GE Healthcare

Data file 18-1174-85 AC

Affinity chromatography

Glutathione Sepharose 4 Fast Flow
GSTPrep FF 16/10
GSTrap FF

Glutathione Sepharose™ 4 Fast Flow is an affinity chromatography medium for the isolation and purification of GST-tagged proteins as well as other glutathione S-transferases and glutathione-binding proteins.

Key features include:

- Fast, one-step purification of GST-tagged proteins
- High binding capacity
- Easy scale-up

With Glutathione Sepharose 4 Fast Flow, GST-tagged proteins produced using the pGEX series of expression vectors (1, 2) can be purified directly from bacterial lysates under mild conditions that preserve protein antigenicity and function. The medium is also available in prepacked HiPrep™ and HiTrap™ columns (Fig 1 and Fig 2) offering the convenience and simplicity of prepacked columns and the flexibility of choosing column type and size.

Chromatography medium characteristics

The glutathione ligand is coupled via a 10-carbon linker to highly cross-linked 4% agarose. The coupling is optimized to give a high binding capacity for GST-tagged proteins and other glutathione-binding proteins.

The total binding capacity is approximately 10 mg of recombinant GST expressed in *E. coli*/ml medium. The dynamic binding capacity varies depending on the flow rate and sample properties. The main characteristics of Glutathione Sepharose 4 Fast Flow are listed in Table 1.

Fig 1. Glutathione Sepharose 4 Fast Flow and prepacked GSTrap FF 1 ml and 5 ml columns.

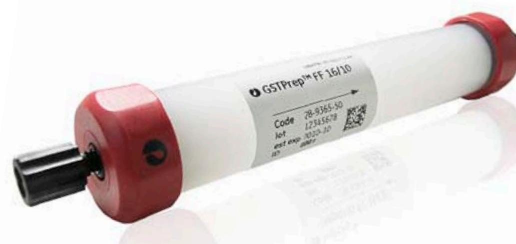


Fig 2. GSTPrep FF 16/10 column for convenient scale-up purification of GST-tagged proteins.

Sepharose

Column characteristics

Glutathione Sepharose 4 Fast Flow is available in convenient prepacked GSTrap™ FF 1 ml and 5 ml columns, and 20 ml GSTPrep™ FF 16/10 columns (Table 2). In combination with ÄKTA™ design systems, which include preset method templates based on these prepacked columns, greater reproducibility is achieved and time is saved.

The prepacked columns are made of transparent polypropylene, which does not react with biomolecules. GSTrap FF and GSTPrep FF 16/10 columns are not designed to be opened or repacked. GSTrap FF columns are delivered with a set of connectors to allow simple connection to a chromatography system such as ÄKTA design or a syringe.

Scale-up

Two or more GSTrap FF 1 ml or 5 ml columns can easily be connected in series, by screwing the end of one into the top of the next column, to increase the binding capacity. Note that connecting columns in series will cause an increase in back pressure. Prepacked GSTPrep FF 16/10 (20 ml) columns are the choice for further scale-up. Bulk quantities are available for packing larger columns when even further scale-up is necessary.

Operation

GSTrap FF and GSTPrep FF 16/10 are quick and easy to use. Both columns are designed for use with pumps or a chromatography system such as ÄKTA design.

Manual purification with GSTrap FF columns is easily conducted with a syringe and the provided Luer connector (Fig 3). Instructions and connectors are included in all packages.

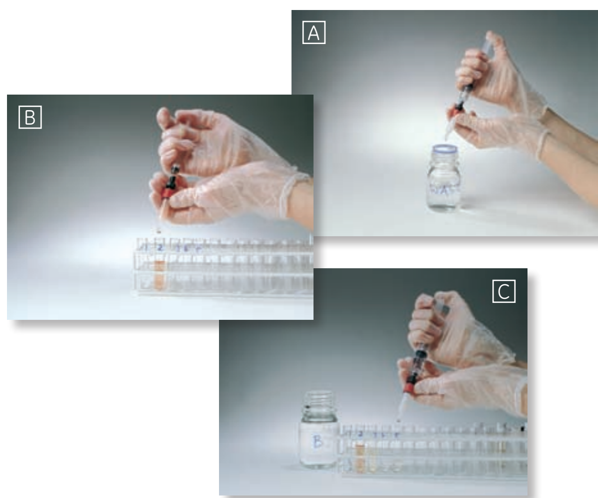


Fig 3. Using GSTrap FF with a syringe. A) Prepare buffers and sample. Remove the column's top cap and snap off the end. Wash and equilibrate. B) Load the sample and begin collecting fractions. C) Elute and continue collecting fractions.

