GE Healthcare
Life Sciences

Instructions 28-9503-68 AC **Prepacked column**

HiScreen™ IMAC FF

HiScreen IMAC FF is a ready to use column for purification of proteins/peptides by immobilized metal ion affinity chromatography (IMAC).

The column is prepacked with 4.7 ml uncharged IMAC Sepharose™ 6 Fast Flow and is ideal for screening of selectivity, binding and elution conditions, as well as small scale purifications.

The columns are used in an optimal way with liquid chromatography systems such as ÄKTA™.

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Please read these instructions carefully before using the HiScreen columns.

Intended use
HiScreen columns are intended for research use only, and shall not be used in any clinical or *in vitro* procedures for diagnostic purposes.

Safety
For use and handling of the product in a safe way, please refer to the Safety Data Sheet.

1 Product description

HiScreen column characteristics

HiScreen columns are made of biocompatible polypropylene that does not interact with biomolecules. The columns are delivered with stoppers at the inlet and outlet. The arrow on the column label indicates the column orientation and the recommended flow direction, see Figure 1.

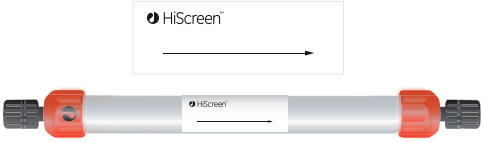

Fig 1. HiScreen column

For easy scaling-up and when a higher bed height is required, two columns can easily be connected in series using a union to give 20 cm bed height (see Section *Scaling up*).

Note: *HiScreen columns cannot be opened or refilled*

Note: *Make sure that the connector is tight to prevent leakage.*

Table 1. Characteristics of HiScreen column

Column volume (CV)	4.7 ml
Column dimensions	0.77 × 10 cm
Column hardware pressure limit [1]	8 bar (0.8 MPa)

[1] The pressure over the packed bed varies depending on a range of parameters such as the characteristics of the chromatography medium and the column tubing used.

Properties of IMAC Sepharose 6 Fast Flow

HiScreen IMAC FF columns are prepacked with IMAC Sepharose 6 Fast Flow, which consists of 90 µm, highly cross-linked agarose beads with a covalently immobilized chelating group. The medium can easily be charged with Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+} or other metal ions.

The high flow rate properties of IMAC Sepharose 6 Fast Flow make HiScreen IMAC FF columns ideal for establishing optimal chromatographic conditions for scaling up.

Table 2. Characteristics of IMAC Sepharose 6 Fast Flow

Matrix	Highly cross-linked 6% agarose
Average particle size (d_{50v}) [1]	90 µm
Metal ion capacity	Approx. 15 µmol Ni^{2+}/ml medium
Dynamic binding capacity [2]	Approx. 40 mg (histidine)6-tagged protein/ml medium (Ni^{2+}-charged). Untagged protein: Approx. 25 mg/ml medium (Cu^{2+} charged), or approx. 15 mg/ml medium (Zn^{2+} or Ni^{2+} charged).
Recommended flow velocity [3]	30 to 300 cm/h
Maximum flow velocity [3]	600 cm/h
pH stability [4] (for medium without metal ion) **Working range** **Cleaning-in-place**	 3 to 12 2 to 14
Compatibility during use	See Table 3.
Chemical stability (for medium without metal ion)	1 M NaOH, 70% acetic acid. Tested for 12 h. 2% SDS. Tested for 1 h. 30% 2-propanol. Tested for 30 min.
Avoid in buffers	Chelating agents, e.g. EDTA, EGTA, citrate (see Table 3).
Storage	4°C to 30°C in 20% ethanol

[1] d_{50v} is the average particle size of the cumulative volume distribution.

[2] Dynamic binding capacity conditions:

Samples:	$(Histidine)_6$-tagged proteins: Capacity data were obtained for a protein (M_r 28 000) bound from an *E. coli* extract, and a pure protein (M_r 43 000; applied at 1 mg/ml in binding buffer; capacity at 10% breakthrough). Untagged protein: Capacity determined at 10% breakthrough for human apo-transferrin applied at 1 mg/ml in binding buffer.
Column volumes:	0.25 or 1 ml
Flow rates:	0.25 or 1 ml/min, respectively
Binding buffer:	20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, (1 mM imidazole for untagged protein), pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, (50 mM imidazole for untagged protein), pH 7.4.

