



Maintenance and cleaning of size exclusion chromatography columns

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Introduction

Size exclusion chromatography (SEC) is a powerful technique for separation of proteins according to differences in size. The prepacked SEC columns—HiLoad™, Tricorn™, and Precision columns—are all produced with high quality in focus where each column is tested regarding column efficiency (N/m), peak symmetry (A_s), and pressure drop over the packed bed (Δp). There are many ways to maintain the high performance of the column and assure a long column life. First, to avoid compressing the packed bed, it is most important to apply the recommended flow rate provided in the column instructions. Also, regular cleaning of the column will contribute to optimal performance. The following guidelines will help you to keep your SEC columns in good condition.

First-time use

Before connecting the column to your system, make sure there is no air in the tubing and valves before the column. It is important to exclude air from entering the column when running SEC.

All SEC columns are delivered in 20% ethanol to prevent microbial growth. The first thing to do before use is to wash out the ethanol. Since ethanol is a viscous solution, a slow flow rate must be used to avoid compressing the packed bed. We recommend using approximately 50% of maximum flow rate at room temperature for your column (see the instructions for your column).

In the instructions for each column, a value for maximum pressure or typical pressure drop over the packed bed is given to protect the packed bed from compression; do not exceed this value at any time. For columns having a given typical pressure value, we recommend that you determine the individual column pressure limit according to the procedure described in the column instructions (see also Appendix 1). By doing this, you will be able to use the full potential of each individual column.

To follow the performance of each column over time, we recommend that you perform an initial efficiency test (see more details in *Column efficiency* and Appendix 2).

Briefly:

1. Connect the column to the system without introducing air
2. Wash out the ethanol at slow flow rate and equilibrate with buffer
3. If needed, determine the individual column pressure limit
4. Perform a column efficiency test
5. Your column is now ready for use

Lower flow rate at low temperature

The maximum flow rate when working at low temperatures, such as in a cold room, is approximately 50% of maximum flow rate at room temperature. Do not exceed the pressure limit of the column. Also, keep buffers and columns at the same temperature during purification to prevent air bubble formation in the column. An example of the relationship between column pressure and flow rate for Superdex™ 200 Increase 10/300 GL column at different temperatures is shown in Figure 1.

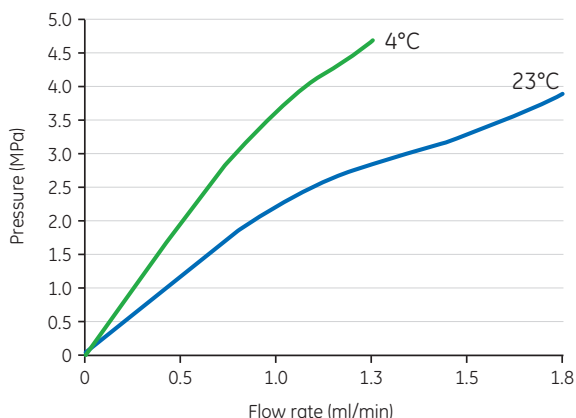


Fig 1. Column pressure at different flow rates and different temperatures on Superdex 200 Increase 10/300 GL column in water.

Column efficiency

A column efficiency test is a quick way to check the column and system performance, see Appendix 2. This test should be performed with the same system setup that is to be used for the column since the result for column efficiency is dependent on the system used, including capillaries and dead volumes. This means that the column efficiency given in the specification or Certificate of Analysis (CofA) for the column will not be exactly the same as your initial column efficiency result.

Regular column efficiency tests are recommended to follow the performance of the column over time, for example, after cleaning.

Sample preparation

Always filter your sample through a 0.22 µm filter (or centrifuge at 10 000 × g for 10 min) to avoid particulate matter contaminating the column (see 29-1132-15, *Guide to laboratory filtration* for information on our available Whatman™ filters). Perform the filtration just before beginning the purification to avoid any changes of the sample during storage. Also, make sure that no precipitation of the sample occurs in contact with the running buffer. Avoid extreme changes in pH or other conditions that might cause precipitation.

Denaturing agents and detergents are useful as solubilizing agents for proteins with low aqueous solubility, such as membrane components. If used, solubilizing agents must be present all the time, both in the running buffer and in the sample.

