



Macrocap SP

Ion exchange chromatography

Instructions for Use

MacroCap™ SP is a cation exchanger designed to purify polyethylene glycol-modified (PEGylated) proteins and other large biomolecules, at high sample load. Mono- PEGylated proteins can be separated to high purity from oligo-PEGylated and non- PEGylated proteins, most often in a single run. Cleaning-in-place (CIP) of MacroCap SP can be performed both in acidic (pH 2) and alkaline (pH 13) conditions. Moreover, the hydrophilic nature of the base matrix reduces nonspecific binding and consequent fouling, thus allowing for long media life time.

MacroCap SP is a BioProcess™ medium, specifically designed to meet the demands of industrial biotechnology. This means that the medium is designed to be scalable from lab to production, is produced in validated manufacturing procedures and can withstand standard cleaning-in-place (CIP) and sanitization-in-place (SIP) procedures. In addition, BioProcess media are supported with regulatory support files and comprehensive documentation, as well as security of supply service.

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1 Characteristics

Table 1. Characteristics of MacroCap SP

Matrix	Cross-linked copolymer of allyl dextran and N,N-methylene bisacrylamide
Ion exchange type	Strong cation
Charged group	-SO ₃ ⁻
Total ionic capacity	0.10 to 0.13 mmol H ⁺ /mL medium
Average particle size	50 µm (d _{50v})
Flow velocity	120 cm/h in BPG 300, 20-cm bed height or 70 cm/h in BPG 300, 30-cm bed height using process buffers with the same viscosity as water at < 3 bar (0.3 MPa).
Recommended separation range	<ul style="list-style-type: none"> • Proteins in excess of 150 000 M_r • Functionalized dextrans or PEGs greater than or equal to 20 000 M_r • PEGylated proteins containing greater than or equal to 10 000 M_r of PEG (total) per conjugate.
pH stability ¹	
short term	2 to 13
working	3 to 12
long term	4 to 11
Storage temperature	4°C to 30°C
Chemical stability	all commonly used aqueous buffers, 0.5 M NaOH, 0.1 M citric acid, 25% ethanol, 30% propanol, 30% methanol, 50% ethylene glycol, 1% Tween™-20, and 1% SDS.

¹ *Short-term pH:* pH interval where the medium can be subjected to cleaning-in-place or sanitization-in-place (accumulated 90 to 400 h at room temperature) without significant change in function.

Working pH: pH interval where the medium binds protein as intended or that needed for elution without adverse long-term effect.

Long-term: pH interval where the medium can be operated without significant change in function.

2 Method design and optimization

The aim of developing a method for the separation of large biomolecules, such as PEGylated proteins, is to secure a high and consistent binding capacity and resolution for various experimental set-ups.

Suggested purification protocol

MacroCap SP operates as a normal cation exchanger. It has been designed for large scale operation, and allows flow velocities of 120 cm/h at 20-cm bed height in a 30 cm i.d. column (70 cm/h at 30-cm bed height). These are common operating flows for processing large, slowly diffusing biomolecules. A faster flow may be possible with lower bed heights. However, residence times of 6 to 15 min or longer may be required to fully use the properties of the medium.

Step	Action
1	Titrate the protein reaction mix to a pH that is 1.0 to 2.0 pH units below the pI of the target molecule. The exact pH has to be determined for each target molecule. Note that the pI of some proteins is altered by PEGylation and that the charge per unit volume of PEGylated proteins is typically lower than for the corresponding non PEGylated protein ¹ .
2	Equilibrate the column with start buffer at an appropriate pH (± 0.5 pH units from the pK_a of the buffering salt).

¹ Results with several different reaction product mixtures relating to proteins modified with different weight PEG polymers suggests that cation exchange should be run 2 to 3 pH units below pI of the corresponding nonPEGylated protein.

Step	Action
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| 3 | Apply the sample to the column. |
| 4 | Wash out unbound sample using start buffer. |
| 5 | Elute the target protein typically using a linear gradient of 0% to 100% B in 10 to 20 column volumes (CV). Buffer B is usually equivalent to buffer A plus a certain amount of eluting salt (e.g., 1 M NaCl). |

PEGylated proteins

Start by running with low sample load and a 10-column volume (CV) linear gradient from 0.01 M to 1.0 M salt at the defined pH. This will identify the elution conductivity range for nontarget oligo-PEGylated proteins and nonPEGylated proteins. Start by using only the nonPEGylated protein to identify conditions where it elutes as late as possible in the gradient. The PEGylated conjugates will always elute earlier than the native protein. The pH can be adjusted depending on initial results. Once the approximate gradient, pH, and conductivity range have been identified, an optimal gradient (linear, or step, or combination) can be developed to resolve target material.