[3] Water at room temperature.
For viscous buffers and samples the flow velocity must be optimized. Starting with a low flow velocity is recommended.

[4] Working range: pH interval where the medium can be handled without significant change in function.
Cleaning-in-place: pH interval where the medium can be subjected to cleaning-in-place without significant change in function.

2 General process development

HiScreen column format is ideal to use for parameter and method optimization and for robustness testing when developing a new purification process. The small column volume, 4.7 ml, and the 10 cm bed height makes it possible to perform scalable experiments at relevant process flow rates. If necessary, two columns can easily be connected in series with a union to give 20 cm bed height (see Section *Scaling up*).

The figure below outlines typical steps during general process development.

Already from start in process development it is necessary to consider process cost, cleaning of the medium and environmental constraints.

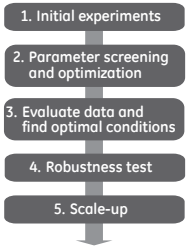

Fig 2. Typical steps during process development.

Design of Experiments (DoE) is an effective tool for investigating the effect of several parameters on protein recovery in order to establish the optimal purification protocol.

A common approach in DoE is to define a reference experiment (center point) and perform representative experiments around that point. Some initial experiments are required in order to define the center point and the variable ranges. DoE may be used for parameter screening and optimization as well as robustness testing.

The robustness of a process is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters, and provides an indication of its reliability during normal usage. An objective of a robustness test is the evaluation of factors potentially causing variability in the responses of the method, for example, purity and yield. For this purpose, small variations in method parameters are introduced.

For scale-up, see Section *Scaling up*.

3 Optimization

General

IMAC is based on the specific interaction between immobilized metal ions and certain amino acid side chains exposed on the surface of proteins (mainly His and to a lesser extent Cys and Trp). The strength of interaction with immobilized metal ions is dependent on the type, number and spatial distribution of the amino acid side chains, and on the nature of the metal ion used. The chromatographic operating conditions (pH, type and concentration of salt, additives, etc.) also contribute to the interaction observed.

Concentration of imidazole in binding/wash buffer

Imidazole at low concentrations is commonly used in binding/wash buffers to minimize the binding of unwanted host cell proteins. For the same reason, it is important to also include imidazole in the sample (generally, at the same concentration as in the binding/wash buffer). At somewhat higher concentrations, imidazole may decrease the binding of histidine-tagged proteins, leading to a lower yield. The concentration of imidazole must therefore be optimized to ensure the best balance of high purity (low binding of unwanted host cell proteins), and high yield (binding all of the histidine-tagged protein).

This optimal concentration is different for different histidine-tagged proteins/target proteins, and is usually slightly higher for IMAC Sepharose 6 Fast Flow than for similar IMAC media on the market.

For untagged target proteins, the concentration of imidazole that should be used are generally much lower than for histidine-tagged proteins, both for binding (sometimes no imidazole is needed) and elution.

Finding the optimal imidazole concentration for a specific target protein is a trial-and-error effort, but 20 to 40 mM in the sample as well as in the binding/wash buffer is a good starting point for many histidine-tagged proteins when using Ni^{2+} or Co^{2+}.

When maximum binding and yield of the histidine-tagged protein (rather than purity) is the main objective, choose a low imidazole concentration for binding and wash, even if that concentration in some cases may lead to suboptimal purity.

Use a high purity imidazole, which has essentially no absorbance at 280 nm.

Choice of metal ion

HiScreen IMAC FF is supplied without bound metal ions and thus needs to be charged with a suitable ion before use. The choice of metal ion is dependent on the type of application and the specific protein to be purified. The metal ions predominantly used in IMAC are Cu^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}.

For purification of untagged proteins, Cu^{2+} ions have frequently been used. When the binding characteristics of an untagged target protein are not known, it is advisable to test also other metal ions (e.g., Zn^{2+}, Ni^{2+}, Co^{2+}) in order to establish the most suitable metal ion to use. In some instances, a weak binding to a metal ion can be exploited to achieve selective elution (higher purity) of a target protein. In some special applications, Fe^{3+} and Ca^{2+} have also been used.

