

If the total back pressure of the system increases during use, repeat the above procedure to determine the cause. If the column back pressure increase is small, and is due to the guard column, the system can be operated normally. If the increase in guard column pressure is greater than 150% of the pressure when new, the guard column should be cleaned or replaced.

If the system increase is caused by the column, the column may be partially clogged and flow rates should be reduced to stay within the operating limits. Occasionally, column pressure increases are caused by clogged frits. Column cleaning and reverse flow may help to reduce the back pressure.

Avoid sudden pressure surges on the column. The packing may compress, which will result in tailing and decreased column efficiency.

3.1 Operating Precautions

Read these precautions carefully before installing the column.

1. Before injecting any samples on the column, generate a chromatogram using the enclosed Gel Filtration Standard (catalog number 151-1901). Duplicate the conditions listed in Section 3.5, Column Testing. Your chromatogram should closely match the test chromatogram provided.

2. If several columns are to be connected in series, connect them in order of decreasing pore size.

3. To keep dead volume to an absolute minimum throughout the system, all components should be coupled as closely as possible.

4. Keep air out of the plumbing system. Thoroughly degas all aqueous and organic solvents prior to use. Before installing the column, flush 20 ml of priming solvent through the system to insure that air, oils, and particulate matter are washed out.

5. Avoid sudden pressure surges on the column. This may compress the packing, which will decrease column efficiency. The flow rate should always be increased gradually over a period of 2-5 minutes. Under no circumstances should the flow rate exceed the maximum rate specified for the column. See Table 1 for flow rate and pressure specifications.

6. Keep the column at a relatively constant temperature. It is possible to heat the column if the following precautions are observed:

   a. The column may be safely operated at temperatures above 10 ^{o}C and below 45 ^{o}C. However, extended operation above 45 ^{o}C will shorten the column life. Below 10 ^{o}C, increases in solvent viscosity will require the use of a lower flow rate for column protection.

   b. The solvents are completely degassed.

   c. After operation, the solvent flow is maintained while the column temperature is being lowered to ambient. If this precaution is not taken, the solvent will contract while cooling and pull air into the column.

7. To extend the life of the Bio-Sil or Bio-Silect SEC column, the use of the appropriate Bio-Sil or Bio-Silect SEC guard is recommended.

3.2 Solvents

1. **Aqueous Salt Solutions and Buffer Solutions.** Bio-Sil and Bio-Silect columns are stable in typical aqueous salt and buffer solutions. Typical aqueous salt solutions include sodium sulfate, potassium dihydrogen phosphate, ammonium acetate, and ammonium formate aqueous solutions.

Typical buffers include phosphate, tris acetate, citrate, and acetate buffers. In general, keep the salt concentration below 0.5 M in order to avoid viscosity rise and salt precipitation. Halogen salts (e.g. sodium chloride, potassium chloride, etc.) are not recommended if using a stainless steel column and/or stainless steel system and tubing. If halide ion-containing solvents are used, it is recommended to rinse the column with deionized water for long-term storage of the column.

2. **Organic Solvents, Solublizing Agents, and Protein Denaturants.** Bio-Sil and Bio-Silect columns are stable in solvents typically used in HPLC such as methanol, ethanol, acetonitrile, DMSO, 6 M guanidine hydrochloride, 0.5% SDS, aqueous urea, or THF. However, there is a tendency toward shorter column life in such systems than with standard aqueous salt and buffer solutions. Avoid using a column that has been used for such systems with other systems. When using higher viscosity solvents, reduce the flow rate accordingly to protect the column. When switching from an aqueous solvent to an organic solvent, first equilibrate the column in 5% organic solution at a low flow rate until a stable baseline is maintained, then increase the organic modifier to the desired concentration. Try to avoid frequent changes in the concentration of organic modifiers in the mobile phase. Be careful of salt precipitation when adding salts to an aqueous solution containing organic solvent.

3. **Solvent and Sample Preparation.** Filter and degas all solvents. Filter or centrifuge the sample to remove particulates. Use only HPLC grade solvents; poor baseline stability is often caused by dirty solvents. In working with the typical buffers used in biological applications, care must be taken to inhibit microbial growth in the mobile phase. Sodium azide (0.05%), 5% methanol in water, or other appropriate bacteriostats should be used. Solvents should be passed through a 0.45 micron filter before use.

4. **pH**. Never expose the column to pHs above or below the pH tolerance limits specified for your column. For extended column life, keep the pH of the mobile phase 1/2 pH unit above the lower specified limit or 1/2 pH unit below the upper specified limit (see Specifications, Section 6.)

3.3 Ionic Strength and Organic Modifier Effects

Protein behavior in solution is very dependent on ionic strength and the presence of organic modifiers.

