HiFLOW GST FPLC Columns

User Guide

HiFLOW GST FPLC columns designed for rapid one-step purification, and ideal for preparative purification and contaminant removal. HiFLOW GST FPLC columns are supplied pre-packed and ready to use with SuperGlu Agarose resin for affinity purification of Glutathione S-transferase (GST) tagged recombinant proteins by affinity chromatography. The SuperGlu Agarose resin provides high binding capacity with high stability, chemical compatibility and reuse.
Compatible with all common liquid chromatography instruments (including ÄKTA™ FPLC's), peristaltic pumps and syringes.

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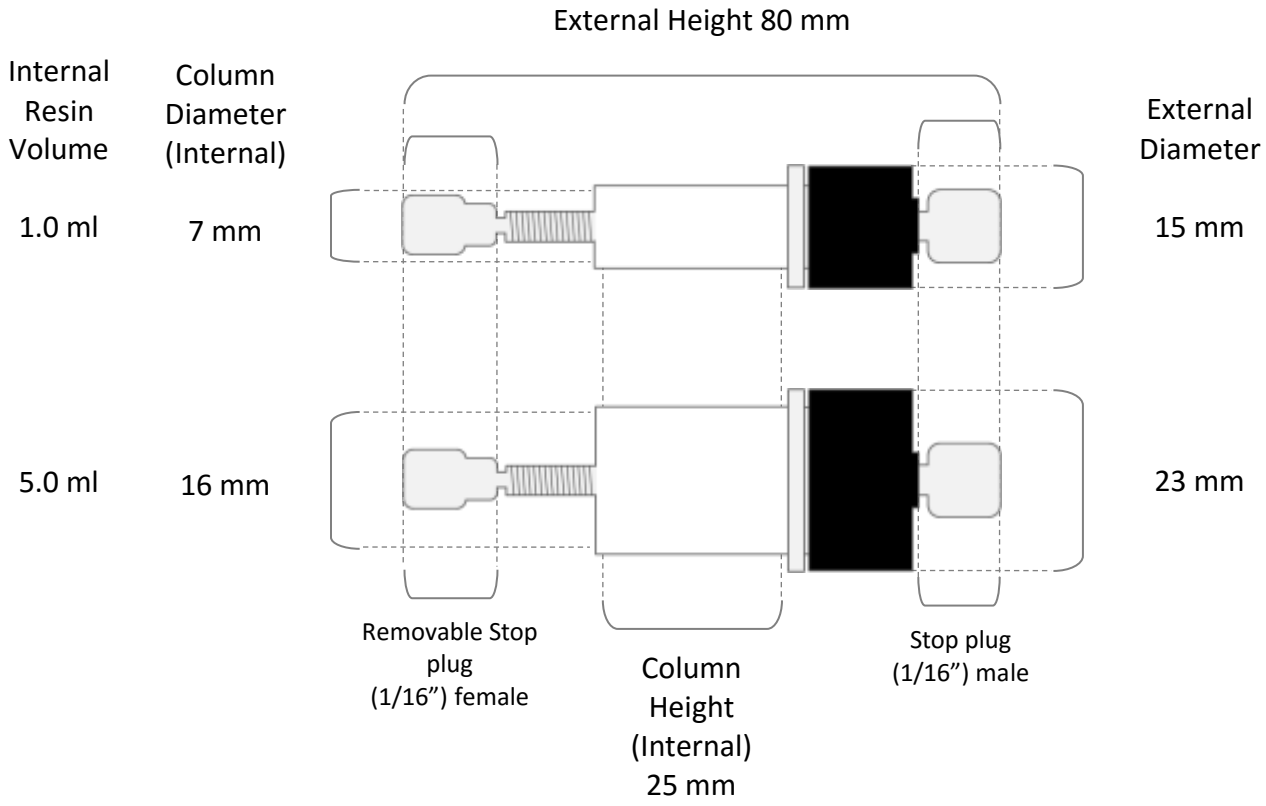
Features of the HiFLOW GST FPLC columns:

- Fast and reliable affinity purification.
- Pre-packed 1 ml and 5 ml columns with SuperGlu Agarose resin.
- Highly stable 7.5% cross linked Agarose with coupled Glutathione ligand provides high buffer stability and broad compatibility.
- High binding capacity for Glutathione S-transferase (GST) tagged recombinant proteins.
- Simple bind-wash-elute procedure.
- Biocompatible polypropylene casing.
- Universal 10.32 (1/16'') UNF threads (Inlet Female/Outlet Male) compatible with all common chromatography instruments (including ÄKTA™ FPLC's).
- Compatible with low pressure pumps (requires a 1/16'' male connector) and syringes (requires a Luer Female – 1/16'' male connector).
- Connect in series for increased capacity.

