cytiva

HiTrap NHS-activated HP

1 ml and 5 ml

Instructions for Use

HiTrap™ NHS-activated HP is a prepacked ready to use, column for preparative affinity chromatography. The special design of the column, together with the medium, provide fast, simple and easy separations in a convenient format.
The column can be operated with a syringe, peristaltic pump or liquid chromatography system such as ÄKTA™.

Table of contents

1. Product description 3
2. Operation 6
3. Measuring of coupling efficiency 9
4. Purification 12
5. Adjusting pressure limits in chromatography system software ... 13
6. Storage 15
7. Ordering information 17

Important

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 x 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

Medium properties

NHS-activated Sepharose™ High Performance is designed for the covalent coupling of ligands containing primary amino groups. The medium is based on highly cross-linked agarose beads with 10 atoms spacer arms attached to the matrix by epichlorohydrin and activated by N-hydroxysuccinimide.

The substitution level is ≈ 10 µmol NHS groups/ml medium. Nonspecific adsorption of proteins to HiTrap columns is negligible due to the hydrophilic properties of the base matrix.

The activated medium is supplied in 100% isopropanol to preserve the stability of the activated medium prior to coupling.

Note: *Do not replace the isopropanol until it is time to couple the ligand.*

The characteristics of the product is summarized in Table 2.

Table 2. HiTrap NHS-activated HP characteristics.

Degree of substitution	≈ 10 µmol NHS groups/ml medium
Spacer arm	10-atom
Bead structure	Highly cross-linked spherical agarose, 6%
Mean particle size	34 µm
Max. flow rates[1]	4 ml/min and 20 ml/min for 1 ml and 5 ml column respectively
Rec. flow rates	1 ml/min and 5 ml/min for 1 ml and 5 ml column respectively
Chemical stability	All common used buffers
Coupling conditions	pH 6.5 to 9, room temperature, 15–30 min or pH 6.5 to 9, 4°C, 4 hours
pH stability[2]	
Long term	3 to 12
Short term	3 to 12

[1] Water at room temperature

[2] Depends on the ligand. The ranges given are estimates based on our knowledge and experience. Please note the following:

pH stability, long term refers to the pH interval where the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance.

pH stability, short term refers to the pH interval for regeneration. Data refers to the coupled product provided that the ligand can withstand the pH given.

2 Operation

Buffer preparation

Water and chemicals used for buffer preparation should be of high purity. It is recommended to filter the buffers by passing them through a 0.45 µm filter before use.

Preparation

The column can be operated with a syringe, peristaltic pump or chromatography system.

Isopropanol (100%) is used to prevent deactivation of the NHS groups.

Note: *Do not wash out the isopropanol until you are ready to do the coupling reaction.*

Standard coupling buffer: 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 Coupling can be done within the pH range 6.5 to 9, maximum yield is achieved at pH ~ 8.

1. Dissolve the desired ligand in the coupling buffer to an accurate concentration (0.5 to 10 mg/ml for proteins) or make a buffer exchange using HiTrap Desalting, HiPrep™ 26/10 Desalting or PD-10 columns. The concentration depends on the ligand being used. Optimal volume is 1 and 5 ml for HiTrap 1 ml and HiTrap 5 ml, respectively.
2. Remove the top-cap and apply a drop of ice cold 1 mM HCl to the top of the column to avoid air bubbles.
3. Connect the HiTrap luer connector (or tubing from a system) to the top of the column.
4. Remove the snap-off end at the column outlet.

Ligand coupling

Coupling is an easy procedure using a syringe (1 or 2 ml syringe for HiTrap 1 ml and 5 or 10 ml syringe for HiTrap 5 ml).

1. Wash out the isopropanol with 1 mM HCl, ice-cold. Use 3 × 2 ml for HiTrap 1 ml and 3 × 10 ml for HiTrap 5 ml. Be sure not to exceed flow rates of 1 ml/min (1/2 drop/sec) for HiTrap 1 ml and 5 ml/min (2 drop/sec) for HiTrap 5 ml at this stage. The medium can be irreversible compressed.
2. Immediately inject 1 ml (HiTrap 1 ml) or 5 ml (HiTrap 5 ml) of the ligand solution onto the column.
3. Seal the column. Then, let it stand for 15 to 30 minutes at 25°C (or 4 hours at 4°C).

If larger volumes of ligand solution are used, recirculate the solution by connecting a second syringe to the outlet of the column and gently pump the solution back and forth for
15 to 30 minutes.

Note: *A Union luerlock female/M6 female, 18-1027-12 is needed,* see *"Ordering information".*

Recirculation can also be performed by connecting a peristaltic pump, e.g. Pump P-1, using the connectors in the supplied connector kit. To measure coupling efficiency, before deactivation, please refer to the section "Measuring of coupling efficiency".