Define optimal loading (mg of protein per mL of medium) that gives the desired result. Evaluate all peaks for recovery and purity and note that the target fraction purity may increase with column loading. We recommend loading the column until the target protein starts to appear in nontarget fractions, including flowthrough.

Subtle changes in loading conductivity or protein concentration may affect column performance. When eluting target PEGylated protein on a gradient, it is not unusual to see several peaks corresponding to PEGylated proteins of similar molecular weight, so called polymer positional isomers or "PEGamers". These proteins can be verified using matrix assisted laser desorption ionization time of flight mass spectroscopy (MALDI-ToF-MS), electrophoresis, or other analytical methods. Make sure that sample concentrations, buffer salts, salt concentrations, and operating temperatures do not promote precipitation of polymer-containing substances.

Optimization

Balancing product recovery against throughput is a major consideration when optimizing a method. The dynamic binding capacity (DBC) for the target substance should be determined by frontal analysis using real process feedstock or reaction mixture. To determine the DBC, apply the sample and follow the breakthrough of the target molecule by a specific method of analysis.

The two main problems that may occur are poor DBC and poor resolution.

Poor DBC can be circumvented by:

- Lowering the pH to increase the net positive charge of the protein and thereby increase the binding of the protein to the ligand.
- Lowering the conductivity of the sample to enhance the interaction between protein and ligand.

- Decreasing the fluid velocity during sample application to allow a larger portion of ligands to interact with the proteins. The breakthrough capacity must be defined over a range of different residence times (flow velocities) to optimize the throughput.

Poor resolution is remedied by:

- Decreasing the slope of the gradient. To avoid long run times and a large number of fractions, the shallow gradient should just be effective at the part of the chromatogram where the target protein is eluted. After an initial run, it should be possible to identify the conductivity at which the target protein is eluted. The altered gradient should start at a conductivity just below where the target protein elutes and extend until the target protein is eluted. A combination of step-wise and linear gradients has shown to be successful.

3 Scale-up

After optimizing the method at laboratory scale, the process can be scaled up.

Step	Action
1	Select the bed volume according to the required binding capacity.
2	Select a column diameter to obtain a bed height of 10 to 30 cm.

Step	Action
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| 3 | Scale-up is typically done by keeping bed height and flow velocity constant, while increasing bed diameter and volumetric flow rate. However, since optimization is preferentially performed using small column volumes, to minimize sample and buffer consumption, some parameters such as the dynamic binding capacity may be optimized using shorter bed heights than those used in the final scale. As long as the residence time is constant, the binding capacity for the target molecule remains the same. Other factors, such as clearance of critical impurities, may change when column bed height is changed and should be validated with the final bed height. The residence time is approximated as the bed height (cm) divided by the flow velocity (cm/h) applied during sample loading. |
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Verification of scale-up

Verification of scale-up can be done in many different ways. Below is a list of some methods that can be used.

- Visually examine the chromatograms and compare the elution volume of the target protein for the different scales. Refractive index is useful for monitoring PEG-containing substances, but it is sensitive to changes in ionic strength.
- Compare the purification factor¹.

¹ The relationship between the target protein and impurities in the starting material and elution peak, expressed as $(M_{\text{stot}} / M_{\text{stp}}) / (M_{\text{etot}} / M_{\text{etp}})$, where M_{stot} is the total mass of proteins in the start material, M_{stp} is the mass of target protein in the start material, M_{etot} is the total mass of proteins in the elution peak and M_{etp} is the mass of target protein in the elution peak.

- Compare the recovery for both scales.
- Analyze the impurity pattern with gel filtration or a high-resolution technique such as polyacrylamide gel electrophoresis (PAGE). Although PAGE can be used with polymer modified proteins, MALDI-ToF-MS or capillary electrophoresis are particularly useful for PEGylated biopharmaceuticals.

It should be noted that some protein assays might be affected by the presence of PEG. If there are small or no differences among the scales for the above mentioned methods, the scale-up can be considered successful.

4 Recommended packing methods

Packing XK 16/20 columns

Materials needed

MacroCap SP
Glass filter funnel
Small spoon or plastic spatula
Filter flask
Measuring cylinder or beaker
20% ethanol
0.15 M NaCl in 20% ethanol

Washing the medium

Equilibrate all materials to room temperature. Mount the glass filter funnel onto the filter flask. Pour the medium into the funnel and wash. Wash with at least 4 mL 0.15 M NaCl per mL medium.

Preparing the medium suspension

The slurry concentration should be 60% in 0.15 M NaCl, measured in a measuring cylinder after settling overnight or after centrifugation at 3000 rpm for 3 min. Wait 5 min after centrifugation before reading.

Equipment needed

Peristaltic Pump, P-1 or High Precision Pump P-500 or P-900.