Table 1. Characteristics of Glutathione Sepharose 4 Fast Flow

Medium	Glutathione Sepharose 4 Fast Flow
Matrix	Highly cross-linked 4% agarose
Mean particle size	90 µm
Ligand	Glutathione
Binding capacity	≈ 10 mg recombinant GST/ml medium (protein-dependent, GST M_r 26 000)
Chemical stability	All commonly used aqueous buffers, e.g. 1 M acetate, pH 4.0, or 6 M guanidine-hydrochloride for 1 h at room temperature
Recommended flow rate[1]	Sample loading: < 100 cm/h (< 3 ml/min using XK 16/20 column) Wash and elution: 100–300 cm/h (3–10 ml/min using XK 16/20 column)
Maximum back pressure	1 bar (0.1 MPa) when packed in XK columns (might vary if used in other columns)
Maximum flow rate	450 cm/h (15 ml/min using XK 16/20 column at room temperature with aqueous buffer)
pH stability	3 to 12
Storage	4°C to 30°C in 20% ethanol

[1] Note: Binding of GST to glutathione is protein- and flow rate dependent. Lower flow rates often increase the binding capacity. This is important to consider during sample loading and elution.

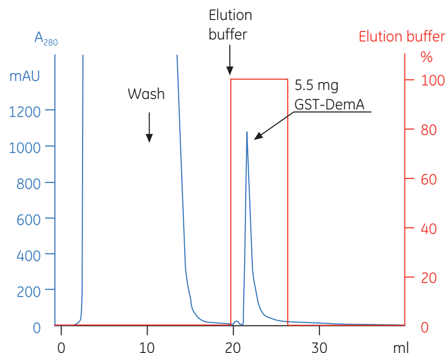
Table 2. Characteristics of GSTPrep FF 16/10 and GSTrap FF columns

	GSTPrep FF 16/10	GSTrap FF
Bed volume	20 ml	1 ml or 5 ml
Bed height	10 cm	2.5 cm
Column i.d.	1.6 cm	0.7 cm (1 ml column) 1.6 cm (5 ml column)
Column hardware	Polypropylene	Polypropylene
Recommended flow rate[1]	1 to 10 ml/min	0.2 to 1 ml/min (1 ml column) 0.5 to 5 ml/min (5 ml column)
Maximum flow rate	10 ml/min (300 cm/h)	4 ml/min (1 ml column) 15 ml/min (5 ml column)
Maximum pressure over the packed bed during operation	1.5 bar (0.15 MPa, 22 psi)	1.5 bar (0.15 MPa, 22 psi)
Column hardware pressure limit	5 bar (0.5 MPa, 73 psi)	3 bar (0.3 MPa, 45 psi)

[1] Note: Binding of GST to glutathione is protein-to-protein and flow rate dependent. Lower flow rates often increase the binding capacity. This is important to consider during sample loading and elution.

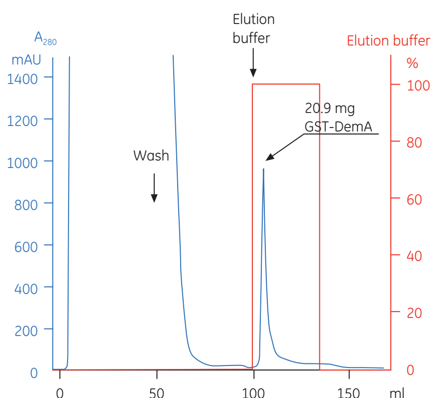
A) **GSTrap FF 1 ml**

Column: GSTrap FF 1 ml
Sample: 10 ml extract from *E. coli* expressing GST-DemA
Binding buffer: PBS, pH 7.4
Elution buffer: 50 mM Tris-HCl, pH 8.0, with 10 mM reduced glutathione
Flow rate: Sample loading: 0.5 ml/min
Washing and elution: 1 ml/min
Procedure: 5 CV (CV = column volumes) binding buffer, 10 ml sample loaded, 10 CV binding buffer, 7 CV elution buffer, and 5 CV binding buffer
System: ÄKTAexplorer 100



