Benzamidine Sepharose 4 Fast Flow (high sub)
HiTrap Benzamidine FF (high sub)

HiTrap™ Benzamidine FF (high sub) are prepacked, ready to use 1 ml and 5 ml columns containing Benzamidine Sepharose™ 4 Fast Flow (high sub). These products are excellent tools for removal of proteolytic activity from a protein or peptide preparation or purification of trypsin and trypsin-like serine proteases such as thrombin and enterokinase (Table 1).

HiTrap Benzamidine FF (high sub) is also convenient to use for removal of enzymes (e.g., thrombin) after cleavage of the tag from a recombinant fusion protein.

Fast, simple and easy separations are provided by the combination of the prepacked column with a high binding capacity affinity medium that has good flow properties. HiTrap Benzamidine FF (high sub) columns can be operated with a syringe, pump or liquid chromatography system.

- Fast and convenient to use
- Very high binding capacity
- Simple operation using a syringe, a peristaltic pump or a chromatographic system such as ÄKTAdesign™
- Scaling-up is easily achieved by connecting several 1 ml or 5 ml columns in series or using lab packages

Fig 1. Benzamidine Sepharose 4 Fast Flow (high sub) and prepacked HiTrap Benzamidine FF (high sub) are designed for removal and/or purification of serine proteases.

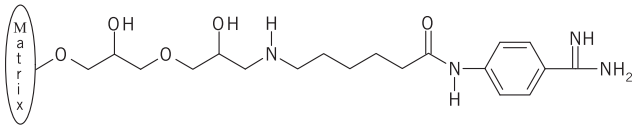


Fig 2. Partial structure of Benzamidine Sepharose 4 Fast Flow (high sub).

Medium characteristics

Benzamidine Sepharose 4 Fast Flow (high sub) is based on highly cross-linked 4% agarose enabling high flow rates without losing binding capacity.

p-Aminobenzamidine (pABA), a synthetic inhibitor of trypsin and trypsin-like serine proteases, is covalently coupled via an amide bond to a long spacer arm attached to Sepharose 4 Fast Flow via a stable ether linkage (Fig 2).

Table 2 shows the main characteristics of HiTrap Benzamidine FF (high sub) and Benzamidine Sepharose 4 Fast Flow (high sub).

Figure 3 shows the pH stability of the medium.

Sepharose

Table 1. Examples of different serine proteases.

Enzyme	Source	M_r	pI
Thrombin	Bovine pancreas	23 345	10.5
Trypsin	Human plasma:		
	chain A	5 700	
	chain B	31 000	7.1
Urokinase	Human urine	54 000	8.9
Enterokinase	Porcine intestine:		
	heavy chain	134 000	4.2
	light chain	62 000	
Plasminogen	Human plasma	90 000	6.4–8.5
Prekallekrein	Human plasma	nd	nd
Kallikrein	Human plasma	86 000	nd
Kallikrein	Human saliva	nd	4.0

Column characteristics

HiTrap Benzamidine FF (high sub) is prepacked 1 ml and 5 ml columns made of polypropylene, a biocompatible material that does not interact with biomolecules. Top and bottom frits are manufactured from porous polyethylene. The column is delivered with a stopper on the inlet and a twist-off end on the outlet. Several columns can be connected in series for easy scale-up. HiTrap columns can not be opened or refilled.

Table 2. Main characteristics of HiTrap Benzamidine 4 FF (high sub) and Benzamidine Sepharose 4 FF (high sub).

Matrix	highly cross-linked 4% agarose
Average particle size	90 µm
Ligand	p-aminobenzamidine (pABA)
Ligand concentration	≥ 12 µmol p-aminobenzamidine/ml medium
Spacer	14-atom
Binding capacity	≥ 35 mg trypsin/ml medium
Column dimensions (i.d.×h)	0.7 × 2.5 cm (1 ml)
	1.6 × 2.5 cm (5 ml)
Column volumes	1 ml and 5 ml
Recommended flow rates	1 ml/min and 5 ml/min for 1 and 5 ml columns respectively
Maximum back pressure	0.3 MPa, 3 bar
Maximum flow rates*	4 ml/min and 20 ml/min for 1 and 5 ml columns respectively
Chemical stability	All commonly used aqueous buffers
pH stability**	
short term	pH 1–9
long term	pH 2–8
Storage temperature	4°C to 8°C
Storage buffer	20% ethanol in 0.05 M acetate buffer, pH 4

* H_2O at room temperature

** The ranges given are estimates based on our knowledge and experience. Please note the following:
pH stability, short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.
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. See Fig 3.