Note: *If a P-1 pump is used a max flow rate of 1 to 3 ml/min can be run on a HiTrap 1 ml column packed with Sepharose High Performance media.*

Washing and deactivation

Deactivate any excess active groups that have not coupled to the ligand, and wash out the non-specifically bound ligands, by following the procedure below:

Buffer A: 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3

Buffer B: 0.1 M sodium acetate, 0.5 M NaCl, pH 4

1. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer A.
2. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer B.
3. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer A.
4. Leave the column for 15 to 30 min in room temp. or approx. 4 hours in 4°C.
5. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer B.
6. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer A.
7. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer B.
8. Finally, inject 2 ml (HiTrap 1 ml) or 10 ml (HiTrap 5 ml) of binding buffer with neutral pH to adjust the pH.

The column is now ready for use. If the column is not going to be used at once, it can be stored in storage solution, e.g., 0.05 M Na_2HPO_4, 0.1% NaN_3, pH 7.

3 Measuring of coupling efficiency

Since the N-hydroxysuccinimide group, which is released during the coupling reaction, shows strong absorbtion at 280 nm (pH above 6), we recommend you to determine the coupling efficiency in either of two ways:

- For ligands with M_r > 5000 and/or acid labile ligands. Use method 1: PD-10 desalting column.
- For ligands with M_r < 5000 and/or acid stable ligands. Use method 2: Acidification to pH 2.5.

Method 1: PD-10 desalting column

Note: *This protocol is a modification of the original instruction for PD-10 columns.*

1 After coupling, the coupling solution is washed out from the column with 3 column volumes of the coupling buffer e.g., 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3.

2 Equilibrate the PD-10 column (Table 3) with a suitable buffer e.g., 0.1 M NaH_2PO_4, 0.15 M NaCl, pH 7.0.

3 Load 0.5 ml of the coupling solution from step 1 and let it run onto the column. Discard the eluent.

4 Load 2.0 ml of equilibration buffer and let it run onto the column. Discard the eluent.

5 Add 1.5 ml of equilibration buffer to elute the high molecular weight component e.g., the protein. Collect eluate and measure the absorbance at 280 nm.

The yield from the PD-10 column with this protocol is normally 90%.

Calculation

Loaded coupling solution, A_{280} × ml:

$$A = A_{280} \times V$$

where

A_{280} = A_{280} of coupling solution
V = loaded volume of coupling solution

Amount not coupled, A_{280} × ml:

$$B = \frac{A_{280} \times 1.5 \times V}{0.5}$$

where

A_{280} = A_{280} of coupling solution after PD-10 run
1.5 = volume collected from PD-10
V = volume post coupling wash
0.5 = volume loaded on PD-10

Coupling yield, %: (A-B)/A × 100

Example:

1 ml of a protein solution with A_{280} = 14 was coupled to a 1 ml HiTrap NHS-activated HP column. After completed coupling, the coupling solution was washed out from the column with 3 ml of coupling buffer (e.g. 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3).

When 0.5 ml of this solution was separated on the PD-10 Desalting column according to above protocol the A_{280} of the eluate was 0.156.

Using this measurement we can calculate the coupling yield according to:

$$A = 14 \times 1 = 14$$

$$B = \frac{0.156 \times 1.5 \times 3}{0.5} = 1.4$$

$$\text{Coupling yield: } = \frac{14 - 1.4}{14} \times 100 = 90\%$$

Method 2: Acidification to pH 2.5

1. After coupling, the coupling solution is washed out from the column with 3 column volumes of the coupling buffer e.g. 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3
2. 1 ml of the solution from step 1 is mixed with 1 ml 2 M glycine-HCl, pH 2.0
3. Measure the absorbance at 280 nm.

With this protocol the contribution to the A_{280} from N-hydroxysuccinimide is < 0.005.

Calculation

Loaded coupling solution, A_{280} × ml:

$A = A_{280} \times V$

where

A_{280} = A_{280} of coupling solution after PD-10 run
V = loaded volume of coupling solution

Amount not coupled, A_{280} × ml:

$B = A_{280} \times V \times 2$

where

A_{280} = A_{280} of coupling solution after PD-10 run
V = loaded volume of coupling solution
2 = dilution when acidified

Coupling yield, %: (A-B)/A × 100

Example:

1 ml of a protein solution with A_{280} = 14 was coupled to a 1 ml HiTrap NHS-activated HP column. After completed coupling, the coupling solution was washed out from the column with 3 ml of coupling buffer (e.g. 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3).

When 1 ml of this solution was mixed with 1 ml 2 M glycine-HCl, pH 2. Absorbance at 280 nm was 0.702.