An ÄKTAexplorer system can be used for packing the column. The inline filter (system filter) unit should be removed due to the high flow velocity used in column packing. Removing the filter decreases the system backpressure.

The columns should be packed without a slurry reservoir as it is important to lock the medium bed quickly when packing is complete.

XK 16/20 is used for 10-cm bed heights.

XK 16/40 with two flow adapters is used for 15-cm bed heights. If one flow adapter and end piece is used, a bed height of 20 cm can be obtained.

Assembling the column

Details of the column parts can be found in the instructions supplied with the column. Before packing, ensure that all parts, particularly the nets, net fasteners, and glass tube are clean and intact.

Step	Action
1	Wet the bottom filter with 20% ethanol with a syringe mounted on the outlet tubing. After the filter is wetted, mount a stop screw on the outlet.
2	Mount the bottom piece in the column tube.
3	Wet the adapter filter with 20% ethanol with a syringe mounted on the inlet tubing. After the filter is wetted, mount a stop screw on the inlet.
4	Keep the adapter in a beaker filled with 20% ethanol.
5	Mount the column vertically on a stand.
6	Fill the glass tube with the 60% medium slurry up to the upper edge of the glass tube.
7	Mount the adapter in the upper part of the glass tube and make sure that no air is trapped below the filter.
8	Tighten the sealing ring and connect the column inlet to the system outlet.

Packing procedure

To pack the column, use 0.15 M NaCl in 20% ethanol and proceed as follows:

Step Action

- 1** Pack the column at 60 cm/h (2.0 mL/min, XK 16) for 30 min or until the bed height and the backpressure are constant. Stop the pump and mount a stop screw on the outlet.
- 2** Increase the flow rate to 150 cm/h (5.0 mL/min) and continue packing for 5 min. Adjust the adapter quickly down to the medium surface and then a further 5 mm into the medium bed. Lock the adapter at that level.

Note:

Do not exceed the operating pressure limit for the medium (3.0 bar not including system effect).

Troubleshooting

If the column does not fulfill the criteria with respect to h and A_s , described in [Evaluation of column packing, on page 13](#), empty the column, and repack according to the table below, and perform a new test.

Situation	Action
$h > 3$ and $A_s < 0.8$	Decrease the flow rate in step 1 of the packing procedure from 2.0 to 1.5 mL/min. Decrease the flow rate in step 2 from 5.0 to 4.0 mL/min.
$h > 3$ and $A_s > 1.5$	Decrease the flow rate in step 1 from 2.0 to 1.5 mL/min. Increase the flow rate in step 2 from 5.0 to 5.5 mL/min.
$h < 3$ and $A_s < 0.8$	Decrease the flow rate in step 2 from 5.0 to 4.0 mL/min
$h < 3$ and $A_s > 1.5$	Decrease the flow rate in step 1 from 2.0 to 1.5 mL/min. Increase the flow rate in step 2 from 5.0 to 5.5 mL/min.

Packing BPG 300 columns

For information, please see:

Evaluation of column packing

Test column efficiency to check the quality of the packing. Tests should be made directly after packing and at regular intervals during the working life of the column as well as when separation performance is seen to deteriorate. The best method of expressing the efficiency of a packed column is in terms of the height equivalent to a theoretical plate (HETP) and the asymmetry factor (A_s). These values are easily determined by applying a sample such as 1% acetone solution to the column. Sodium chloride can also be used as a test substance. Use a concentration of 2.0 M NaCl in water with 0.5 M NaCl in water as eluent.

Note: *The calculated plate number will vary according to the test conditions and it should only be used as a reference value. It is important that test conditions and equipment are kept constant so that results are comparable. Changes of solute, solvent, eluent, sample volume, flow velocity, liquid pathway, temperature, etc. will influence the results.*

For optimal results, the sample volume should be at maximum 2.5% of the column volume and the flow velocity between 15 and 30 cm/h. If an acceptance limit is defined in relation to column performance, the column plate number can be used as one of the acceptance criteria for column use.

Method for measuring HETP and A_s

Calculate HETP and A_s from the UV curve (or conductivity curve if NaCl is used as sample) as follows:

$$\text{HETP} = L/N$$

$$N = 5.54(V_e/W_h)^2$$

where

$$L = \text{Bed height (cm)}$$

$$N = \text{Number of theoretical plates}$$

$$V_R = \text{Peak elution distance}$$

$$W_h = \text{Peak width at half peak height}$$

V_R and W_h are in the same units. These values are illustrated in [Fig. 1, on page 15](#).

To facilitate comparison of column performance, the concept of reduced plate height is often used. Reduced plate height is calculated as:

$$h = \text{HETP}/d_{50v}$$

where d_{50v} is the mean diameter of the bead, using the same unit as for HETP.

As a guideline, a value of $h < 3$ is normally acceptable at the optimal test conditions presented above.