Specification:

Item:	HiFLOW1-GST	HiFLOW5-GST
Column Volume:	1 ml	5 ml
Resin:	SuperGlu Agarose	SuperGlu Agarose
Base Matrix:	7.5% cross-linked Agarose	7.5% cross-linked Agarose
Coupled ligand:	Glutathione	Glutathione
Typical Binding Capacity:	10 mg	50 mg
Mean Bead Size:	40 µm	40 µm
Recommended flow rate:	1 ml/min	1-5 ml/min
Max. operating pressure:	0.5 MPa (72 psi)	0.5 MPa (72 psi)
External Dimensions:	15 mm D. x 80 mm H.	23 mm D. x 80 mm H.
Column Dimensions (internal):	7 mm D. x 25 mm H.	16 mm D. x 25 mm H.
Column Construction:	Polypropylene	Polypropylene
Inlet Port:	10-32 (1/16") Female	10-32 (1/16") Female
Outlet Port:	10-32 (1/16") Male	10-32 (1/16") Male
Storage (2-8°C):	20% Ethanol	20% Ethanol

HiFLOW GST FPLC column schematic:



NOTE:	HiFLOW FPLC columns are supplied with a moulded removable (1/16") female stop plug attached to the (1/16") male outlet port. Remove prior to use, reverse and use to seal the column for storage.
NOTE:	HiFLOW FPLC columns cannot be opened or repacked.

Chemical compatibility:

Buffer compatibility	
Standard buffers:	Common aqueous buffers and salts
pH range:	3.0-12.0
Chelating agents	
EDTA:	1 mM
Sulfhydril reagents	
β-mercaptoethanol:	5 mM
DTT:	10 mM
Denaturants	
Urea:	4 M
Guanidinium hydrochloride:	3 M
Detergents	
DDM (n-Dodecyl-β-Dmaltoside):	0.1%
OG (n-Octyl-β-Dglucopyranoside):	5%
Triton® X-100:	Up to 2 %
Tween® 20:	Up to 2%
NP-40:	0.2%
SDS:	Up to 0.03% (w/v)
Cetyltrimethylammonium bromide (CTAB):	UP to 1%
C12E8:	0.05%
Brij 35:	0.1%
Cholate:	5%
Deoxycholate:	2%
CHAPS :	1%
Other additives	
NaCl	1 M
NaOH	0.1 M
HCl	0.1 M
Ethanol:	70% (v/v)

Principles of GST affinity chromatography:

Glutathione S-transferase (GST) is a 26 kDa protein whose DNA sequence is frequently integrated into expression vectors as a terminal tag for the production of recombinant proteins. The expression of which produces a GST-tagged protein in which the functional GST protein is fused to the N or C-terminus of the recombinant protein. GST rapidly folds into a stable and highly soluble protein helping to promote greater expression, solubility, and folding of the recombinant proteins. In addition, the GST enzyme/tag can be detected and affinity purified by binding to its substrate glutathione (a Glu-Cys-Gly tripeptide). The simplicity of GST affinity purification is extremely attractive as the immobilised glutathione substrate lends itself to a simple bind-wash-elute mode of operation using lysate samples without any prior treatment (e.g. buffer exchange steps) providing the appropriate buffer formulations are used.

GST affinity resin:

Reduced glutathione tripeptide (Glu-Cys-Gly) covalently immobilised onto 7.5% cross-linked agarose beads via an 11 atom spacer through its sulfhydryl group.

Application drivers for GST chromatography:

Screening expression clones for high levels of GST-tagged proteins.
Purification of recombinant proteins for raising antibodies.
Purification of recombinant proteins for activity and/or structural studies.
Removal of cleaved Glutathione S-transferase tag from protein samples.