Buffers

Always use filtered and degassed buffers. Include 150 to 300 mM sodium chloride in the buffer to reduce any nonspecific interactions. Select buffer conditions that are compatible with protein stability to prevent sample precipitation. When using buffers with increased viscosity (for example containing urea or glycerol) it is necessary to reduce the flow rate to avoid compressing the packed bed. See the column instructions for recommended additives and buffers. In Figure 2, the relationship between viscosity and pressure is illustrated for 1 M sodium chloride, 8 M urea, and 30% 2-propanol.

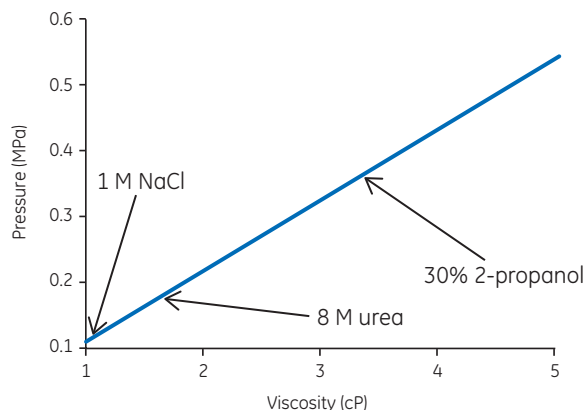


Fig 2. Relationship between pressure on the column and viscosity of the buffer used.

Regular column cleaning

Routine cleaning of the SEC column, for example after every 20 runs (depending on sample), is highly recommended. Cleaning is performed in order to remove any precipitated proteins or other contaminants that can build up on the column. Cleaning is also necessary if a colored band is seen at the top of the column, when loss in resolution is observed, or if a significant increase in back pressure is detected.

Reversing the flow during cleaning of Superdex and Superose™ columns is recommended. However, reversed flow during cleaning of HiPrep™ Sephacryl™ columns should only be considered under cases of severe contamination.

Cleaning is performed at low flow rates using 1 CV of sodium hydroxide (500 mM sodium hydroxide for Superdex and Superose columns and 200 mM sodium hydroxide for Sephacryl columns). Note that the column should never be stored in sodium hydroxide. Equilibrate the column immediately after the cleaning with 2 CV of water followed by 2 CV of running buffer.

If cleaning using sodium hydroxide is not sufficient, additional cleaning using for example 500 mM acetic acid can be useful. Check the instruction for your specific column on details of the cleaning procedure.

Figure 3 shows the effect on resolution of a “dirty” column and the effect after cleaning the column with sodium hydroxide. For more information on this topic, please refer to Application note 18-1124-57, *Use of sodium hydroxide for cleaning and sanitation of chromatography media and systems*.

Storage of SEC columns

If the column is to be stored for more than 2 d after use, wash it with 2 CV of water and then equilibrate with at least 2 CV of 20% ethanol (for HiLoad Superdex 30 pg and Superdex 75 pg, use 200 mM sodium acetate in 20% ethanol).

Note: Use a lower flow rate for viscous 20% ethanol.

Store the column in 20% ethanol to prevent any microbial growth. Connect the transport tool to the column outlet, to prevent air entering the column. Fill the transport tool up to approximately 50% of its total volume.