Using this measurement we can calculate the coupling yield according to:

$A = 14 \times 1 = 14$

$B = 0.702 \times 3 \times 2 = 4.2$

$$\text{Coupling yield: } = \frac{14 - 4.2}{14} \times 100 = 70\%$$

4 Purification

Conditions for binding and elution depend on the type of ligand used.

Perform a blank run before the experiment to wash off all the loosely bound ligand.

1. Prepare the column by washing with:
   - **a** 3 ml (HiTrap 1 ml) or 15 ml (HiTrap 5 ml) binding buffer.
   - **b** 3 ml (HiTrap 1 ml) or 15 ml (HiTrap 5 ml) elution buffer.
2. Equilibrate the column with 10 column volumes of the binding buffer.
3. Sample preparation. The sample should be adjusted to the composition of the binding buffer. This can be done by either diluting the sample with binding buffer or by buffer exchange using HiTrap Desalting, HiPrep 26/10 Desalting, or PD-10 Desalting column (Table 3). The sample should be filtered through a 0.45 µm filter or centrifuged just before it is applied to the column.

4 Apply the sample, using a syringe fitted to the luer connector or by pumping it onto the column. Recommended flow rate 0.2 to 1 ml/min (HiTrap 1 ml) or 1 to 5 ml/min (HiTrap 5 ml), the optimal flow rate is dependent on the binding constant of the ligand.

5 Wash with binding buffer, 5 to 10 column volumes or until no material appears in the effluent. Excessive washing should be avoided if the interaction between the protein of interest and the ligand is weak, since this may decrease the yield.

6 Elute with elution buffer. 1 to 3 column volumes is usually sufficient but larger volumes may be necessary.

7 The purified fractions can be desalted using HiTrap Desalting, HiPrep 26/10 Desalting or PD-10 columns if necessary (Table 3).

8 Re-equilibrate the column by washing with 5 to 10 column volumes binding buffer.

Note: *If a P1-pump is used a max flow rate of 1 to 3 ml/min can be run on a HiTrap 1 ml column packed with Sepharose High Performance media.*

5 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 3. 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.*

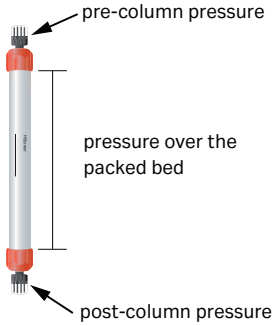

Fig 3. Pre-column and post-column measurements.

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:

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.*

6 Storage

The columns are delivered in 100% isopropanol and should be stored in this solution until use. After ligand coupling store the column in for example 0.05 M Na_2HPO_4, 0.1% NaN_3, pH 7. The storage buffer depends on the ligand coupled.

Note: *Sudden changes in temperature may cause small dark spots to appear against the light background of the gel bed. This phenomenon disappears when the column reaches room temperature or during equilibration.*
The appearance and disappearance of these spots does not affect the performance of HiTrap NHS-activated HP.

Table 3. Prepacked columns for desalting and buffer exchange

Column	Loading volume	Elution volume
HiPrep 26/10 Desalting [1]	2.5 to 15 mL	7.5 to 20 mL
HiTrap Desalting [2]	0.25 to 1.5 mL	1.0 to 2.0 mL
PD-10 Desalting [3]	1.0 to 2.5 mL[4] 1.75 to 2.5 mL[5]	3.5 mL Up to 2.5 mL
PD MiniTrap™ G-25	0.1 to 2.5 mL[4] 0.2 to 0.5 mL[5]	1.0 mL Up to 0.5 mL
PD MidiTrap™ G-25	0.5 to 1 mL[4] 0.75 to 1 mL[5]	1.5 mL Up to 1 mL

[1] Prepacked with Sephadex™ G-25 Fine and requires a pump or a chromatography system to run.

[2] Prepacked with Sephadex G-25 Superfine and requires a syringe or pump to run.

[3] Prepacked with Sephadex G-25 and can be run by the gravity flow or centrifugation.

[4] Volumes with gravity elution.

[5] Volumes with centrifugation

7 Ordering information

Product	No. supplied	Code No.
HiTrap NHS-activated HP	5 × 1 ml	17-0716-01
	1 × 5 ml	17-0717-01

Related products	No. supplied	Code No.
HiPrep 26/10 Desalting	1 × 53 ml	17-5087-01
	4 × 53 ml	17-5087-02
HiTrap Desalting	1 × 5 ml	29-0486-84
	5 × 5 ml	17-1408-01
PD-10 Desalting Column	30	17-0851-01

Accessories	Quantity	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
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.
Affinity Chromatography Handbook, Principles and Methods	18-1022-29
Affinity Chromatography Columns and Media, Selection Guide	18-1121-86
Prepacked chromatography columns for ÄKTA design systems, Selection Guide	28-9317-78

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