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HiTrap Benzamidine FF (high sub)

1 ml and 5 ml

Instructions for Use

Abstract

HiTrap™ Benzamidine FF (high sub) are prepacked 1 ml and 5 ml columns for convenient, one-step removal and/or purification of trypsin, trypsin-like serine proteases, and zymogens including urokinase and prekallikrein. Removal of serine proteases is easily done directly from serum, monoclonal cell supernatants and bacterial lysates.
The medium, Benzamidine Sepharose™ 4 Fast Flow (high sub), is also available as lab packages and is an excellent choice for scale-up. The columns can be operated using syringe, peristaltic pump or liquid chromatography system such as ÄKTA™.

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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

Benzamidine Sepharose 4 Fast Flow (high sub) is designed for removal and/or purification of serine proteases. Removal of serine proteases from serum, monoclonal cell supernatants, and bacterial lysates can be easily performed using HiTrap Benzamidine FF (high sub) in order to protect the produced protein from degradation. The benzamidine ligand is covalently coupled via an amide bond to a long spacer arm attached to highly crosslinked 4% agarose via a stable ether linkage. The coupling is optimized to give high binding capacity. Total binding capacity is
≥ 35 mg trypsin/ml medium.

The characteristics of HiTrap Benzamidine FF (high sub) are summarized in Table 2.

Table 2. Characteristics of HiTrap Benzamidine FF (high sub)

Ligand	p-aminobenzamidine (pABA)
Spacer	14-atom
Ligand concentration	≥ 12 µmol p-aminobenzamidine/ml medium
Binding capacity	≥ 35 mg trypsin/ml medium
Mean particle size	90 µm
Bead structure	Highly cross-linked agarose, 4%
Recommended flow rates	1 ml/min and 5 ml/min for 1 and 5 ml columns respectively
Maximum flow rates[1]	4 ml/min and 20 ml/min for 1 and 5 ml columns respectively
Chemical stability	All commonly used aqueous buffers
pH stability[2] short term long term	 pH 1 to 9 pH 2 to 8
Storage temperature	4 to 8°C
Storage buffer	20% ethanol in 0.05 M acetate buffer, pH 4

[1] Water at room temperature

[2] 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, cleaning-in-place, and sanitization procedures.

2 Operation

The columns can be operated using a syringe, peristaltic pump or liquid chromatography system.

Buffer preparation

Water and chemicals used for buffer preparation should be of high purity. We recommend filtering the buffers by passing them through a 0.45 µm filter and de-gassing them before use.

Recommended buffers

Binding and wash buffers

Since HiTrap Benzamidine FF (high sub) has some ionic binding characteristics, we recommend the use of a salt concentration of at least 0.5 M and pH 7.4 to 8, *for example*

0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4.

Note: *If a lower salt concentration is used, include a high salt wash step before further elution takes place to wash out proteins bound due to ionic forces.*

Elution buffers

Elution can be performed using either a step or a continuous gradient. Substances bound through ionic interactions can be eluted by increasing the salt concentration to 1.0 M. Affinity bound substances can be eluted in different ways:

Low pH elution buffers:

0.05 M glycine, pH 3.0 or

10 mM HCl, 0.5 M NaCl, pH 2.0

Note: *To each collection tube, add 60–200 µl 1 M Tris-HCl, pH 9 per ml of fraction to be collected, in order to prevent eluted protein denaturing at low pH.*

Competitive elution buffer:

20 mM p-aminobenzamidine in binding buffer.

Note: *This elution buffer has a very high A_{280}. The eluted protein must be detected by other methods, such as activity*

measurement (if possible), total protein, or SDS-PAGE analysis. The advantage of competitive elution is that pH can be kept constant during the run.

Other possible elution buffers:

Denaturing agents such as 8 M urea or 6 M guanidine hydrochloride.

Sample preparation

The sample should be centrifuged and/or filtered through a 0.45 µm filter immediately before it is applied to the column. If the sample is too viscous, dilute it with binding buffer to prevent column clogging.

Purification

Recommended flow rates: 1 ml/min (1 ml column) and 5 ml/min (5 ml column).

1. Fill the pump tubing or syringe with distilled water. Connect the column to the syringe (use the adaptor supplied) or pump tubing "drop-to-drop" to avoid introducing air into the column.
2. Remove the snap-off end at the column outlet.
3. Wash the column with 5 column volumes of distilled water to remove the storage buffer (0.05 M acetate buffer, pH 4 containing 20% ethanol).
4. Equilibrate the column with 5 column volumes of binding buffer.
5. Apply the sample using a syringe fitted to the luer connector or by pumping it onto the column.
6. Wash with 5–10 column volumes of binding buffer or until no material appears in the eluent.
7. Do a second wash with high salt, if preferred.
8. Elute with 5–10 column volumes of elution buffer of choice (see page 5).

The purified fractions can be buffer exchanged using HiTrap Desalting, HiPrep™ 26/10 Desalting or PD-10 Desalting columns.