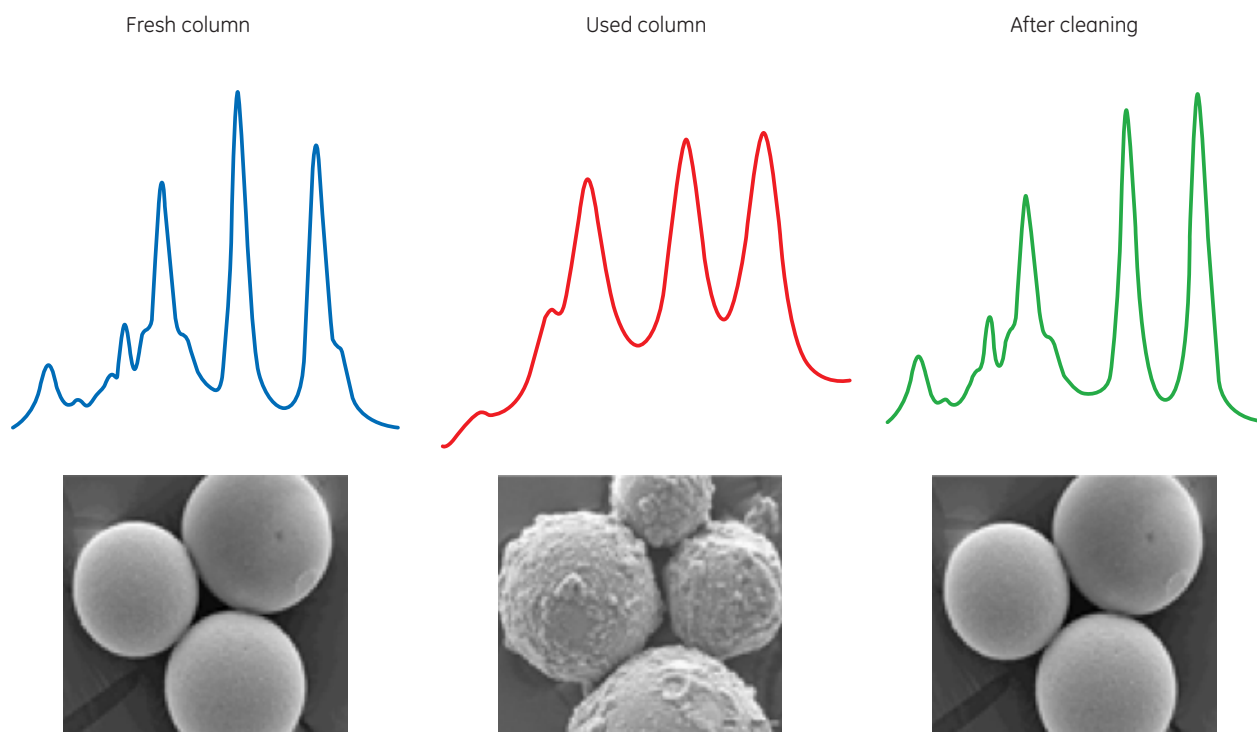




Fig 3. Separation resolution on a new column, used column, and column cleaned with sodium hydroxide.

Troubleshooting

The following are recommended actions when specific problems have occurred.

Situation	Possible cause	Action
Air bubbles in the medium bed.	Column packed or stored at cool temperature and then warmed up. Buffers not properly degassed.	Remove small air bubbles by passing degassed buffer through the column. Make sure that buffers, system, and columns have the same temperature especially if buffers are used after storage in a fridge or cold room. Repack column if necessary ¹ .
Cracks in the medium bed. 	Air leakage in column.	Check all connections for leaks. Repack column if necessary ¹ .
Space between medium bed and adapter. 	Flow rate used for viscous buffers is too high. Flow rate used in cold room is too high. Bed insufficiently packed.	Turn down the adapter to the medium bed. Perform a column efficiency test. Repack column if necessary ¹ .
Increased back pressure ² or Reduced flow through the column.	Clogged on-line filter in system. Blocked column filter. Protein precipitation. Medium bed compressed by a flow rate that is too high.	Replace on-line filter. Replace the column filter if possible ¹ . Modify the running buffer to maintain stability of the sample. Repack column if possible ¹ .

¹ Note that repacking or exchange of top filter is not possible on HiPrep columns prepacked with Sephacryl or Precision column 3.2/300. See Appendix 3 for the procedure for changing the top filter.

² Check back pressure at the same stage during each run, since the value can vary within a run, for example during sample injection or when changing to a different buffer.

Appendix 1. Principle for setting the column pressure limit

Pressure is generated by the flow through the chromatographic system. For optimal chromatography functionality, it is important to understand the principle of the pressure drop over the different parts of a system (Fig 4).

- Δp_{before} does not affect the column.
- The pressure on the column hardware is the sum of Δp_{after} and Δp . Do not exceed the column hardware limit!
- Δp is individual and needs to be determined for each column.

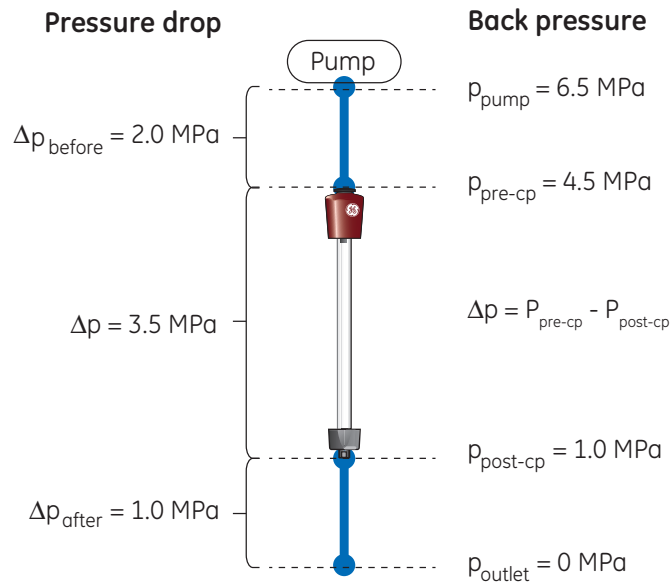


Fig 4. Example of the pressure in different parts of a system during run of a column. Note that the pressure values are only used to illustrate the principle.