B) **GSTrap FF 5 ml**

Column: GSTrap FF 5 ml
Sample: 50 ml extract from *E. coli* expressing GST-DemA
Binding buffer: PBS, pH 7.4
Elution buffer: 50 mM Tris-HCl, pH 8.0, with 10 mM reduced glutathione
Flow rate: Sample loading: 2.5 ml/min
Washing and elution: 5 ml/min
Procedure: 5 CV binding buffer, 50 ml sample loaded, 10 CV binding buffer, 7 CV elution buffer, and 5 CV binding buffer
System: ÄKTAexplorer 100



C) **GSTPrep FF 16/10***

Column: GSTPrep FF 16/10
Sample: 200 ml extract from *E. coli* expressing GST-DemA
Binding buffer: PBS, pH 7.4
Elution buffer: 50 mM Tris-HCl, pH 8.0, with 10 mM reduced glutathione
Flow rate: Sample loading: 5 ml/min
Washing and elution: 10 ml/min
Procedure: 5 CV binding buffer, 200 ml sample, 10 CV binding buffer, 7 CV elution buffer, and 5 CV binding buffer
System: ÄKTAexplorer 100

Note: Data was obtained using first-generation GSTPrep FF 16/10 columns.

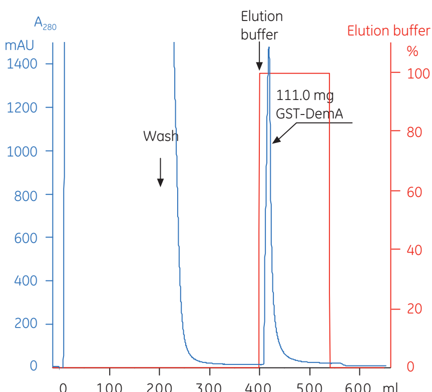


Fig 4 A-C. Purification and scale-up of GST-DemA on GSTrap FF 1 ml, GSTrap FF 5 ml, and GSTPrep FF 16/10.

Applications

1. Purification and scale-up of two GST-tagged proteins

Glutathione Sepharose 4 Fast Flow is easy to use for one-step purification of GST-tagged proteins. Figures 4 and 6 show scale-up studies on GSTrap FF 1 ml, GSTrap FF 5 ml, and GSTPrep FF 16/10. Two different GST-tagged proteins were purified: GST-DemA and GST-Purα. The gene encoding DemA was isolated from *Streptococcus dysgalactiae*. DemA is a fibrinogen-binding protein that shows both plasma protein binding properties and sequence similarities with the M and M-like proteins of other Streptococcal species.

Purα is a sequence-specific, single-stranded DNA and RNA binding protein that binds to purine-rich promoter regions with a $(GGN)_n$ consensus sequence called PUR elements. Purα has been shown to function in transcriptional regulation by: 1) interacting with several promoter response elements; 2) enhancing transcription through an RNA element; and 3) transcriptional transactivation through promoter response elements, protein-protein interactions, and protein-RNA interactions.

Preparation of cytoplasmic extract

E. coli expressing the GST-tagged proteins was resuspended in PBS[1] (1 g/5 ml) supplemented with 1 mM PMSF, 1 mM DTT, 100 mM $MgCl_2$, 1 U/ml RNase A and 13 U/ml DNase I. The cells were sonicated on ice with a Vibra-Cell™ ultrasonic processor for 3 minutes at 50% amplitude. Cell debris was removed by centrifugation at 48 000 × g, 4°C for 30 minutes. The supernatant was applied to the column after passage through a 0.45 µm filter.

[1] 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4

Lanes
1. LMW markers, reduced (LMW-SDS Marker Kit)
2. Extract of *E. coli* expressing GST-DemA, 1 g cell paste/5 ml
3. Flowthrough from GSTrap FF 1 ml
4. GST-DemA eluted from GSTrap FF 1 ml
5. Extract of *E. coli* expressing GST-DemA, 1 g cell paste/5 ml
6. Flowthrough from GSTrap FF 5 ml
7. GST-DemA eluted from GSTrap FF 5 ml
8. Extract of *E. coli* expressing GST-DemA, 1 g cell paste/5 ml
9. Flowthrough from GSTPrep FF 16/10
10. GST-DemA eluted from GSTPrep FF 16/10