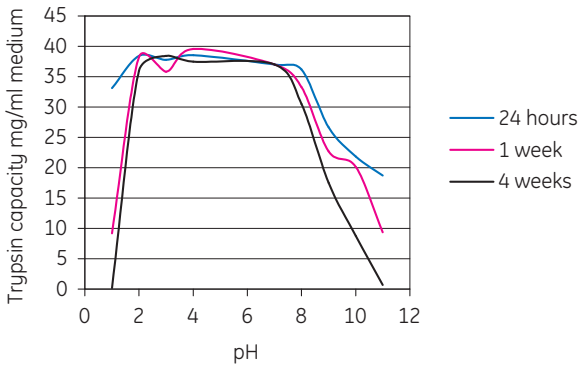


Fig 3. Benzamidine Sepharose 4 Fast Flow (high sub) has been tested for trypsin capacity after being stored at pH 1–11 at ambient temperature for 24 h, 1 week and 4 weeks.

Operation

Like all HiTrap columns, HiTrap Benzamidine FF (high sub) is quick and convenient to use. Instructions and connectors are included with each pack of columns. In general, the separation can be easily achieved with a syringe (using the luer adapter provided). Figure 4 a–c illustrates this technique. Alternatively, the column can be operated using a laboratory pump or a chromatography system.

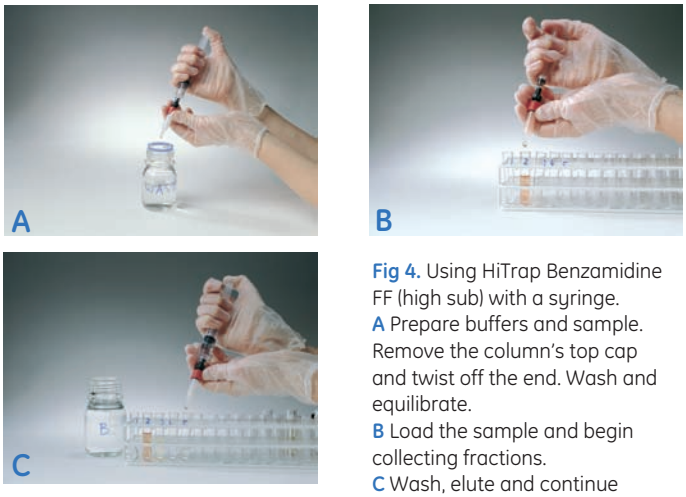


Fig 4. Using HiTrap Benzamidine FF (high sub) with a syringe.
A Prepare buffers and sample. Remove the column's top cap and twist off the end. Wash and equilibrate.
B Load the sample and begin collecting fractions.
C Wash, elute and continue collecting fractions.

Scaling-up

HiTrap Benzamidine FF (high sub) is available as 1 ml and 5 ml columns. Two or more columns can easily be connected in series by screwing the end of one into the top of the next for fast scaling-up. Note that the backpressure will increase. Benzamidine Sepharose 4 Fast Flow (high sub) is also available as lab packages for further scaling-up.

Applications

Removal of trypsin-like serine proteases from human plasma

Proteases included in human plasma can damage the sample if not removed. This application shows the use of HiTrap Benzamidine FF (high sub) for removal of trypsin-like serine proteases in one step. The 1 ml column was equilibrated with 10 ml binding buffer, 1 ml human plasma was loaded and the column was washed with 10 ml binding buffer before a one step elution using 5 ml elution buffer.

The results show that according to the activity test, almost all trypsin-like serine protease activity is removed from the human plasma sample and bound to the column.