For more information, refer to the *ÄKTA Lab-scale Chromatography Systems Instrument Management Handbook*, 29-0108-31.

To protect the column hardware and the packed bed of the chromatographic medium, it is important to set limits that must not be exceeded during the run. There are two important pressure limits that must be taken into consideration:

1. **To protect the column hardware:** Column hardware pressure limit (Fig 5), which is the maximum pressure the hardware can withstand without being damaged. This value is fixed for each column type. Leakage from the column could be a sign of excessive pressure on the column hardware. The column hardware pressure limit is included in the instructions and column UNICORN™ list for each column type, respectively.

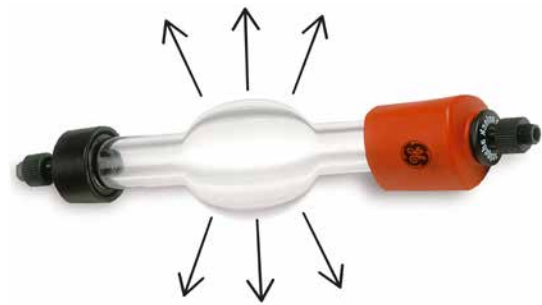


Fig 5. Column hardware pressure limit is the maximum pressure the column can withstand without damage.

2. **To protect the packed bed:** The pressure drop over the packed bed (Δp), generated at maximum flow rate at room temperature, is the maximum pressure at which the packed bed can be used without risking gap formation (Fig 6) or bed collapse. This value varies depending on conditions. A typical value for Δp or maximum pressure drop over the packed bed is provided for each column type in the instructions and UNICORN column list. Note however that Δp is individual for each column and needs to be determined. The procedure for doing this is described in Instructions 29-0272-71. The packed bed is protected by controlling the flow rate. Use lower flow rates for high-viscosity solutions and/or low temperature (Table 1).

Table 1. Example of flow rate limits at different viscosity and temperature, Superose 6 Increase 10/300 GL

Temperature	Flow rate (ml/min)
20°C to 25°C	Maximum flow rate, water
	Maximum flow rate, 20% ethanol
4°C to 8°C	Maximum flow rate, water
	Maximum flow rate, 20% ethanol



Fig 6. The maximum pressure over the packed bed is the maximum pressure the packed bed of chromatography medium can withstand without gap formation. This is not a fixed value.

The pressure over the packed bed depends on several parameters including:

- Flow rate
- Viscosity of sample and eluent
- Running temperature
- Chromatography medium particle properties
- Column packing

Appendix 2. Checking column efficiency

Column performance should be checked at regular intervals by determining the column efficiency in terms of the number of theoretical plates per meter (N/m) and peak symmetry (A_s). Note that the result for column efficiency is dependent on the system used, including the capillaries and dead volumes. This means that the column efficiency given in the specification for the column (tested on another system) will not be exactly the same as your initial column efficiency result.

Typical values for column performance:

Superdex prep grade: Column efficiency, N/m > 10 000
Peak symmetry, $A_s = 0.70$ to 1.30

Sephacryl HR: N/m > 9000
 $A_s = 0.80$ to 1.50

1. Equilibrate the packed column in distilled water at the recommended flow rate given in the instructions.
2. Inject acetone (20 mg/ml in water) in a volume equivalent to 0.2% of the total packed column volume.
3. Monitor UV absorbance 280 nm from the time of injection until the acetone peak has eluted and the signal has returned to baseline.
4. Calculate efficiency, N/m:

$$N/m = 5.54 (V_e / W_{1/2})^2 / L$$

where

V_e = peak elution (retention) volume

$W_{1/2}$ = peak width at half peak height

L = bed height (m)

V_e and $W_{1/2}$ are in same units

Calculate the peak symmetry, A_s :

$$A_s = b/a$$

where

a = first half peak width at 10% peak height

b = second half peak width at 10% peak height

Note: The column efficiency test can also be performed in 20% ethanol instead of water. See also Application note 28-9372-07, *Column efficiency testing*.