1. Protein solubility in the mobile phase should be determined before an injection is made. Biochemical reactions are occasionally done under one set of conditions and subsequent separations under another. Be sure the mobile phase and the sample to be injected are compatible; if possible, dissolve or dialyze the sample into the mobile phase prior to injection on the column.

2. Buffer salts reduce ionic interactions between the gel filtration matrix and sample components, while organic modifiers reduce hydrophobic interactions. If some components seem to be adhering to the gel, the problem can often be resolved by increasing the ionic strength of the buffer, or by adding increasing concentrations of organic modifiers such as DMSO, methanol, or acetonitrile.

3. Denaturing conditions will often cause proteins to unfold and exhibit much greater effective molecular size, so proteins which may be included under normal physiological conditions may be excluded when fully denatured. Thus, be careful in selecting the column pore size for the measurement of denatured proteins. When calibrating a column, always use the same mobile phase and sample solvent systems with the calibration standard as with the sample to be analyzed to ensure reliable quantitative results.

3.4 Gel Filtration Standard

Included with the column is one vial of a lyophilized protein test mix (catalog number 151-1901) containing thyroglobulin, gamma globulin, ovalbumin, myoglobin, and vitamin B_{12} (cyanocobalamin). Both myoglobin and vitamin B_{12} serve as colored markers.

Table 2. Bio-Rad's Gel Filtration Standard

Component	**MW (daltons)**
Thyroglobulin, 5.0 mg	670,000
Bovine gamma globulin, 5.0 mg	158,000
Chicken ovalburnin, 5.0 mg	44,000
Equine myoglobin, 2.5 mg	17,000
Vitamin B_{121} 0.5 mg	1,350

3.5 Column Testing

Instructions

Rehydrate the Gel Filtration Standard by adding 0.5 ml of deionized HPLC grade water. Swirl gently to mix and allow the vial to stand for 2-3 minutes. Centrifuge the standard before application to remove any fine particulates. Swirl the vial again and apply the appropriate volume of standard to the column. The test mix must be refrigerated following rehydration and should be stored frozen if kept longer than 2 weeks. Store at -70 ^{o}C if possible. If only occasional standard injections are desired, best results are achieved by using a freshly prepared standard each time. **Examine the gel filtration standard vial for precipitate before every injection.**

Use the following test conditions to simulate the enclosed chromatogram **before any other injections are made.**

Injection:	20 microliters, Gel Filtration Standard
Eluant:	0.05 M NaH_2P0_4
	0.05 M Na_2HP0_4
	0.15 M NaCl
	0.01 M NaN_3
	pH 6.8
Temperature:	Ambient
Detector:	UV absorbance at 280 nm, 1.28 AUFS
Flow rate:	1.0 ml/min (7.5 mm ID and 7.8 mm ID columns)
	6.0 ml/min (21.5 mm ID columns)

Section 4
Column Maintenance Procedures

4.1 Column Backflushing

Silica-based resins are resilient. However, if there is evidence of bed compression (refer to the Troubleshooting Guide) gentle backwashing can help to restore a collapsed bed to its original configuration or allow entrained air spaces to redissolve. If bed compression is severe, the column may not return to original performance. Backwashing is also a cost effective means of clearing a blocked column frit, and solves the problem about one time out of three cases.

1. Shut off the pump and let the column bed "relax" for about 15 minutes.
2. Reverse the flow direction and backwash the column at 0. 1 ml/min with the running solvent for at least 4 hours (if necessary, run overnight).
3. Return the column to original operating conditions.
4. Observe the suggested maximum flow rate.

4.2 Column Wash

Prolonged operation with complex mixtures may lead to the gradual accumulation of strongly ionic or hydrophilic sample components. These compounds will decrease the resolution of the column and alter the column's elution profile of standard solutions. The adsorbed compounds can usually be removed with the following wash procedure.

Start this procedure with Step 1, followed by each of the next steps if necessary. Remember, most back pressure problems are caused by "dirty" or old guard columns which should be either cleaned (following the procedure below) or replaced. Clean the guard and analytical (or preparative) columns separately.

1. Invert the guard or column and wash with five column volumes of HPLC grade deionized water at 0.3 ml/min (1.5 ml/min for 21.5 mm ID columns).
2. Return the column to its normal operating orientation. At the flow rates recommended in step 1, wash with 5 column volumes of 0.3 M NaH_2PO_4, pH 3.5 (to remove hydrophilically adsorbed material) or run a 5 column volume 0-100% acetonitrile or methanol gradient (to remove hydrophobically adsorbed material).
3. If there is little or no improvement in column performance and/or back pressure after completing step 2, wash with five column volumes of 6 M guanidine-HCI.
4. If Step 3 proves ineffective, wash with 5 column volumes of 0.1% trypsin in 0.01 M phosphate, 0.15 M NaCl, pH 6.8, as a final resort.