Sample:	1 ml human plasma filtered through a 0.45 µm filter
Column:	HiTrap Benzamidine FF (high sub), 1 ml
Binding buffer:	20 mM Tris-HCl, 0.5 M NaCl, pH 7.4
Elution buffer:	50 mM glycine, pH 3.0 0-100% elution buffer in one step
Flow rate:	1.0 ml/min
Instrumentation:	ÄKTAexplorer™ 10
Protease activity:	S-2288 from Chromogenix, Heamochrom Diagnostica AB A_{405} measurement. The activity is presented as the proteolytic activity/mg protein

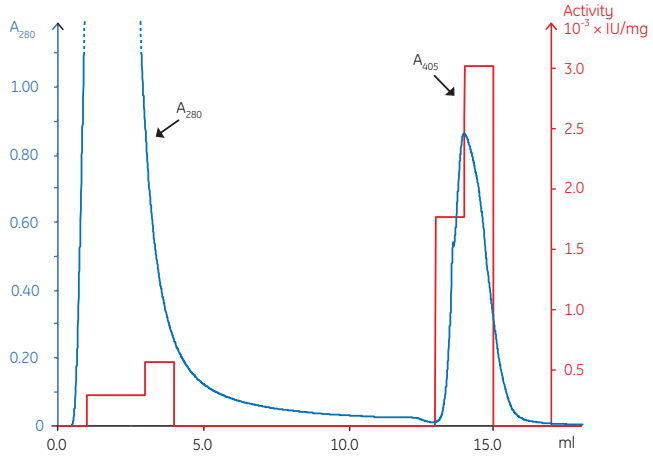


Fig 5. Removal of trypin-like serine proteases from human plasma using HiTrap Benzamidine FF (high sub), 1 ml.

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

1. Isolation of GST-tagged protein on GSTrap FF

The SH2-GST fusion protein sample (2 ml, clarified *E. coli* homogenate) was loaded on a GSTrap™ FF 1 ml column equilibrated in binding buffer. Non-binding proteins were washed out using 10 ml binding buffer.

2. On-column cleavage using thrombin

Between the SH2 protein and the GST-tag, a thrombin cleavage site was incorporated. To separate the protein from its GST-tag, the serine protease, thrombin, was dissolved in binding buffer (1 ml, 20 units/ml) and applied to the column using a syringe. The column was sealed with the supplied connectors and left to incubate for 2 hours at room temperature.

Note: Cleavage conditions have to be optimized for each case. For a sensitive protein fast cleavage may be preferred over complete cleavage.

3. Sample purification

HiTrap Benzamidine FF (high sub) 1 ml column was equilibrated with water and binding buffer before being placed after the GSTrap FF column. Placed in series, the columns were washed with 7 ml binding buffer and later with 5 ml high salt buffer. This procedure allowed the cleaved SH2 protein and thrombin to be washed out from the GSTrap FF column and thrombin to bind when passing the HiTrap Benzamidine FF (high sub) column. Collected fractions contained pure cleaved SH2 protein (Fig 7).

4. Elution of HiTrap Benzamidine FF (high sub)

HiTrap Benzamidine FF (high sub) 1 ml column was eluted competitively using 10 ml 20 mM p-aminobenzamidine in binding buffer. Since p-aminobenzamidine by itself gives a high absorbance at 280 nm an enzymatic activity assay for detection of thrombin was used (Fig 6).

5. Elution of GSTrap FF

GSTrap FF 1 ml column was eluted using 10 ml 20 mM reduced glutathione in 50 mM Tris, pH 8.0 (reduced glutathione has a slight absorbance at 280 nm).

Thrombin activity assay

A small amount of thrombin was used in this on-column cleavage of SH2-GST. The presence of thrombin could neither be determined by SDS-PAGE analysis nor by absorbance (280 nm), due to the small amount and the usage of p-aminobenzamidine for elution. Therefore the chromogenic substrate S-2238 (Chromogenic, Heamochrom Diagnostica AB) was used to determine the thrombin activity and thereby verify the removal of protease.