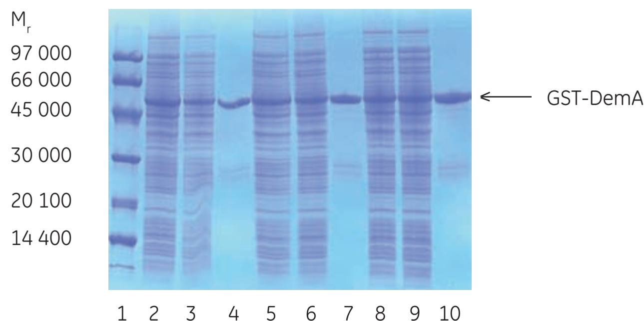
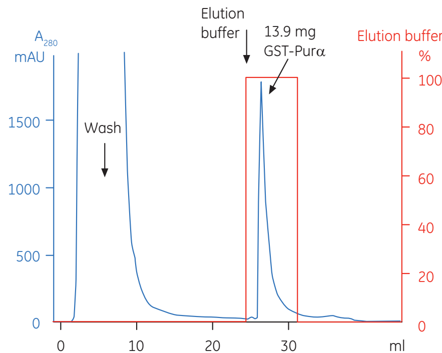


Fig 5. SDS-PAGE analysis of GST-DemA on ExcelGel™ Homogeneous 12.5% using Multiphor™ II followed by Coomassie™ staining. Due to the low turnover rate of GST, some of the applied protein was found in the flowthrough.

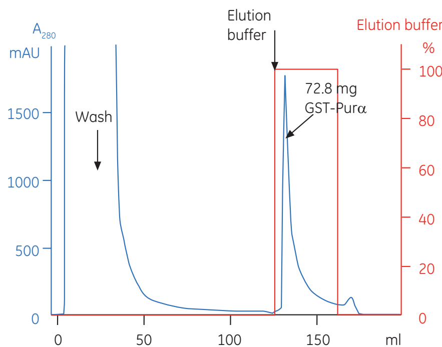
A) **GSTrap FF 1 ml**

Column:	GSTrap FF 1 ml
Sample:	5 ml extract from *E. coli* expressing GST-Purα
Binding buffer:	PBS, pH 7.4
Elution buffer:	50 mM Tris-HCl, pH 8.0, with 10 mM reduced glutathione
Flow rate:	Sample loading: 0.5 ml/min Washing and elution: 1 ml/min
Procedure:	5 CV binding buffer, 5 ml sample, 20 CV binding buffer, 7 CV elution buffer, and 5 CV binding buffer
System:	ÄKTAexplorer 100



B) **GSTrap FF 5 ml**

Column:	GSTrap FF 5 ml
Sample:	25 ml extract from *E. coli* expressing GST-Purα
Binding buffer:	PBS, pH 7.4
Elution buffer:	50 mM Tris-HCl, pH 8.0, with 10 mM reduced glutathione
Flow rate:	Sample loading: 2.5 ml/min Washing and elution: 5 ml/min
Procedure:	5 CV binding buffer, 25 ml sample, 20 CV binding buffer, 7 CV elution buffer, and 5 CV binding buffer
System:	ÄKTAexplorer 100



C) **GSTPrep FF 16/10***

Column:	GSTPrep FF 16/10
Sample:	100 ml extract from *E. coli* expressing GST-Purα
Binding buffer:	PBS, pH 7.4
Elution buffer:	50 mM Tris-HCl, pH 8.0, with 10 mM reduced glutathione
Flow rate:	Sample loading: 5 ml/min Washing and elution: 10 ml/min
Procedure:	5 CV binding buffer, 100 ml sample, 20 CV binding buffer, 7 CV elution buffer, and 5 CV binding buffer
System:	ÄKTAexplorer 100

Note: Data was obtained using first-generation GSTPrep FF 16/10 columns.

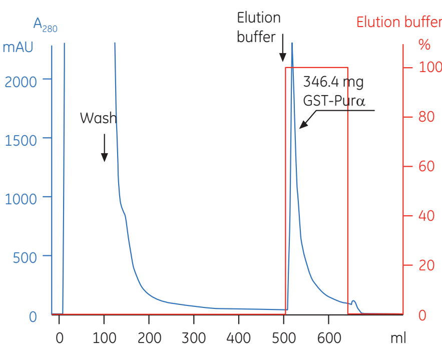


Fig 6 A-C. Purification and scale-up of GST-Purα on GSTrap FF 1 ml, GSTrap FF 5 ml, and GSTPrep FF 16/10.

