Instructions 28-9063-51 AC

HiTrap™ Capto adhere, 1 ml and 5 ml

Capto™ adhere is a multimodal BioProcess™ medium for intermediate purification and polishing of monoclonal antibodies after capture on Protein A medium by packed bed chromatography. In combination with Protein A medium (i.e., MabSelect™ family), Capto adhere offers a robust chromatography platform for the development of monoclonal antibody manufacturing processes.

Capto adhere improves yield, productivity and process economy with:

- High capacity in flow-through mode
- Contaminant removal to formulation levels in post Protein A purification:
    - Leached Protein A
    - Antibody dimers and aggregates
    - Host cell proteins
    - Nucleic acids
    - Viruses
- Wider operational window of pH and conductivity
- Savings in time and operating costs with a two steps chromatographic process

HiTrap Capto adhere are prepacked 1 ml and 5 ml columns for screening of loading and elution conditions, and method scouting. The column design, together with a modern chromatography medium, provides fast, reproducible separations in a convenient format. The columns are best used with liquid chromatography systems such as ÄKTA™, but can be operated with a syringe or peristaltic pump.

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Please read these instructions carefully before using HiTrap columns.

Intended use

HiTrap 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

HiTrap column characteristics

The columns are made of biocompatible polypropylene that does not interact with biomolecules.

The columns are delivered with a stopper at the inlet and a snap-off end at the outlet. Table 1 lists the characteristics of HiTrap columns.

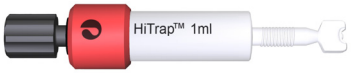

Fig 1. HiTrap, 1 ml column.

Fig 2. HiTrap, 5 ml column.

Note: *HiTrap columns cannot be opened or refilled.*

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

Table 1. Characteristics of HiTrap columns.

Column volume (CV)	1 ml	5 ml
Column dimensions	0.7 × 2.5 cm	1.6 × 2.5 cm
Column hardware pressure limit	5 bar (0.5 MPa)	5 bar (0.5 MPa)

Note: *The pressure over the packed bed varies depending on a range of parameters such as the characteristics of the chromatography medium, sample/liquid viscosity and the column tubing used.*

Supplied Connector kit with HiTrap column

Connectors supplied	Usage	No. supplied
Union 1/16" male/ luer female	For connection of syringe to HiTrap column	1
Stop plug female, 1/16"	For sealing bottom of HiTrap column	2, 5 or 7

Chromatography medium properties

Capto adhere is a strong anion exchanger with multimodal functionality (Fig 3). The multimodal functionality gives a different selectivity compared to traditional anion exchangers. Capto adhere is designed for post Protein A purification of monoclonal antibodies. Removal of leached Protein A, aggregates, host cell proteins, nucleic acids and viruses from monoclonal antibodies is performed in flow-through mode at which the antibodies pass directly through the column while the contaminants are adsorbed.

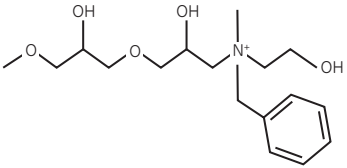

Fig 3. The Capto adhere ligand, N-Benzyl-N-methyl ethanol amine, exhibits many functionalities for interaction. The most pronounced are ionic interaction, hydrogen bonding and hydrophobic interaction.

Table 2. Characteristics of Capto adhere

Matrix	Highly cross-linked agarose
Functional group	Multimodal strong anion exchanger
Total ionic capacity	0.09 to 0.12 mmol Cl^-/ml medium
Particle size[1]	75 µm (d_{50v})
Flow velocity[2]	At least 600 cm/h in a 1 m diameter column with 20 cm bed height at 20°C using process buffers with the same viscosity as water at < 3 bar (0.3 MPa)
pH-stability[3]	
short term	2 to 14
long term	3 to 12
Working temperature[4]	4°C to 30°C
Chemical stability[5]	All commonly used aqueous buffers, 1 M acetic acid, 1 M sodium hydroxide.
Avoid	Oxidizing agents, anionic detergents
Storage	4°C to 30°C in 20% ethanol

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

[2] The capacity for selective removal of some key contaminants may decrease at high flow velocity.

[3] **Short term pH:** pH interval where the medium can be subjected to, for cleaning- or sanitization-in-place.
Long term pH: pH interval where the medium can be operated without significant change in function.