Results

GSTrap FF efficiently bound and purified the SH2-GST protein. On-column cleavage using thrombin was almost complete in only 2 hours. (To achieve full cleavage the incubation time or amount of protease can be increased). HiTrap Benzamidine FF (high sub) 1 ml bound the total added thrombin, as shown by the activity measurement in Figure 6, resulting in pure cleaved SH2 protein (2 mg) without contamination of used protease (Fig 7). This whole procedure is completed in less than one day.

Sample:	2 ml clarified *E. coli* homogenate expressing SH2-GST (M_r 37 000) with a thrombin cleavage site
Columns:	GSTrap FF, 1 ml HiTrap Benzamidine FF (high sub), 1 ml
Binding buffer:	20 mM Na phosphate, 0.15 M NaCl, pH 7.5
High salt wash buffer:	20 mM Na phosphate, 1.0 M NaCl, pH 7.5
HiTrap Benzamidine FF (high sub) elution buffer:	20 mM p-aminobenzamidine in binding buffer
GSTrap FF elution buffer:	50 mM Tris, 20 mM reduced glutathione, pH 8.0
Flow rate:	0.5 ml/min
System:	ÄKTAprime™
Protease treatment:	1 ml, 20 units/ml thrombin (GE Healthcare) for 2 hours at room temperature
Thrombin activity:	S-2238 from Chromogenix, Heamochrom Diagnostica AB, A_{405} measurement

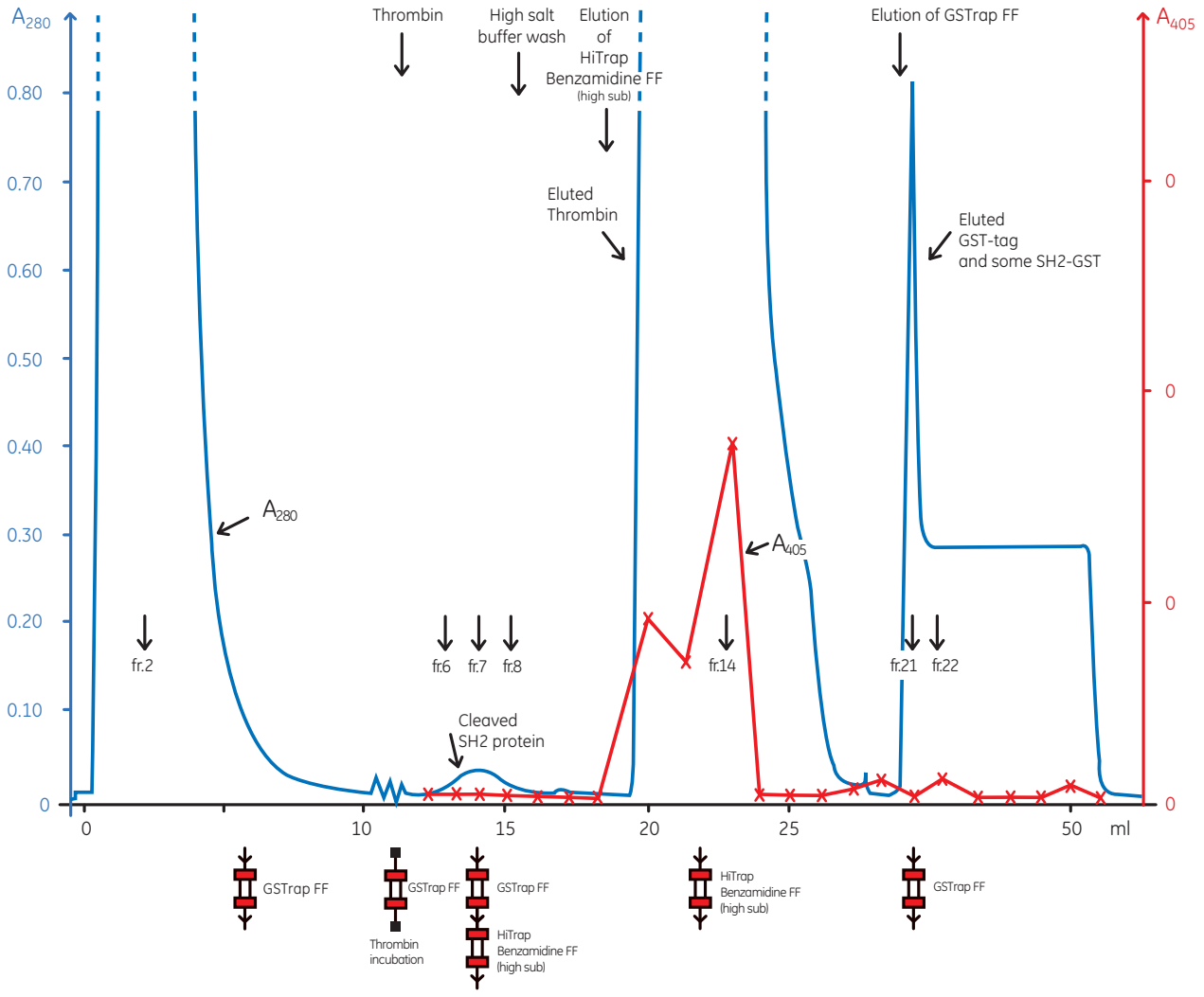
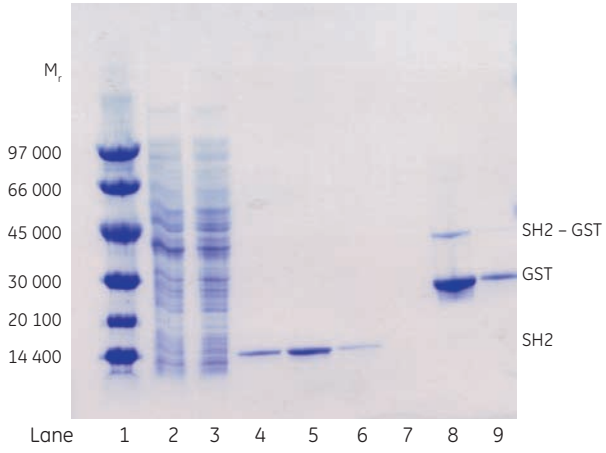