Purification and scale-up

The purification of GST-Purα and GST-DemA was performed using an ÄKTAexplorer™ 100 chromatography system. After the columns were equilibrated, sample was loaded on GSTrap FF 1 ml (Figs 4A and 6A), GSTrap FF 5 ml (Figs 4B and 6B), and GSTPrep FF 16/10 (Figs 4C and 6C). The columns were washed, and the proteins were eluted according to the procedures described. Finally, the eluted proteins were analyzed by SDS-PAGE (Figs 5 and 7).

Summary

The main parameter in this scale-up study was the residence time (i.e., the period of time the sample is in contact with the chromatography medium). The residence time was the same for the GSTrap FF 1 ml and 5 ml columns, while it was twice as long for the GSTPrep FF 16/10 column (due to the difference in column length versus column diameter). The amount of bound protein differed between GST-DemA and GST-Purα. This was due to protein-dependent binding characteristics. Some of the applied protein was found in the flowthrough, an effect of the low turnover rate of GST. The amount of eluted GST-tagged proteins increased proportionally with increased column volume and sample load.

Lanes
1. LMW markers, reduced (LMW-SDS Marker Kit)
2. Extract of *E. coli* expressing GST-Purα, 1 g cell paste/5 ml
3. Flowthrough from GSTrap FF 1 ml
4. GST-Purα eluted from GSTrap FF 1 ml
5. Extract of *E. coli* expressing GST-Purα, 1 g cell paste/5 ml
6. Flowthrough from GSTrap FF 5 ml
7. GST-Purα eluted from GSTrap FF 5 ml
8. Extract of *E. coli* expressing GST-Purα, 1 g cell paste/5 ml
9. Flowthrough from GSTPrep FF 16/10
10. GST-Purα eluted from GSTPrep FF 16/10

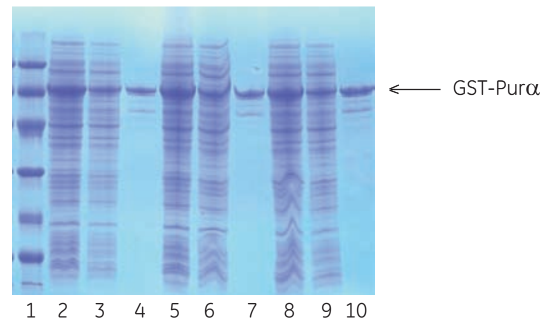
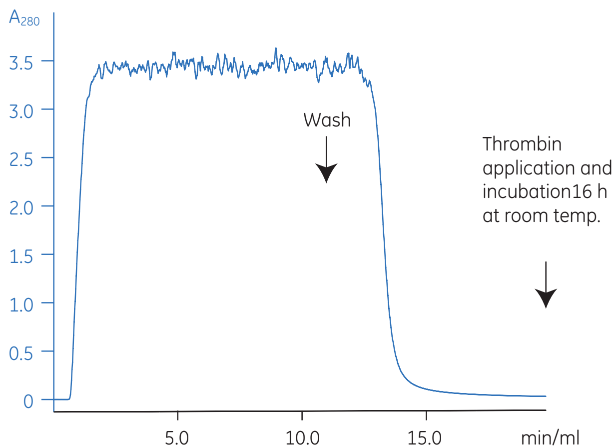


Fig 7. SDS-PAGE analysis of GST-Purα on ExcelGel SDS Homogeneous 12.5% using Multiphor II followed by Coomassie staining. Due to the low turnover rate of GST, some of the applied protein was found in the flowthrough.

A)

Column: GSTrap FF 1 ml
Sample: 10 ml cytoplasmic extract from *E. coli* expressing GST-tagged protein
Binding buffer: PBS, pH 7.3
Elution buffer: 50 mM Tris-HCl, pH 8.0, with 10 mM reduced glutathione
Flow rate: 1 ml/min
Procedure (Fig 8A): 4 CV binding buffer, 10 ml sample, 10 CV binding buffer, and filling the column with 1 ml thrombin solution using a syringe
System: ÄKTAexplorer 10



B)

Procedure (Fig 8B): 8 CV binding buffer (elution of cleaved target protein)
5 CV elution buffer (elution of free GST and non-cleaved GST-tagged proteins)
5 CV binding buffer