3 Application

Note: *After any cleavage reaction, or for any protein sample, a similar protocol as described below, using HiTrap Benzamidine FF (high sub), can be used to remove trypsin-like serine proteases.*

Removal of thrombin after on-column cleavage of a GST-tagged protein

Binding buffer:	20 mM sodium phosphate, 0.15 M NaCl, pH 7.5
High salt wash buffer:	20 mM sodium phosphate, 1.0 M NaCl, pH 7.5

Purification and cleavage of tagged protein

A GST-tagged protein was expressed with a thrombin cleavage site incorporated. The GST-tagged protein was bound to GSTrap™ FF 1 ml column (see Instructions for GSTrap FF) and contaminating proteins were removed by washing with binding buffer. To cleave the GST-tagged protein from its GST-tag, thrombin (a serine protease) was dissolved in binding buffer and applied to the column by a syringe. The column was sealed and left to incubate according to the recommendations for the specific enzyme (2 to 16 h at 22ºC to 25ºC).

Sample clean-up

A HiTrap Benzamidine FF (high sub) 1 ml column was washed with water and equilibrated with binding buffer. The column was then placed in series directly after the GSTrap FF 1 ml column, which had been loaded with GST-tagged protein, washed and incubated with thrombin (as described).

While situated in series, the columns were washed with binding buffer. This allowed the cleaved over-expressed protein and thrombin to wash out fromthe GSTrap FF column directly onto the HiTrap Benzamidine FF (high sub).

Cleaved, expressed protein (now without GST-tag) passed through the HiTrap Benzamidine FF (high sub) column while thrombin was bound. The collected fractions thereby contained the pure protein of interest.

It is also possible to do a second wash step with a buffer including a high concentration of salt (e.g. 1 M NaCl) to wash out proteins bound due to ionic forces.

Elution

GSTrap FF 1 ml and HiTrap Benzamidine FF (high sub) 1 ml columns were eluted one by one according the instructions for each of them. In order to check for thrombin activity without taking into account any denaturing effects (due to low pH), HiTrap Benzamidine FF (high sub) was eluted using competitive elution
(20 mM p-aminobenzamidine in binding buffer).

Thrombin detection

Very small amounts of serine proteases are used in these types of cleavages. The chromogenic substrate S-2238 (Chromogenix, Heamochrom Diagnostica AB) can be used to determine the presence or absence of small quantities of thrombin in different fractions. In other words, it can be used to verify the removal of protease from expressed, purified, and cleaved protein.

4 Scaling up

HiTrap Benzamidine FF (high sub) is available as 1 ml and 5 ml columns. For fast scaling up, two or more columns can be connected in series by screwing the end of one into the top of the next (the back pressure will increase).

Benzamidine Sepharose 4 Fast Flow (high sub) is also available in bulkand is an excellent choice for scaling up due to high flow rate and binding properties. See Ordering information.

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.*

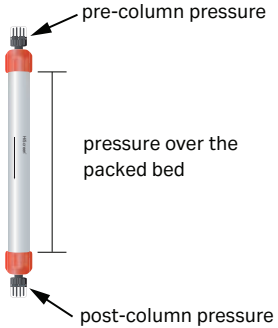

Fig 3. Pre-column and post-column measurements.

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

HiTrap Benzamidine FF (high sub) is supplied, and should be stored in 0.05 M acetate buffer, pH 4 containing 20% ethanol as a bacteriostat.

Storage temperature 4ºC to 8ºC.

7 Further information

Visit cytiva.com/hitrap for further information. Useful handbooks are available, see Ordering information.

8 Ordering information

Product	No. Supplied	Product code
HiTrap Benzamidine FF (high sub)	2 × 1 ml	17-5143-02
HiTrap Benzamidine FF (high sub)	5 × 1 ml	17-5143-01
HiTrap Benzamidine FF (high sub)	1 × 5 ml	17-5144-01

Related products	No. Supplied	Product code
Benzamidine Sepharose 4 Fast Flow (high sub)	25 ml	17-5123-10
HiTrap Desalting	1 × 5 ml	29-0486-84
HiTrap Desalting	5 × 5 ml	17-1408-01
HiTrap Desalting	100 × 5 ml*	11-0003-29
HiPrep 26/10 Desalting	1 × 53 ml	17-5087-01
HiPrep 26/10 Desalting	4 × 53 ml	17-5087-02
GSTrap FF	5 × 1 ml	17-5030-01
GSTrap FF	2 × 1 ml	17-5030-02
GSTrap FF	100 × 1 ml*	17-5130-05
GSTrap FF	1 × 5 ml	17-5031-01
GSTrap FF	100 × 5 ml*	17-5131-05
GSTrap 4B	1 × 1 ml	29-0486-09
GSTrap 4B	5 × 1 ml	28-4017-45
GSTrap 4B	100 × 1 ml*	28-4017-46
GSTrap 4B	1 × 5 ml	28-4017-47
GSTrap 4B	5 × 5 ml	28-4017-48
GSTrap 4B	100 × 5 ml*	28-4017-49
GSTrap HP	5 × 1 ml	17-5281-01

Related products	No. Supplied	Product code
GSTrap HP	100 × 1 ml*	17-5281-05
GSTrap HP	1 × 5 ml	17-5282-01
GSTrap HP	5 × 5 ml	17-5282-02
GSTrap HP	100 × 5 ml*	17-5282-05
Thrombin	500 Units	27-0846-01
Factor Xa	400 Units	27-0849-01

* Special pack size delivered on specific customer order.

Accessories	Quantity	Product code
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	Product code
Affinity Chromatography Handbook, Principles & Methods	18-1022-29
Recombinant Protein Purification Handbook, Principles & Methods	18-1142-75
Affinity Chromatography, Columns and Media, Selection guide	18-1121-86
Prepacked chromatography columns for ÄKTAdesign systems, Selection guide	28-9317-78

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