Ni^{2+} is usually the first choice for purifying most histidine-tagged recombinant proteins, and is also the metal ion most generally used. Nevertheless, it is not always possible to predict which metal ion will be the most suitable for a given protein. The strength of binding between a protein and an immobilized metal ion is affected by several factors, including the length, position, and exposure of the affinity tag on the protein, the type of metal ion used, and the pH, etc. of buffers. Some proteins may therefore be easier to purify with metal ions other than Ni^{2+} (e.g., Zn^{2+}, Co^{2+}, or Cu^{2+}).

4 Operation

Prepare buffers

Start buffer

20 mM sodium phosphate, 0.5 M NaCl, 20 to 40 mM imidazole, pH 7.4

The optimal imidazole concentration is protein-dependent. 20 to 40 mM is suitable for many histidine-tagged proteins when using Ni^{2+} or Co^{2+}.

Elution buffer

20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4

The imidazole concentration required for elution is protein-dependent.

Note: *Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.22 µm or a 0.45 µm filter before use.*

Note: *Compared to histidine-tagged proteins, untagged, naturally occurring proteins bind immobilized metal ions with lower affinity. Thus, the concentrations of imidazole that should be used with untagged proteins are lower than the above recommended, both for binding (sometimes no imidazole needs to be added) and elution.*

An alternative, especially for untagged target proteins, is elution at low pH (e.g., a linear gradient from pH 7.4 to pH 4). Like imidazole elution buffer, also low-pH elution buffers should contain 0.5 M NaCl.

Example: First stepwise elution with 50 mM sodium acetate, 0.5 M NaCl, pH 6, followed by a linear gradient to 50 mM sodium acetate, 0.5 M NaCl, pH 4.

Table 3. IMAC Sepharose 6 Fast Flow charged with Ni^{2+} is compatible with the following compounds, at least at the concentrations given

Reducing agents [1]	5 mM DTE
	5 mM DTT
	20 mM ß-mercaptoethanol
	5 mM TCEP (Tris[2-carboxyethyl] phosphine)
	10 mM reduced glutathione
Denaturing agents	8 M urea [2]
	6 M Gua-HCl [2]
Detergents	2% Triton™ X-100 (nonionic)
	2% Tween™ 20 (nonionic)
	2% NP-40 (nonionic)
	2% cholate (anionic)
	1% CHAPS (zwitterionic)
Other additives	500 mM imidazole
	20% ethanol
	50% glycerol
	100 mM Na_2SO_4
	1.5 M NaCl
	1 mM EDTA [3]
	60 mM citrate [3]
Buffer substances	50 mM sodium phosphate, pH 7.4
	100 mM Tris-HCl, pH 7.4
	100 mM Tris-acetate, pH 7.4
	100 mM HEPES, pH 7.4
	100 mM MOPS, pH 7.4
	100 mM sodium acetate, pH 4 [2]

1 See *Blank run* in this section.

2 Tested for one week at 40°C.

3 The strong chelator EDTA has been used successfully in some cases at 1 mM. Generally, chelating agents should be used with caution (and only in the sample, not the buffer). Any metal-ion stripping may be counteracted by adding a small excess of $MgCl_2$ before centrifugation/filtration of the sample. Note that stripping effects may vary with the applied sample volume.

Charging with metal ions

Step	Action
1	Charge the water-washed column by loading at least 2.5 ml 0.1 M metal-ion solution in distilled water. Chlorides, sulfates, etc. can be used. For choice of metal ion, see Section *Optimization*.
2	Wash with 25 ml distilled water and 25 ml start buffer (washing with start buffer to adjust pH should be done even if the metal-charged column is only to be stored in 20% ethanol)

In some cases, a blank run may be needed.

Note: *The column does not have to be stripped and recharged between each purification if the same protein is going to be purified; it may be sufficient to strip and recharge it after approximately five purifications, depending on the sample properties, sample volumes, metal ion, etc.*

Optional: Blank run

Note: *Perform a blank run without reducing agents before applying buffers/samples containing reducing agents. Likewise, a blank run is recommended for critical purifications where metal ion leakage during purification must be minimized.*

Use binding buffer and elution buffer without reducing agents.