The peak should be symmetrical with an asymmetry factor as close as possible to 1 (values between 0.8 and 1.5 are usually acceptable). A change in the shape of the peak is usually the first indication of bed deterioration due to use. Peak asymmetry factor is calculated as:

$$A_s = b/a$$

where

$$a = \text{1}^{\text{st}} \text{ half peak width at 10\% of peak height}$$

$$b = \text{2}^{\text{nd}} \text{ half peak width at 10\% of peak height.}$$

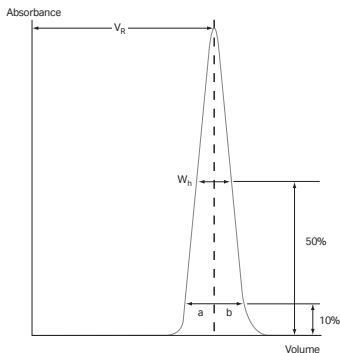


Fig 1. A typical test chromatogram showing the parameters used for HETP and A_s calculations.

5 Maintenance

For the best performance from MacroCap SP over a long working life, follow the procedures described below.

Equilibration

After packing, and before a chromatographic run, equilibrate the column with start buffer by washing with at least 5 column volumes, or until the column effluent shows stable conductivity and pH values.

Regeneration

After each separation, elute any reversibly bound material with a high ionic strength solution (e.g. 0.5 to 2 M NaCl in buffer) and at the same time increase pH to about 10 to 11. Re-equilibrate the medium by washing with at least 5 column volumes of start buffer, or until the column effluent shows stable conductivity and pH values.

Cleaning-in-place

Cleaning-in-place (CIP) is a procedure that removes contaminants such as lipids, endotoxins, and precipitated or denatured proteins that remain in the packed column after regeneration. This type of contamination occurs frequently when working with crude feedstock. Regular CIP prevents the buildup of contaminants in the medium bed and helps to maintain the capacity, flow properties, and general performance of MacroCap SP. A specific CIP protocol should be designed for each process according to the type of contaminants present. The frequency of CIP depends on the nature and the condition of the feedstock, but for capture steps, CIP is recommended after each cycle.

It should be noted that PEG polymers tend to self associate and precipitate from aqueous solutions at room temperature in the presence of high concentration of various salts.

Table 2. CIP-protocol

Precipitated, hydrophobically bound proteins or lipoproteins	Wash with 0.5 M NaOH at 40 cm/h with reversed flow direction. Contact time 1 to 2 h, dependent on feed. Apply a CIP-cycle using NaOH pH 13 followed by an acidic solution pH 2. It is recommendable to wash with water in between the basic and acidic solutions.
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Ionically bound proteins	Wash with 0.5 to 2 column volumes of 0.5 to 2 M NaCl with reversed flow direction. Contact time 10 to 15 min.
Lipids and very hydrophobic proteins	Wash with 2 to 4 column volumes of 25% ethanol ¹ or 30% propanol with reversed flow direction. Contact time 1 to 2 h, dependent on feed. Alternatively, wash with 2 to 4 column volumes of 0.1% nonionic detergent with reversed flow direction. Contact time 1 to 2 h, dependent on feed.

¹ Specific regulations may apply when using ethanol since it can require the use of explosionproof areas and equipment. Note also that polyethylene glycol and other polymers may not be soluble in some mixed mode solvent and water solutions.

Sanitization

To reduce microbial contamination in the packed column, sanitize with 0.5 M NaOH with a contact time of 1 h. The CIP protocol for precipitated, hydrophobic bound proteins or lipoproteins removes bound contaminants and sanitizes the medium effectively.

Storage

Store unused medium in its original container at a temperature of 4 to 30°C. Ensure that the screw top is fully tightened. Packed columns should be equilibrated in 20% ethanol in 20 mM sodium acetate pH 4.5 to prevent microbial growth and to buffer the ligand. After storage, equilibrate with at least 5 column volumes of starting buffer before use.

6 Ordering information

Product	Pack size	Code No
MacroCap SP	25 mL	17544010
	100 mL	17544001
	1 L	17544002
	5 L	17544003

All bulk media products are supplied in suspension in 20% ethanol with 0.2 M sodium acetate. For additional information, please contact your local Cytiva representative.

Related products	Quantity	Code No
Tricorn™ 5/100 column	1	18116310
Tricorn 10/100 column	1	18116315
XK 16/20 column	1	18877301

Accessories	Quantity	Code No
Tricorn 5/100 Glass Tube	1	18115306
Tricorn Packing Connector 5-5	1	18115321
Tricorn 5 Coarse Filter Kit	1	11001253
Tricorn 10 Coarse Filter kit	1	11001254
Tricorn Packing Equipment 10/100	1	18115325

Literature	Code No
Data file	28400584

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