Fig 6. Removal of thrombin after on-column cleavage of a GST-tagged protein.

SDS-PAGE analysis, see page 5.



Lane 1 Low Molecular Markers
Lane 2 Sample, clarified *E. coli* homogenate expressing SH2-GST
Lane 3 Flow through from GSTrap FF, (fr. 2)
Lane 4 SH2 (GST-tag cleaved off), washed out through both columns, (fr. 6)
Lane 5 -"- , (fr. 7)
Lane 6 -"- , (fr. 8)
Lane 7 Elution of thrombin, HiTrap Benzamidine FF (high sub), (fr. 14)
Lane 8 Elution of GST-tag and some non-cleaved SH2-GST, GSTrap FF, (fr. 21)
Lane 9 -"- , (fr. 22)

Fig 7. SDS-PAGE on ExcelGel™ SDS Gradient 8-18%, Coomassie™ staining.

Cleaning

The cleaning protocol has to be designed for each protein. General recommendation is to use a solution of guanidine hydrochloride to remove precipitated or denatured substances. For hydrophobically bound substances a solution of nonionic detergent or ethanol is recommended. Short term use of pH 1–9 is possible. For example, more than half of the trypsin capacity is available after 1 week (168 h) contact time at pH 9 (Fig 3). However, prolonged exposure to pH greater than 8 and lower than 2 should be avoided due to hydrolysis of the ligand at high pH and decomposition of the matrix at low pH.

Storage

HiTrap Benzamidine FF (high sub) and Benzamidine Sepharose 4 Fast Flow (high sub) are supplied in 0.05 M acetate buffer, pH 4 containing 20% ethanol as a bacteriostat. Storage temperature is 4°C to 8°C.