General considerations for selecting optimal binding conditions for the GST resin:

The key parameters affecting the binding of the GST-Tagged recombinant protein to the affinity resin are the flow rate over the column and the structural integrity of the Glutathione-S-transferase enzyme tag. Glutathione-S-transferase shows slow kinetics for its Glutathione substrate and it's important to maintain a low flow rate over the resin during loading of the lysate to achieve the maximum binding capacity. Binding is also dependent upon preserving the native structure and function of the Glutathione *S*-transferase enzyme. Binding is most effective under physiologic conditions (or neutral buffers) such as Tris-buffered saline (TBS) pH 7.5 and is not compatible with protein denaturants.

If the binding efficiency is found to be poor and the lysis buffer differs significantly from the pre-equilibration buffer, it is recommended that the lysate be dialysed, titrated with a concentrated stock solution, or buffer exchanged using an ultrafiltration device with a more appropriate pre-equilibration buffer.

It is imperative that the lysate is completely clear prior to loading on the column as any particulate matter (e.g. cell debris) may partially foul and clog-up the resin resulting in an increased back pressure and reduced flow rates. This will significantly increase the binding, washing and elution times and effect the final purify of the eluted protein. It is recommended that the cleared lysate be filter just prior to loading even if it has been previously filtered several days before. Ideally samples should be processed rapidly and, if the need arises, the protein purified at 4°C. It is also recommended that number of freeze/thaw cycles be minimised during storage to reduce the amount of aggregation/precipitation of the proteins.

Optimal buffer conditions for binding the target molecule to a resin are critical for successful purification of the protein. If the binding conditions are not optimal with respect to pH, salt concentration, detergent ...etc, purification can be adversely affected.

Protein purification conditions:

This protocol describes the purification of recombinant glutathione-S-transferase (GST) tagged proteins from an *E. coli* cell pellet under native conditions using HiFLOW GST FPLC Columns. Reagent amounts given apply to IPTG-induced bacterial culture of a well-expressed protein (approximately 10-50 mg/l). Cells are lysed with lysozyme because it is an inexpensive and efficient method for cells that have been frozen. However other lysis methods based on physical disruption (e.g. sonication or homogenization) or detergents (e.g. CHAPS) can also be used. The GST-tagged protein is then purified from the cleared lysate under native conditions in a bind-wash-elute procedure. This method is most efficient when the GST-tag is available, correctly folded and accessible. It is recommended that the bind-wash-elute conditions be tested and optimised in order to achieve the optimum conditions for purification. All volumes are given in column bed volume (CV).

Example: Buffers for purifications using reduced glutathione elution.

Lysis buffer:

125 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% Triton X-100, pH 7.4

Wash buffer:

125 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.4

ATP wash buffer:

50 mM Tris-HCl, 2 mM *ATP, 10 mM $MgSO_4$, pH 7.4

Elution buffer:

125 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1% Triton X-100, 50 mM reduced glutathione, pH 7.4

NOTE:	Optimal buffer conditions may vary depending on the protein of interest. Proteins may require addition of protease inhibitor cocktail, EDTA, 1-5 mM DTT, 1% BSA, or detergents such as 0.5-1% Igepal CA-630 (Nonindet P-40) or 0.5-1% Tween-20.
*NOTE:	Add ATP to the 'ATP wash buffer' immediately before use.

Procedure

1. Thaw the *E. coli* cell pellet on ice.

NOTE: Freezing the cell pellet at –20°C for 30 min prior to incubation at room temperature improves lysis by lysozyme.

2. Resuspend the cell pellet in 'Lysis buffer' (50 ml / litre cell media) supplemented with 1 mg/ml Lysozyme.

NOTE: Benzonase® is recommended to reduce the viscosity caused by the nucleic acid (3 U/ml bacterial culture) if required.
NOTE: Add 1 protease inhibitor table to reduce protease activity

3. Incubate at room temperature for >30 min (or > 1 hour at 4°C) on an end-over-end shaker.

NOTE: Mechanical lysis can also be used by repeated freeze/thaw, vortexing, homogenization, sonication or french press if required.

4. Centrifuge the lysate for 30 min at 4°C and 10,000 x g. Collect the supernatant.

NOTE: If the supernatant remains cloudy then repeat step 4.