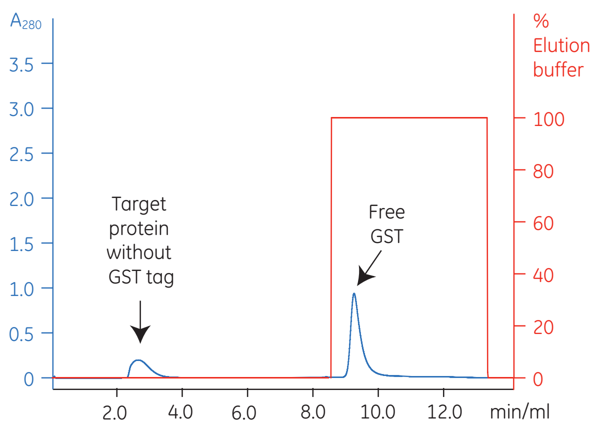


Fig 8. A) Elution of cleaved target protein (without the GST tag) and free GST. B) Sample application, washing, and start of thrombin incubation.

2. Purification of GST-tagged proteins and on-column cleavage with thrombin

The GST tag sometimes has the advantage of increasing the yield of soluble recombinant proteins, but removal of the GST tag from the target protein might be required, for example, for protein crystallography studies. The tagged protein can be digested with an appropriate site-specific protease while bound to Glutathione Sepharose 4 Fast Flow, or alternatively, after elution. On-column cleavage eliminates the extra step of separating the released protein from GST, since the GST tag remains bound to the column.

Thrombin was used for on-column cleavage of the GST tag from a fusion protein with a thrombin cleavage site between the GST tag and the target protein.

After equilibration, sample application, and washing, the column was filled by a syringe with 1 ml of thrombin solution (20 U/ml PBS), which was prepared according to the instructions (Fig 8A). The column was sealed using the supplied connectors and left for 16 h at room temperature. After incubation, the target protein, without the GST tag, was eluted using PBS and the bound GST was eluted using elution buffer (Fig 8B).

The cleavage reaction yield was 100%. Analysis by SDS-PAGE (Fig 9, lanes 5 and 6) shows that no non-cleaved GST tagged protein could be detected in the bound material eluted from the column (Fig 9, lane 6).

The amount of the site-specific protease required and the time for the cleavage reaction depend on the properties and the amount of the specific GST-tagged protein. For best results, this should be checked for each individual cleavage.

Lanes
1. LMW markers, reduced (LMW-SDS Marker Kit)
2. Cytoplasmic extract *of E.coli* expressing GST-tagged protein, 1 g cell paste/10 ml
3. GST-tagged protein eluted from GSTrap FF 1 ml (not shown)
4. GST-tagged protein eluted from GSTrap FF 5 ml (not shown)
5. GST-free target protein eluted from GSTrap FF 1 ml after 16 h thrombin cleavage (Fig 8B)
6. Free GST eluted from GSTrap FF 1 ml after thrombin cleavage (Fig 8B)
7. Thrombin solution (20 U/ml)
8. LMW markers, reduced (LMW-SDS Marker Kit)

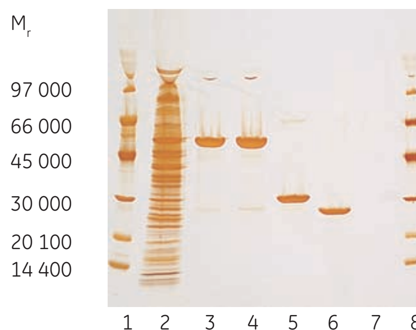


Fig 9. SDS-PAGE on ExcelGel SDS Gradient 8-18 using Multiphor II followed by silver staining.

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

Isolation of GST-tagged proteins on GSTrap FF

The SH2-GST fusion protein sample (2 ml of 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 of binding buffer (Fig 10).

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 U/ml) and loaded on the GSTrap FF column using a syringe. The column was sealed with the supplied connectors and incubated for 2 h at room temperature (Fig 10).

Note: Cleavage conditions have to be optimized in each case. For sensitive proteins, fast cleavage may be preferred over complete cleavage.