[4] Capto adhere can be used under cold-room conditions, but the capacity for some key contaminants may decrease.

[5] No significant change in ionic binding capacity and carbon content after 1 week storage in 1 M NaOH at 40°C.

2 Method design and optimization

Capto adhere is as multimodal ion exchanger designed to be used as a second or third step in monoclonal antibody (MAb) purification processes, (i.e., after capture on Protein A medium). Removal of leached Protein A, antibody dimers and aggregates (D/A), host cell proteins (HCP), viruses and nucleic acids is preferably performed in flow-through mode where the antibodies pass directly through the column while the contaminants are adsorbed.

General purification protocol

- Adjust pH and conductivity of the sample to loading conditions for flow-through mode.
- Equilibrate the column with loading buffer of the same pH and conductivity as the sample.
- Apply the sample onto the column. Collect the flow-through fraction.
- Wash out unbound material with loading buffer and collect together with the flow-through fraction.
- Regenerate column to elute bound material.
- Clean-in-place (CIP).
- Re-equilibrate.

Sample preparation

Before sample loading, pH and conductivity of the sample should to be adjusted to desired loading conditions. This is done either by buffer exchange or by direct adjustment of pH and conductivity. Buffers normally used for ion exchange chromatography can also be used also for Capto adhere (Table 3).

Table 3. Recommended buffers.

pH interval	Buffer[1,2]	Concentration[3]
4–5	Acetate	20–100 mM
4–6	Citrate	20–200 mM
5.5–6.5	Bis-TRIS	20–50 mM
6–7.5	Phosphate	50–200 mM
7.5–8.5	TRIS	20–50 mM
8.5–	Glycin-NaOH	20–100 mM

[1] The choice of buffer systems and salts may influence both yield and contaminant clearance.
[2] Buffers in the interval 5.5–8 will normally be most efficient for contaminant removal.
[3] Conductivity can be adjusted by addition of salt or by varying the buffer concentration.

Buffer exchange

For preparation of samples for optimization of loading conditions in lab scale, buffer exchange can be performed on HiPrep™ 26/10 Desalting. A typical chromatogram is shown in Figure 4.

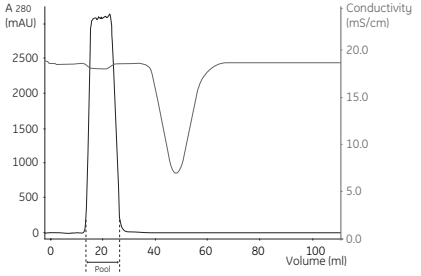

Fig 4. Buffer exchange on HiPrep 26/10 Desalting.

Sample:	MabSelect SuRe™ eluated pool
Sample volume:	10 ml
Buffer:	25 mM BIS-Tris, 175 mM NaCl, pH 7.0
Flow rate:	15 ml/min
Pooled desalted material:	13 ml

Conductivity and pH adjustment

For larger volumes of feed, sample preparation is preferably performed by diafiltration or directly by adjustment of pH and conductivity[1].

[1] Buffer exchange may result in reduction of HCP levels and improve column performance.

Initial screening of loading conditions

Balancing product yield against product purity is the major consideration when optimizing a method. When running in flow-through mode, loading conditions will usually be a compromise between conditions favoring yield and conditions favoring contaminant clearance. By adjusting pH and conductivity of the sample as well as the sample load, conditions can be obtained where most contaminants are adsorbed while the monomeric antibodies pass through the column. Optimization of loading conditions is preferably performed by using Design of Experiments (DoE). A common approach in DoE is to define a reference experiment (center point) and perform representative experiments around that point. To be able to define the center point and the variable ranges some initial experiments are required.

To find conditions suitable for the DoE, initial experiments can be performed in binding mode, using a pH gradient for elution (Fig 5, left). The elution position, that is pH at peak maximum, defines the lower pH in the design. The upper pH in the design should normally be about two pH units higher. Experiments can also be performed in flow-through mode, keeping the conductivity constant at a moderate level. A comparison of chromatograms is shown in Figure 5. At high pH (i.e., close to pI for the antibodies) the breakthrough during sample load is delayed, the breakthrough and wash curves are shallow and significant amounts of MAb binds to the adsorbent. A decrease in pH (i.e., further from pI) results in weaker electrostatic interaction between the antibodies and the adsorbent, steeper breakthrough and wash curves and increased yield.