5. Connect the HiFLOW GST column to an FPLC/syringe/pump and wash with 3-5 CV's of distilled water to remove the 20% ethanol before equilibrating with 10 CV's of 'Wash buffer'.

6. Filter the cleared lysate through a 0.2 µm syringe filter directly before loading onto the column at the recommended flow rate.

NOTE: For optimal binding reduce the flow rate over the column to maximise the interaction time with the resin.
1 ml HiFLOW GST column= flow rate 0.2-1 ml/min
5 ml HiFLOW GST column= flow rate 1-5 ml/min

7. After loading wash the column with 'Wash buffer' until the measured absorbance (OD=280nm) reaches a stable baseline.

NOTE: This will typically take 10-15 CV's.
NOTE: Keep the wash fractions for SDS-PAGE analysis if required.

8. To remove chaperone contaminants wash the column with 3-5 CV's of 'ATP wash buffer'.

NOTE: ATP shows high absorbance at 280nm preventing monitoring of the ATP wash.
NOTE: Retain the ATP wash fractions for SDS-PAGE analysis.

9. Elute the GST-tagged protein with 3-5 CV's of 'Elution buffer' or until the measured absorbance (OD=280nm) reaches a stable baseline.

NOTE: Additional elution's with increased reduced glutathione concentrations may be required.
NOTE: Collect the eluate in separate tubes for SDS PAGE analysis and protein concentration determination.

9. Analyze all fractions by SDS-PAGE.

NOTE: Western Blot experiment using an anti-His antibody can be performed if required.

NOTE:	Volumes and buffer conditions are protein depended and may require scouting and optimising.
NOTE:	It is recommended that each column is re-used for identical protein samples to avoid cross-contamination.

Desalting and concentrating the purified protein:

Reduced glutathione, EDTA, or detergents should be removed by diafiltration using ultrafiltration concentrators or rapid dialysis against an appropriate buffer for your downstream application.

Column washing and regeneration conditions:

HiFLOW GST FPLC columns should be carefully washed with PBS (wash buffer) and stored in 20% ethanol after each run. If the column shows increased back pressure or loss of binding capacity then this may be due to the accumulation of precipitated, denatured, or non-specifically bound substances and proteins. This protocol delineates washing and regeneration procedures for the 1 and 5 ml HiFLOW GST FPLC column to remove these substances and proteins. All volumes are given in column bed volume (CV).

Example: Buffers washing and regeneration.

Regeneration buffer #1:
6M Guanidine hydrochloride
Regeneration buffer #2:
70% Ethanol
Regeneration buffer #3:
1% Triton™ X-100
Wash buffer:
10-100 mM Sodium phosphate, 2.7 mM potassium chloride, 0.137 M NaCl, pH 7.4 (PBS)
Storage buffer:
20% Ethanol

Wash and regeneration procedure 1: removal of precipitated and denatured substances and proteins.

1. Connect the HiFLOW GST column to an FPLC/syringe/pump and wash with 5 CV's of 'Wash buffer'.

2. Wash the column with 2 CV's 'Regeneration buffer #1'.

3. Immediately wash the column again with 5 CV's of 'Wash buffer'.

4. Wash the column with 3-5 CV's of 'storage buffer' for long term storage at 4°C.

NOTE: The column is now ready for re-use if required.

NOTE: If wash procedure 1 does not fully resolve the problem then repeat or proceed to wash procedure 2.

Wash and regeneration procedure 2: removal of hydrophobic substances and proteins.

1. Connect the HiFLOW GST column to an FPLC/syringe/pump and wash with 5 CV's of 'Wash buffer'.

2. Wash the column with 3-4 CV's of 'Regeneration buffer #2' (or 2 CV's of 'Regeneration buffer #3').

3 Immediately wash the column again with 5 CV's of 'Wash buffer'.

4. Wash the column with 3-5 CV's of 'storage buffer' for long term storage at 4°C.

NOTE: The column is now ready for re-use if required.

NOTE: If wash procedure 2 does not fully resolve the problem then repeat the procedure with the alternative 'Regeneration buffer'.

Performance data:

Purification of GST-tagged protein from *E.coli* lysate.