Lanes

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

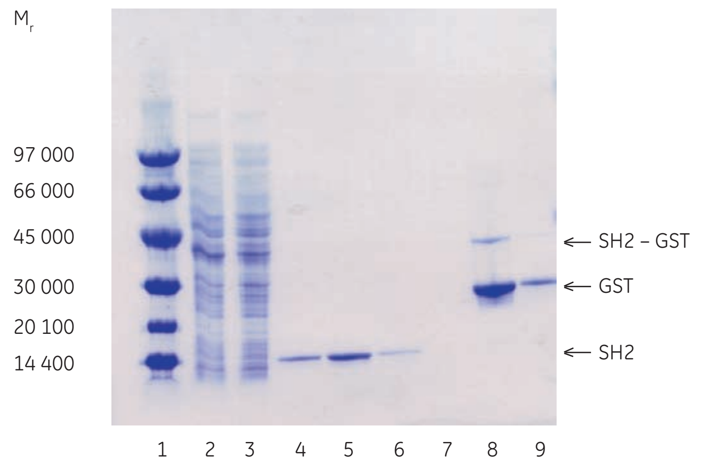


Fig 11. SDS-PAGE on ExcelGel SDS Gradient 8-18 followed by Coomassie staining.

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

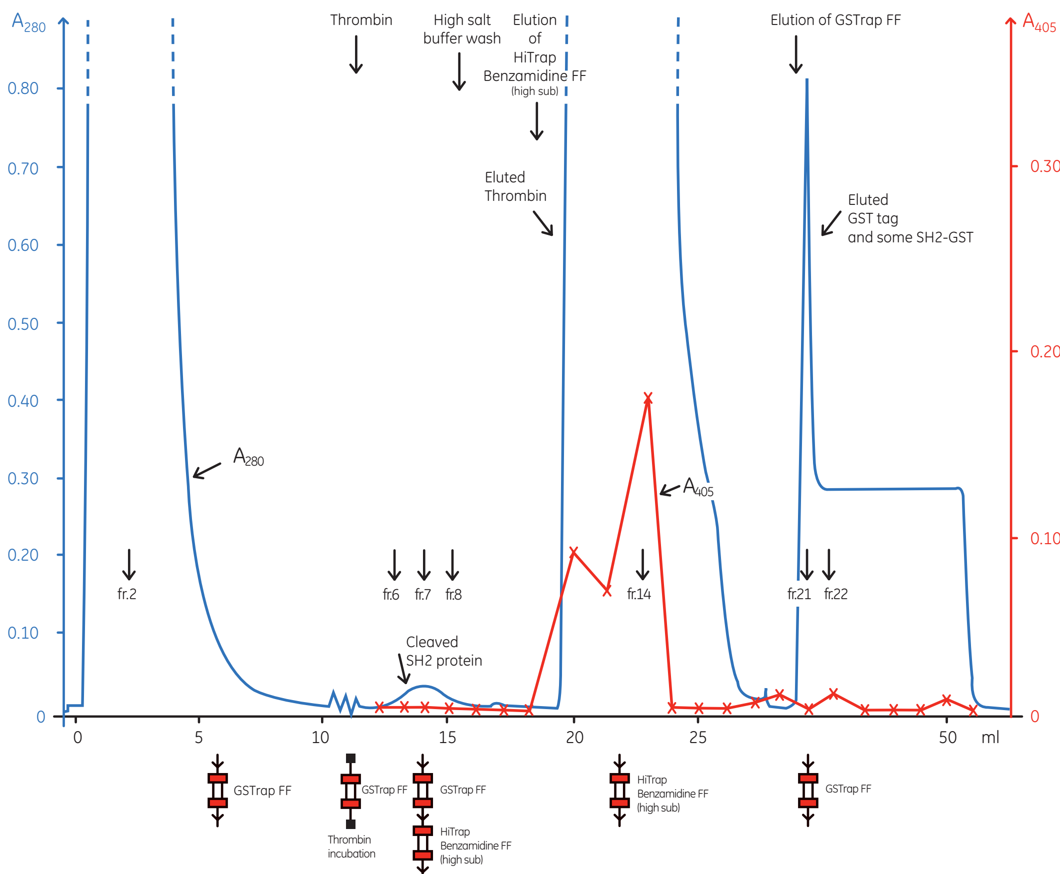


Fig 10. Removal of thrombin after on-column cleavage of a GST-tagged protein.

Sample purification

HiTrap Benzamidine FF (high sub) 1 ml column (see data file 18-1139-38) 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 the thrombin to bind when passing the HiTrap Benzamidine FF (high sub) column (Fig 10). Collected fractions contained pure, cleaved SH2 protein (Fig 11).