In the DoE, pH, conductivity and load must be included. It is important to include conditions at the higher pH range resulting in lower yield and higher purity as well as conditions at lower pH

range resulting in higher yield and lower purity. An example is given below.

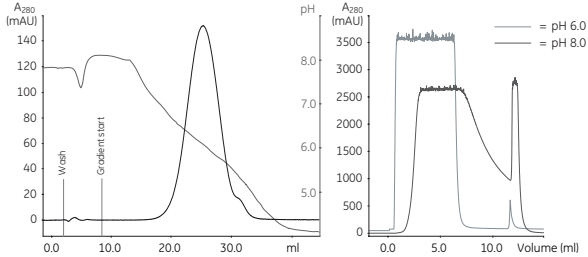

Fig 5.

Sample:	rProtein A elution pool, after buffer exchange on HiPrep 26/10 Desalting.
Binding mode (left):	Sample loading at pH 7.8. Load: 1 mg/ml medium. Elution performed in a pH gradient from 7.8 to 4.0.
Flow-through mode (right):	Comparison of chromatograms obtained at different pH. Load 75 mg MAb/ml medium. Conditions: pH 8.0, 2 mS/cm and pH 6.0, 2 mS/cm.

Design of experiments for optimization of loading conditions

Design of experiments (DoE) is recommended for optimization of loading conditions. By systematically varying important parameters (i.e., pH, conductivity and sample load) response surfaces can be obtained for yield and for clearance of key contaminants. The following is an example of how to set up a full factorial DoE with three parameters.

1. Work prior to actual setup of the design. Perform initial loading experiments at varying pH, as described above. Choose parameters to include and define parameter ranges and responses.
2. Choose design for screening or optimization. Full factorial design is commonly used in both screening and optimization.

A full factorial DoE in 3 parameters will give $2^3 = 8$ experiments + center points

3 Choose center points for the design. Center points are important in DoE, because they give an indication if there is curvature in the data. Three replicated center points are recommended. A full factorial design in three parameters with three center points gives a total of 11 experiments

4 Systematic variation of the parameters. Limiting values, high and low, should be used of each parameter. The high and low values should be combined in a way that makes the parameters independent of each other to be able to separate effects. For further information, see *www.umetrics.com*

Example of design of experiments

Start material: Monoclonal IgG_1 expressed in CHO cell culture supernatant, initially purified on rProtein A medium.

Sample characteristics: pI ~ 9, leached Protein A 36 ppm, D/A 3.3%[1] and HCP 210 ppm. The experimental setup was a full factorial design[2] in three variables, load, pH and conductivity, with additional points to resolve curvature effects (Table 4). In all, the number of experiments included in the model was 14, and the measured responses were yield (%) and Protein A (ppm), dimer/aggregate (%) and HCP (ppm) in the flow-through pool. For each response a separate model was calculated. The models were fitted to MLR (multiple linear regression) and are well explained and show good stability to cross validation.

[1] As determined by analytical size exclusion chromatography on Superdex™ 200 10/300 GL.

[2] Experiments were designed and evaluated using Modde 7.0 software (Umetrics, Sweden). www.umetrics.com.

Table 4. Design setup[1]

Load (mg MAb/ml)	pH	Conductivity (mS/cm)
75	6	2
300	6	2
75	8	2
300	8	2
75	6	15
300	6	15
75	8	15
300	8	15
187.5	**7**	**8.5**
187.5	**7**	**8.5**
75	7	15
300	7	15
187.5	7	2
187.5	7	15

[1] The design setup includes 2 center points (bold) and 4 additional points at pH 7 to resolve curvature effects.

Parameters affecting the yield

The parameters that affect the yield are shown in the coefficient plot[1] (Fig 6). The plot shows that high sample load, low pH and high conductivity results in high yield. The interaction effects (load × pH and load × conductivity) are also significant for the yield response.

The response surfaces also show that higher loads will give larger pH window with yield > 90%.