Always remove the guard before proceeding with the column wash.

4.3 Column Storage

If the column will not be used for several days, it should be stored as follows:

1. After rinsing with several column volumes of distilled water, replace the mobile phase with 0.05% sodium azide or 5% methanol in water. Use a low flow rate (0.3 ml/min for 7.5 and 7.8 mm ID columns; 1.5 ml/min for 21.5 mm ID columns) during solvent replacement.
2. Keep the ends of the column tightly capped. Use the protective nuts or end plugs originally furnished with the column. **Do not allow the resin to dry out.**

   The column can be safely stored for short duration (1-2 days) in HPLC grade deionized water. If the column is used daily, it is safe to leave the buffer in the column overnight as long as the buffer is not corrosive. It is highly recommended to maintain a low flow (< 0.5 ml/min) of solvent through the column to prevent precipitation of salts.

Section 5
Troubleshooting

Table 3. Troubleshooting Guide

Problem	Symptom	Solution	See Section
1. Collapsed bed (void formation)	Poor peak shape or peak splitting; loss of efficiency; possible increase in pressure	1) Backflush column 2) If no improvement, discard column	4.1
2. Strongly adsorbed sample	Gradual increase in pressure during use; loss of efficiency; decreased retention	1) Backflush column 2) If necessary, follow wash procedure	4.1 4.2
3. Air in column or system	Recorder will not zero; spikes on baseline	Disconnect column at detector; purge column with mobile phase; prime pump(s)	3.1, 4
4. Microbial contamination	Increase in pressure; noisy baseline; poor column performance	1) Backflush column 2) Follow wash procedure 3) Discard column if no improvement	4.1 4.2
5. Bed degradation from extreme pH exposure	Gradual increase in pressure during use	No solution; discard column	-
6. Blocked column frit	Gradual or sudden pressure increase	1) Backflush column 2) If necessary, follow wash procedure	4.1 4.2
7. Inability to match test chromatogram	Low efficiency; poor resolution; increase in retention	Excessive dead volume 1) Check system fittings for leaks 2) Minimize intersystem tubing lengths Run conditions not exactly duplicated	 2.2 3.1 3.5

Section 6
Column Specifications

6.1 Bio-Sil Stainless Steel SEC HPLC Columns

Catalog Number	Column	Dimensions (mm)	MW Range (proteins)	Particle Size (µm)	Capacity (protein, mg)	pH Range
125-0060	Bio-Sil SEC 125-5 Column	300 x 7.8	5,000-100,000	5	0.01-1.5	2-8
125-0062	Bio-Sil SEC 250-5 Column	300 x 7.8	10,000-300,000	5	0.01-1.5	2-8
125-0064	Bio-Sil SEC 400-5 Column	300 x 7.8	20,000-1,000,000	5	0.01-1.5	2-8
125-0066	Bio-Sil SEC 250 Colurrm	600 x 7.5	10,000-300,000	10	0.01-1.5	2-7
125-0072	Bio-Sil SEC 125 Guard	80 x 7.8	5,000-100,000	5	0.5	2-8
125-0073	Bio-Sil SEC 250 Guard	80 x 7.8	10.000-300.000	5	0.5	2-8
125-0074	Bio-Sil SEC 400 Guard	80 x 7.8	20,000-1,000,000	5	0.5	2-8
155-0201	Bio-Sil SEC 250 Column	600 x 21.5	10,000-300,000	13	10-100	2-7
155-0203	Bio-Sil SEC Prep Guard	75 x 21.5	_	13	_	2-7

6.2 Bio-Silect Biocompatible PEEK SEC HPLC Columns

Catalog Number	Column	Dimensions (mm)	MW Range (proteins)	Particle Size (µm)	Capacity (protein, mg)	pH Range
125-0475	Bio-Silect SEC 125-5 Column	300 x 7.8	5,000-100,000	5	0.01-1.5	2-8
125-0476	Bio-Silect SEC 250-5 Column	300 x 7.8	10,000-300,000	5	0.01-1.5	2-8
125-0477	Bio-Silect SEC 400-5 Column	300 x 7.8	20,000-1,000,000	5	0.01-1.5	2-8
125-0478	Bio-Silect SEC 125 Guard	50 x 7.8	5,000-100,000	5	0.01-1.5	2-8
125-0479	Bio-Silect SEC 250 Guard	50 x 7.8	10,000-300,000	5	0.01-1.5	2-8
125-1480	Bio-Silect SEC 400 Guard	50 x 7.8	20,000-1,000,000	5	0.01-1.5	2-8

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