Ordering information

Product	Quantity	Code No.
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
Benzamidine Sepharose 4 Fast Flow (high sub)	25 ml	17-5123-10

Related products	Quantity	Code No.
HiTrap Desalting	5 × 5 ml	17-1408-01
HiPrep™ 26/10 Desalting	1 × 53 ml	17-5087-01
HiPrep 26/10 Desalting	4 × 53 ml	17-5087-02
GSTrap FF	2 × 1 ml	17-5130-02
GSTrap FF	5 × 1 ml	17-5130-01
GSTrap FF	1 × 5 ml	17-5131-01
GSTrap FF	5 × 5 ml	17-5131-02
Thrombin	500 units	27-0846-01
Factor Xa	400 units	27-0849-01

Accessories	Quantity	Code No.
1/16" male/Luer female*	2	18-1112-51
Tubing connector flangeless/M6 female*	2	18-1003-68
Tubing connector flangeless/M6 male*	2	18-1017-98
Union 1/16" female/M6 male*	6	18-1112-57
Union M6 female /1/16" male	5	18-3858-01
Union Luerlock female/M6 female	2	18-1027-12
HiTrap/HiPrep, 1/16" male connector for ÄKTAdesign	8	28-4010-81
Stop plug female, 1/16"†	5	11-0004-64
Fingertight stop plug, 1/16"‡	5	11-0003-55

* One connector included in each HiTrap package

† Two, five, or seven female stop plugs included in HiTrap packages, depending on products

‡ One fingertight stop plug is connected to the top of each HiTrap column

Related literature	Quantity	Code No.
The Recombinant Protein Purification Handbook, Principles and Methods	1	18-1142-75
Affinity Chromatography Handbook, Principles and Methods	1	18-1022-29
Affinity Columns and Media, Selection Guide	1	18-1121-86
Convenient Protein Purification, HiTrap Column Guide	1	18-1129-81

www.gehealthcare.com/hitrap

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

GE Healthcare Europe GmbH
Munzinger Strasse 5, D-79111 Freiburg, Germany

GE Healthcare UK Ltd
Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK

GE Healthcare Bio-Sciences Corp
800 Centennial Avenue, P.O. Box 1327, Piscataway,
NJ 08855-1327, USA

GE Healthcare Bio-Sciences KK
Sanken Bldg. 3-25-1, Hyakunincho, Shinjuku-ku,
Tokyo 169-0073, Japan

Asia Pacific T +85 65 62751830 F +85 65 62751829 • Australasia T +61 2 8820 8299 F +61 2 8820 8200 • Austria T 01 /57606 1613 F 01 /57606 1614 • Belgium T 0800 73 890 F 02 416 8206 • Canada T 1 800 463 5800 F 1 800 567 1008 • Central & East Europe T +43 1 972 720 F +43 1 972 722 750 • Denmark T +45 70 25 24 50 F +45 45 16 2424 • Eire T 1 800 709992 F +44 1494 542010 • Finland & Baltics T +358 9 512 3940 F +358 9 512 39439 • France T 01 69 35 67 00 F 01 69 41 98 77 Germany T 0800 9080 711 F 0800 9080 712 • Greater China T +852 2100 6300 F +852 2100 6338 • Italy T 02 26001 320 F 02 26001 399 • Japan T 81 3 5331 9336 F 81 3 5331 9370 • Korea T 82 2 6201 3700 F 82 2 6201 3803 • Latin America T +55 11 3933 7300 F +55 11 3933 7304 • Middle East & Africa T +30 210 96 00 687 F +30 210 96 00 693 • Netherlands T 0800-82 82 82 1 F 0800-82 82 82 4 • Norway T +47 815 65 777 F +47 815 65 666 • Portugal T 21 417 7035 F 21 417 3184 • Russia, CIS & NIS T +7 495 956 5177 F +7 495 956 5176 • Spain T 902 11 72 65 F 935 94 49 65 • Sweden T 018 612 1900 F 018 612 1910 • Switzerland T 0848 8028 10 F 0848 8028 11 • UK T 0800 515 313 F 0800 616 927 • USA T +1 800 526 3593 F +1 877 295 8102

imagination at work

18-1139-38 AC 01/2007