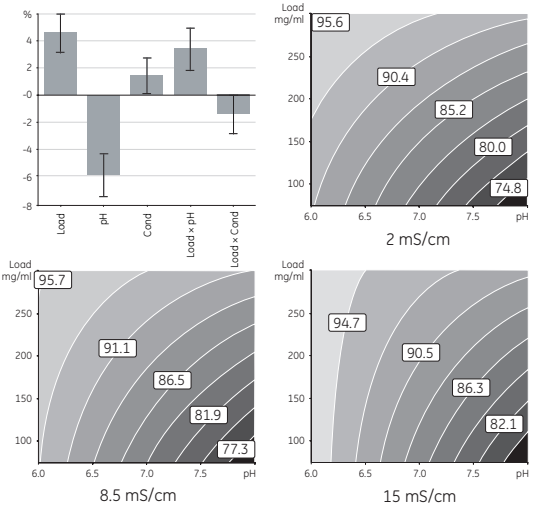

Fig 6. Coefficient plot and response surfaces for the yield model. Load versus pH at different conductivities.

[1] The coefficient plot describes the impact on the response from the investigated parameters. In the case above it can be seen that load is positively correlated to the response, meaning that a higher load will give a higher value on the response, pH is negatively correlated to the response, meaning that a lower pH will give a higher value on the response and conductivity is positively correlated to the response, but smaller, meaning that a higher conductivity will also give a higher response value, but not to the same extent as the effect of Load.

The interaction effects that are present in the coefficient plot (Load × pH and Load × Conductivity) means that if, for example pH is changed the response will not only be changed with the effect of pH but also with the effect of Load at that specific pH. The same goes for the Load × Conductivity interaction.

Parameters affecting the Protein A clearance

The coefficient plot shows that high pH will give good Protein A clearance. The conductivity by itself did not significantly affect the response, but there is a significant interaction effect for pH ×

conductivity (Fig 7). If this term is high, the Protein A clearance will be low. Load was not a significant factor for this response.

The response surfaces show that high pH and low conductivity will give high Protein A clearance.

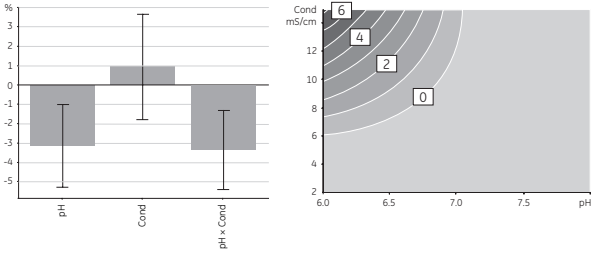

Fig 7. Coefficient plot and response surfaces for the Protein A model. Conductivity versus pH. Protein A concentration expressed in ppm.

Parameters affecting dimer/aggregate clearance

The coefficient plot (Fig 8) shows that pH is the most important parameter and that high pH will give low D/A concentration in the flow-through pool. The load parameter is also significant, but very small. The load should be low to give high D/A-clearance. There is also a significant curvature effect assigned to pH. If pH is too high or too low the D/A response will increase. The conductivity did not significantly affect D/A-clearance. The response curve shows that the load has only small affect on D/A-clearance, so only pH has to be considered.

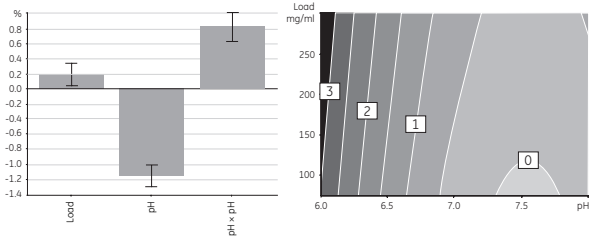

Fig 8. Coefficient plot and response surfaces for the D/A model. Load versus pH. D/A concentration expressed in %.

Parameters affecting host cell proteins (HCP) clearance

The coefficient plot (Fig 9) and response curves shows that low sample load, low conductivity and high pH will give low HCP values.