Elution of the columns

HiTrap Benzamidine FF (high sub) 1 ml column was eluted competitively using 10 ml of 20 mM p-aminobenzamidine in binding buffer. Since p-aminobenzamidine by itself gives a high absorbance at 280 nm, an enzymatic activity assay was used to detect thrombin (Fig 10). After elution and washing, the column is ready for reuse. GSTrap FF 1 ml column was eluted using 10 ml of 20 mM reduced glutathione in 50 mM Tris, pH 8.0 (reduced glutathione has a slight absorbance at 280 nm, see Fig 10).

Thrombin activity assay

Because of the small amount of thrombin used in the cleavage and the usage of p-aminobenzamidine for elution, the presence of thrombin could neither be determined by SDS-PAGE analysis nor by absorbance (280 nm). Therefore, the chromogenic substrate S-2238 (Chromogenix, Haemochrom Diagnostica AB) was used to determine the thrombin activity and thereby verify the removal of protease (Fig 10). After elution and washing, the column is ready for reuse.

Summary

GSTrap FF efficiently bound and purified the SH2-GST protein. On-column cleavage using thrombin was almost complete in only 2 h. Note that 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 10, resulting in pure, cleaved SH2 protein (2 mg) without contamination of used protease (Fig 11). The whole procedure was completed in less than one day.

Storage

Glutathione Sepharose 4 Fast Flow, GSTrap FF 1 ml and 5 ml, and GSTPrep FF 16/10 are supplied in 20% ethanol. The recommended storage temperature is 4°C to 30°C.

Acknowledgement

We would like to thank Dr. D. Birse (GE Healthcare, Piscataway, USA) and Biovitrum (Stockholm, Sweden) for fruitful discussions and kindly contributing the samples.

References

1. *GST Gene Fusion System*, GE Healthcare, 18-1157-58, Edition AA (2002).
2. *The Recombinant Protein Purification Handbook:* Principles and Methods, GE Healthcare, 18-1142-75, Edition AD (2009).

Ordering information

Products	Quantity	Code No.
Glutathione Sepharose 4 Fast Flow	25 ml	17-5132-01
	100 ml	17-5132-02
	500 ml[1]	17-5132-03
GSTPrep FF 16/10	1 × 20 ml	28-9365-50
GSTrap FF	2 × 1 ml	17-5130-02
	5 × 1 ml	17-5130-01
	100 × 1 ml[2]	17-5130-05
	1 × 5 ml	17-5131-01
	5 × 5 ml	17-5131-02
	100 × 5 ml[2]	17-5131-05

[1] Larger quantities are available. Please contact your GE Healthcare representative.
[2] Special pack size delivered upon specific customer order.

Related products	Quantity	Code No.
HiTrap Benzamidine FF (high sub)	2 × 1 ml	17-5143-02
	5 × 1 ml	17-5143-01
	1 × 5 ml	17-5144-01
HiPrep 26/10 Desalting	1 × 53 ml	17-5087-01
	4 × 53 ml	17-5087-02
HiTrap Desalting	5 × 5 ml	17-1408-01
PreScission™ Protease	500 units	27-0843-01
Thrombin	500 units	27-0846-01
Factor Xa	400 units	27-0849-01
GST Detection Module	50 reactions	27-4590-01
GST MultiTrap™ FF	4 × 96-well plates	28-4055-01
Anti-GST Antibody	0.5 ml	27-4577-01

Accessories	Quantity	Code No.
1/16” male/Luer female[1]	2	18-1112-51
Tubing connector flangeless/M6 female[1]	2	18-1003-68
Tubing connector flangeless/M6 male[1]	2	18-1017-98
Union 1/16” female/M6 male[1]	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 ÄKTA design	8	28-4010-81
Stop plug female, 1/16”[2]	5	11-0004-64
Fingertight stop plug, 1/16”[3]	5	11-0003-55

[1] One connector included in each HiTrap package.
[2] Two, five, or seven female stop plugs included in HiTrap packages, depending on the products.
[3] One fingertight stop plug is connected to the top of each HiTrap column.

Related literature	Code No.
GST Gene Fusion System Handbook	18-1157-58
Recombinant Protein Purification Handbook, Principles and Methods	18-1142-75
Affinity Chromatography Handbook, Principles and Methods	18-1022-29
Affinity Chromatography Columns and Media, Selection Guide	18-1121-86
Glutathione Sepharose, Selection Guide	28-9168-33
HiTrap Column Guide	18-1129-81
Prepacked chromatography columns for ÄKTA design systems, Selection Guide	28-9317-78

For local office contact information, visit
www.gelifesciences.com/contact

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