Step	Action
1	If the column has been stored in 20% ethanol after metal ion charging, wash it with 25 ml of distilled water.
2	Wash with 25 ml of the buffer that has been chosen for protein elution, e.g., imidazole elution buffer or low-pH elution buffer.
3	Equilibrate with 25 to 50 ml of start buffer. Imidazole equilibration can be monitored by absorbance, e.g., at 220 nm.

Prepare the sample

Step	Action
1	Adjust the sample to the composition of the start buffer, using one of these methods: • Dilute the sample with start buffer. • Exchange buffer using a HiPrep™ 26/10 Desalting, HiTrap™ Desalting or PD-10 Desalting column (see Table below).
2	Filter the sample through a 0.45 µm filter or centrifuge immediately before loading it to the column. This prevents clogging and increases the longevity of the column when loading large sample volumes.

Note: *To minimize the co-adsorption of unwanted host cell proteins, it is essential to include imidazole at a low concentration in the sample and start buffer, see Section Optimization.*

Table 4. Prepacked columns for desalting

Column	Code No.	Loading volume	Elution volume	Comments	Application
HiPrep 26/10 Desalting	17-5087-01	2.5 to 15 ml	7.5 to 20 ml	Prepacked with Sephadex™ G-25 Fine. Requires a laboratory pump or a chromatography system to run.	For desalting and buffer exchange of protein extracts (M_r > 5000).
HiTrap Desalting	17-1408-01	0.25 to 1.5 ml	1.0 to 2.0 ml	Prepacked with Sephadex G-25 Superfine. Requires a syringe or pump to run.	
PD-10 Desalting	17-0851-01	1.0 to 2.5 ml[1] 1.75 to 2.5 ml[2]	3.5 ml[1] up to 2.5 ml[2]	Prepacked with Sephadex G-25 Medium. Runs by gravity flow or centrifugation	For desalting, buffer exchange, and cleanup of proteins and other large biomolecules (M_r > 5000).
PD MiniTrap™ G-25	28-9180-07	0.1 to 0.5 ml[1] 0.2 to 0.5 ml[2]	1.0 ml[1] up to 0.5 ml[2]		
PD MidiTrap™ G-25	28-9180-08	0.5 to 1.0 ml[1] 0.75 to 1.0 ml[2]	1.5 ml[1] up to 1.0 ml[2]		

[1] Volumes with gravity elution
[2] Volumes with centrifugation

Recommended flow rates

Table 5. Recommended flow rates for HiScreen IMAC FF.

Column	Flow velocity (cm/h)	Flow rate (ml/min)
HiScreen IMAC FF	30 to 300	0.25 to 2.3

Purification

Flow rate: See Table 5.

Column tubing: Choose the optimal tubing kit for the column and the application you intend to run. (i.d.: 0.25, 0.50 or 0.75 [mm]). A tubing with wider inner diameter gives broader peaks whereas a tubing with a smaller inner diameter gives higher back pressure.

Step	Action
1	After the column preparation (charging with metal ions), equilibrate with at least 5 column volumes (CV) of binding buffer. Avoid introducing air into the column. ***Note:*** *To prevent leakage, ensure that the connectors are tight. Use fingertight 1/16" connector (28-4010-81).* ***Note:*** *In some cases, we recommend a blank run before final equilibration/sample application.*
2	Adjust the sample to the chosen starting conditions and load on the column.
3	Wash with 5 to 10 CV of start buffer until the UV trace of the effluent returns to near baseline.

Step	Action
4	Elute either by linear gradient elution or a step elution at recommended flow rates. If required, the collected eluted fractions can be buffer exchanged or desalted using columns listed in Table 4. • Linear gradient elution Elute with 0% to 100% elution buffer (up to 500 mM imidazole) in 10 to 20 CV. • Step elution Elute with 5 CV of elution buffer including imidazole at chosen concentration. Repeat at higher imidazole concentrations until the target protein has been eluted.
5	If required, strip the column from metal ions and perform a CIP to clean the column.
6	Re-equilibrate the column with 5 to 10 CV of start buffer or until the UV baseline, eluent pH, and conductivity reach the required values. ***Note:*** *Do not exceed the maximum recommended flow and/or back pressure for the column.* ***Note:*** *If imidazole needs to be removed from the eluted protein, use HiTrap Desalting, a PD-10 Desalting Column, or HiPrep 26/10 Desalting depending on the sample volume, see Table 4.*