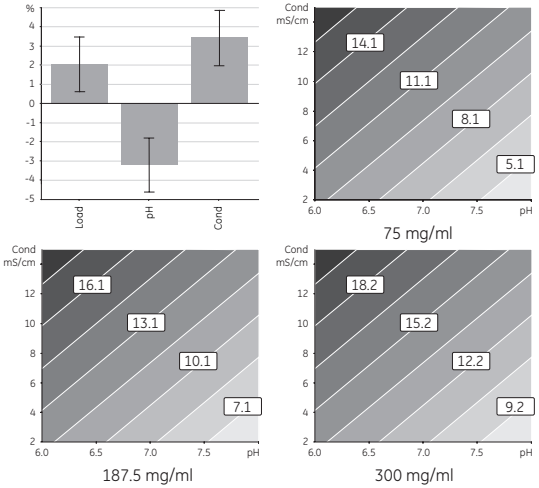

Fig 9. Coefficient plot and response surfaces for the HCP model. Conductivity versus pH. HCP concentration expressed in ppm.

Conclusions

The response surfaces above show the influence of sample load, pH and conductivity on four different responses (yield of monomeric MAb and clearance of Protein A, dimer/aggregates and HCP respectively), and how to reach desired values for each of them. Even though the optimal conditions for each response is not the same, there is quite a large area where acceptable values can be obtained for all four responses.

Suggested loading conditions for this MAb could be a sample load of 200 mg/ml, pH 7 and conductivity 8.5 mS/cm.

General trends

Each monoclonal antibody is unique, and the level of contaminants varies between different cell lines and differences in previous purification steps. This implies that it may be difficult to predict optimal loading conditions.

However, based on design of experiments performed with several different antibodies some general trends have been identified (Fig 10).

- For best yield load should be high, the pH low and conductivity high.
- For the best D/A-clearance the pH should be high, while load and conductivity should be low. D/A-clearance is often less affected by conductivity than Protein A and HCP clearance.
- For the best Protein A- and HCP-clearance the pH should be high and conductivity low.

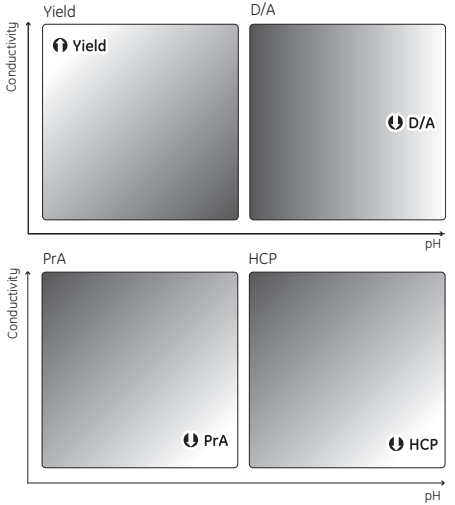

Fig 10. General trends with respect to loading conditions for yield, D/A-, Protein A- and HCP-clearance.

Loading conditions will therefore be a compromise between conditions favoring yield and conditions favoring contaminant clearance. Optimal loading conditions will be a balance between load, pH and conductivity. Consequently, for optimization of the loading step, all three parameters should be varied in the same experimental series.

In Table 5 optimal loading conditions for five different antibodies are shown. As can be seen, pH should normally be below the isoelectric point, while optimal conductivity is harder to predict.

Table 5. Optimal loading conditions for different MAbs.

MAb	pI	pH	Conductivity (mS/cm)
MAb 1	~ 9	7.0	8
MAb 2	8.3–8.9	5.5	3
MAb 3	7.5–8.4	6.0	2
MAb 4	7.7–8.0	7.0	20
MAb 5	6.5–9.0	7.7	20

Flow velocity and residence time

For efficient removal of contaminants in flow-through mode, residence time[1] should be 2 minutes or more. Longer residence time may result in more efficient contaminant removal.

Bed height in HiTrap Capto adhere 1 ml and 5 ml columns is 2.5 cm. This implies flow rates of ≤ 0.5 ml/min (75 cm/h) for HiTrap 1 ml and ≤ 2.5 ml/min for HiTrap 5 ml.

In production scale bed heights of 10–20 cm are recommended. Recommended flow velocity is between 150–600 cm/h.

[1] Residence time, $\tau = L/u$, where L is the bed height in cm and u is the linear velocity (cm/h).

Wash out unbound material

After sample load, continue to wash out unbound MAb with loading buffer until the UV-curve starts to level off. The wash fractions can normally be pooled together with the flow-through

fractions. How much of the wash fractions that is collected is a balance between yield and dilution of the pooled material.