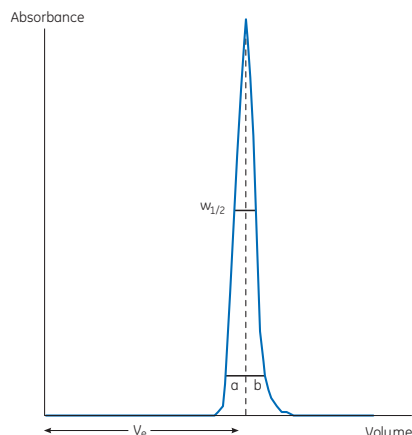


Fig 7. Determination of column efficiency by number of theoretical plates and peak symmetry.

Appendix 3. Replacing the top filter

This instruction is valid for Tricorn columns. For HiLoad columns, please follow the instructions supplied with your column.

Before replacing the top filter, try cleaning the column medium first as recommended in the instructions supplied with the medium. Replace the top filter if you still observe increased back pressure, loss of resolution, or reduced sample recovery after cleaning the column.

If the top filter is clogged and needs to be replaced and/or the medium bed surface becomes contaminated, proceed as follows:

1. Disconnect the column from the system and insert a stop plug into the bottom unit (see the column instructions).
2. Lift the adapter lock and remove the adapter unit.
3. Using a hooked filter tool (Fig 8) to carefully remove the filter from the surface of the medium bed.

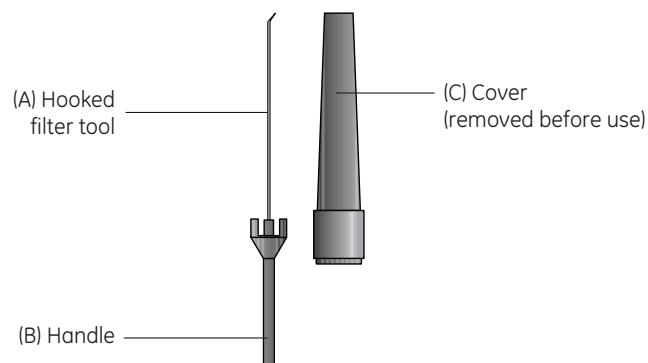


Fig 8. Filter tool.

4. Fill the column tube with fluid and carefully stir the top 1 mm of the medium bed surface with a Pasteur pipette and remove the suspended medium particles.
5. Fill the column tube with fluid and remove all air bubbles.
6. Place a new, prewetted top filter on top of the fluid in the column.
7. Screw the guiding ring inside the adapter unit down to its end position so that it is level with the bottom of the adapter unit (Fig 9). Screw the guiding ring back 1.5 turns.

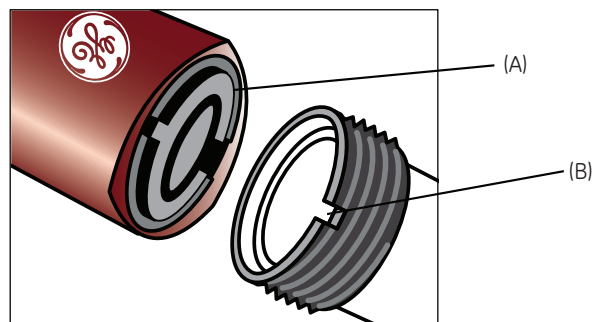


Fig 9. (A) Adapter unit and (B) slot on column tube threads.

8. Screw the adapter unit (Fig 9A) onto the column tube, ensuring the inner part of the guiding ring fits into the slot on the column threads (Fig 9B). Make sure that there are no air bubbles.
9. Screw the adapter halfway down. Press the adapter lock down into locked position. It is now not possible to turn the adapter if the adapter unit has been mounted correctly.
10. Unlock the adapter lock and screw the adapter unit down until the adapter meets the medium bed surface. Screw the adapter unit down a further 360° so that the adapter is positioned slightly below the medium surface.
11. Press the adapter lock down into the locked position.
12. The column is now ready for use.

Note: If the column is not to be used immediately, fit a stop plug into the adapter unit.

Note: Do not tighten the adapter unit using a wrench, only by hand.

Note: We do not recommend changing the bottom filter without repacking the column as this can lead to a loss of efficiency. This will also mean that the top filter should be replaced.

Note: See the column instructions for changing the adapter net ring.

For further details, see Instruction 56-3154-70, *Tricorn filters*.
For more hints and tips to get the most out of your SEC column, download the *Size Exclusion Chromatography Handbook, Principles and Methods* 18-1022-18 from www.gelifesciences.com/handbooks.

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29-1407-60 AA 02/2015