5 Stripping and re-charging

Stripping buffer

20 mM sodium phosphate, 0.5 M NaCl, 50 mM EDTA, pH 7.4

Step	Action
1	Wash with at least 5 to 10 CV stripping buffer
2	Wash with at least 5 to10 CV start buffer
3	Wash with at least 5 to 10 CV distilled water
4	Clean the column, see Section *Cleaning-in-place (CIP)* and/or recharge with metal ions according to the method previously described, see Section *Operation*.

Note: *The column does not have to be stripped and recharged between each purification cycle if the same protein is to be purified; it may be sufficient to strip and recharge it after 5 to 7 purifications, depending on the metal ion, sample properties, sample volume, target protein, etc.*

6 Cleaning-in-place (CIP)

General description

Correct preparation of samples and buffers should maintain columns in good condition. However, reduced performance, increased back pressure or blockage indicates that the column medium needs cleaning.

CIP removes very tightly bound, precipitated or denatured substances from the medium. If such contaminants are allowed to accumulate, they may affect the chromatographic properties of the prepacked column, reduce the capacity of the medium and, potentially, come off in subsequent runs. If the fouling is severe, it may block the column, increase back pressure and reduce flow rate.

CIP should be performed regularly to prevent the build-up of contaminants and to maintain the capacity, flow properties and general performance of prepacked columns. CIP is usually performed after the elution.

It is recommended to perform a CIP:

- When an increase in back pressure is seen.
- If reduced column performance is observed.
- Before first time use or after long term storage.
- Between runs when the same column is used for purification of different proteins to prevent possible cross-contamination.
- After every run with real feed.

CIP protocol

The **metal ion stripped** column can be cleaned by the following procedures.

To remove...	Then...
Ionically bound proteins	1 Wash with several column volumes (CV) of 1.5 M NaCl. 2 Wash with at least 3 CV distilled water.
Precipitated proteins, hydrophobically bound proteins, and lipoproteins	1 Wash the column with 1 M NaOH, contact time usually 1 to 2 h (longer time may be required to inactivate endotoxins). 2 Wash with approximately 3 to 10 CV start buffer. 3 Wash with 5 to 10 CV distilled water.
Hydrophobically bound proteins, lipoproteins, and lipids.	1 Wash with 5 to 10 CV 30% isopropanol for at least 15 to 20 min. 2 Wash with approximately 10 CV distilled water. or 1 Wash with 2 CV detergent in a basic or acidic solution. Use, for example, 0.1 to 0.5% nonionic detergent in 0.1 M acetic acid, contact time 1 to 2 h. 2 Remove residual detergent by washing with at least 5 CV 70% ethanol. 3 Wash with 3 to 10 CV start buffer.

7 Scaling up

After optimizing the method at laboratory scale, the process is ready for scaling up.

For quick scale-up of purification, two HiScreen columns can easily be connected in series with a union (18-1120-93) to give 20 cm bed height. Note that the back pressure will increase. This can easily be addressed by lowering the flow rate.

Factors, such as clearance of critical impurities, may change when column bed height is modified and should be validated using the final bed height.

Scale-up is typically performed by keeping bed height and flow velocity (cm/h) constant while increasing bed diameter and flow rate (ml/min or l/h).

Bulk media is available for further scale-up, see Section *Ordering information*.

8 Adjusting pressure limits in chromatography system software

Pressure generated by the flow through a column affects the packed bed and the column hardware, see Figure below. Increased pressure is generated when running/using one or a combination of the following conditions:

- High flow rates
- Buffers or sample with high viscosity
- Low temperature
- A flow restrictor

Note: *Exceeding the flow limit (see Table 2) may damage the column.*

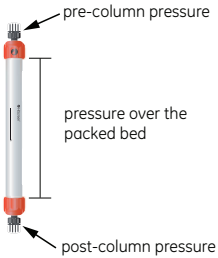

Fig 3. Pre-column and post-column measurements.

ÄKTA avant

The system will automatically monitor the pressures (pre-column pressure and pressure over the packed bed, Δp). The pre-column pressure limit is the column hardware pressure limit (see Table 1).