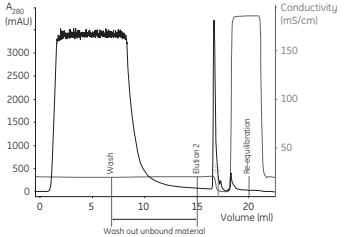

Fig 11. Example of wash after sample application.

3 Regeneration and cleaning

Regeneration

After each step, elute any reversibly bound material with low pH (e.g., 0.1 M acetate, pH 3.0). Regenerate the medium by washing until the column effluent shows stable conductivity and pH values. Recommended flow rate: 3.8 ml/min (600 cm/h) for HiTrap 1 ml and 20 ml/min for HiTrap 5 ml.

Cleaning-in-place

Cleaning-in-place (CIP) is recommended after each cycle. A standard protocol for CIP is to wash with 1 M NaOH using a contact time of 15 minutes. Recommended flow rate: 1 ml/min (150 cm/h) for HiTrap 1 ml and 5 ml/min for HiTrap 5 ml.

For further information about CIP, *see* instruction for HiTrap Capto adhere (28-9064-05)

Re-equilibration

At the end of each purification cycle, and after regeneration and cleaning-in-place, the column should be re-equilibrated with 5 bed volumes of loading buffer, or until the column effluent shows stable conductivity and pH values. Recommended flow rate:

3.8 ml/min (600 cm/h) for HiTrap 1 ml and 20 ml/min for HiTrap 5 ml.

Storage

Wash with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Store at 4ºC to 30ºC.

Do not freeze. Ensure that the column is sealed well to avoid drying out.

After storage, equilibrate with at least 5 column volumes start buffer before use.

4 Adjusting pressure limits in chromatography system software

Pressure generated by the flow through a column affects the packed bed and the column hardware, see Fig 12. 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.*

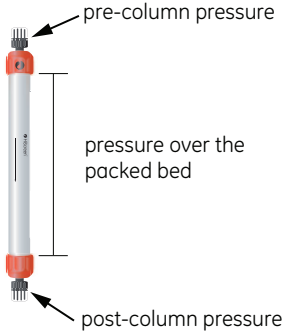

Fig 12. Pre-column and post-column measurements.

ÄKTA avant and ÄKTA pure

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:

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.*

5 Ordering information

Product	Pack size	Code No
HiTrap Capto adhere	5 × 1 ml	28-4058-44
HiTrap Capto adhere	5 × 5 ml	28-4058-46

Related products	Pack size	Code No
HiTrap Capto adhere	25 ml	17-5444-10
	100 ml	17-5444-01
HiTrap MabSelect	5 × 1 ml	28-4082-53
	5 × 5 ml	28-4082-56
HiTrap MabSelect SuRe	1 × 1 ml	29-0491-04
	5 × 1 ml	11-0034-93
	5 × 5 ml	11-0034-95
HiTrap MabSelect Xtra™	5 × 1 ml	28-4082-58
	5 × 5 ml	28-4082-56
HiPrep 26/10 Desalting	1 × 53 ml	17-5087-01
	4 × 53 ml	17-5087-02

Accessories	Pack size	Code No.
1/16" male/luer female *(For connection of syringe to top of HiTrap column*)	2	18-1112-51
Tubing connector flangeless/M6 female (*For connection of tubing to bottom of HiTrap column)*	2	18-1003-68
Tubing connector flangeless/M6 male *(For connection of tubing to top of HiTrap column)*	2	18-1017-98
Union 1/16" female/M6 male *(For connection to original FPLC System through bottom of HiTrap column)*	6	18-1112-57
Union M6 female /1/16" male *(For connection to original FPLC System through top of HiTrap column*)	5	18-3858-01

Accessories	Pack size	Code No.
Union luerlock female/M6 female	2	18-1027-12
HiTrap/HiPrep, 1/16" male connector for ÄKTA design	8	28-4010-81
Stop plug female, 1/16" *(For sealing bottom of HiTrap column)*	5	11-0004-64
Fingertight stop plug, 1/16"	5	11-0003-55

Related literature	Code No
Data file Capto adhere	28-9078-88
Application note: Optimization of loading conditions on Capto adhere using design of experiments	28-9078-89
Application note: Two step purification of monoclonal IgG1 from CHO cell supernatant	28-9078-92
Application note: Selective removal of aggregates with Capto adhere	28-9078-93

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