The maximum pressure the packed bed can withstand depends on media characteristics and sample/liquid viscosity. The measured value also depends on the tubing used to connect the column to the instrument.

ÄKTAexplorer™, ÄKTApurifier™, ÄKTAFPLC™ and other systems with pressure sensor in the pump

To obtain optimal functionality, the pressure limit in the software may be adjusted according to the following procedure:

Step	Action
1	Replace the column with a piece of tubing. Run the pump at the maximum intended flow rate. Note the pressure as *total system pressure*, P1.
2	Disconnect the tubing and run the pump at the same flow rate used in step 1. Note that there will be a drip from the column valve. Note this pressure as P2.
3	Calculate the new pressure limit as a sum of P2 and the column hardware pressure limit (see Table 1). Replace the pressure limit in the software with the calculated value. The actual pressure over the packed bed (Δp) will during run be equal to actual measured pressure - *total system pressure* (P1).

Note: *Repeat the procedure each time the parameters are changed.*

9 Storage

Store HiScreen IMAC FF columns equilibrated with 5 to 10 CV 20% ethanol at 4°C to 30°C. Do not freeze.

Ensure that the column is tightly sealed to avoid drying out.

10 Troubleshooting

Problem	Possible cause/*corrective action*
High back pressure during the run	The column is clogged. *Clean the column, see Section Cleaning-in-place (CIP).* High viscosity of solutions. *Use lower flow rate.*
Unstable pressure curve during sample loading	Air bubbles trapped in the sample pump. *If possible, degas the sample using a vacuum degasser.*
Gradual broadening of the eluate peak	Insufficient elution and CIP, caused by contaminants accumulating in the column. *Optimize the elution conditions, the CIP protocol and/or perform CIP more frequently.*
Gradual decrease in yield	Insufficient elution and CIP. *Optimize the elution conditions, the CIP protocol and/or perform CIP more frequently.*
Precipitation during elution	Sub-optimal elution conditions and CIP. *Optimize the elution conditions, the CIP protocol and/or perform CIP more frequently.*
Gradual increase in CIP peaks	Sub-optimal elution conditions and CIP. *Optimize the elution conditions, the CIP protocol and/or perform CIP more frequently.*
High back pressure during CIP	Proteins precipitated in column. *Optimize elution conditions and/or run high salt wash before CIP or use lower flow rate.*
Reduced column performance despite optimized elution and CIP	*Change to a new column. The longevity of the column depends mainly on the sample and sample preparation.*

11 Ordering information

Product	Quantity	Code No.
HiScreen™ IMAC FF	1 × 4.7 ml	28-9505-17

Related products	Quantity	Code No
HiTrap IMAC FF	5 × 1 ml	17-0921-02
	1 × 5 ml	17-0921-04
HiPrep IMAC FF 16/10	1 × 20 ml	28-9365-52
IMAC Sepharose 6 Fast Flow	25 ml	17-0921-07
	100 ml [1]	17-0921-08
HiTrap Desalting	5 × 5 ml	17-1408-01
HiPrep 26/10 Desalting	1 × 53 ml	17-5087-01
	4 × 53 ml	17-5087-02

[1] Process-scale quantities are available. Please contact your local representative.

Accessories HiScreen	Quantity	Code No
HiTrap/HiPrep, 1/16" male connector for ÄKTA *(For connection of columns with 1/16" fittings to ÄKTA)*	8	28-4010-81
Union 1/16" male/1/16" male with 0.5 mm i.d. *(For connecting two columns with 1/16" fittings in series)*	2	18-1120-93
Fingertight stop plug, 1/16" [1] *(For sealing a HiScreen column)*	5	11-0003-55

[1] One fingertight stop plug is connected to the inlet and the outlet of each HiScreen column at delivery.

Related literature	Code No.
Affinity Chromatography Handbook, Principles and Methods	18-1022-29
Recombinant Protein Purification Handbook, Principles and Methods	18-1142-75
Affinity Chromatography Columns and Media, Selection Guide	18-1121-86
Ni Sepharose and IMAC Sepharose, Selection Guide	28-4070-92
Prepacked chromatography columns for ÄKTA systems, Selection Guide	28-9317-78

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