Glutathione affinity purification of recombinant GST-tagged protein from 1 ml cleared *E.coli* lysate using a 1 ml HiFLOW GST FPLC column on an FPLC. The eluted fraction contained >95% pure protein according to SDS-PAGE analysis (Figure 1B).

Sample:	1 ml *E.coli* lysate
Column:	1 ml HiFLOW GST FPLC Column
Instrument:	FPLC
Flow rate:	1 ml/min
Binding buffer:	PBS pH 7.5
Elution Buffer:	50 mM Tris, 10mM reduced glutathione, pH8.0
Eluted Protein:	0.7 mg

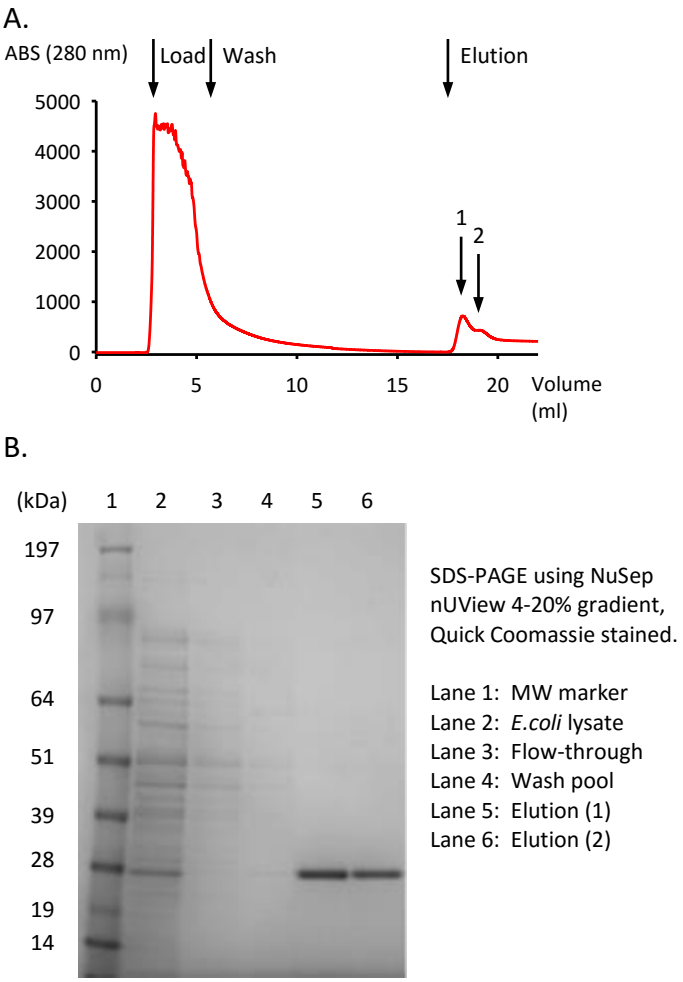


Figure 1. Purification of GST-tagged protein from *E.coli* lysate on a 1 ml HiFLOW GST FPLC column. (A) FPLC chromatogram (B) SDS-PAGE analysis.

Storage conditions:

Item:	HiFLOW1-GST	HiFLOW5-GST
Shipping:	20 % Ethanol at room temperature	20 % Ethanol at room temperature
Short-term storage:	Equilibration buffer	Equilibration buffer
Long-term storage:	20% Ethanol at 4°C	20% Ethanol at 4°C

NOTE: Do not freeze or store the column in buffer or water for long periods. Each column is stored in 20 % ethanol and reusable for up to 2 years from the date of resin manufacture. Please see the label for the recorded expiry date.

Questions and answers:

1. *What is the shelf-life of the HiFLOW GST FLPC Column?*
   The resin is guaranteed for 2 years after the date of manufacture provided they are stored at 2-8°C.

2. *Do I need to filter the buffers prepared in my laboratory?*
   It is good laboratory practice to filter all buffers.

3. *How should I prepare my sample for GST affinity separation?*
   Many chromatographic procedures demand that the sample is pre-conditioned prior to loading. We recommend that all samples are filtered to at least 0.45 µm (preferably 0.20 µm) pore size. High viscosity is mostly attributed to contaminating DNA or RNA. The intrinsic viscosity of a lysate can be reduced by either drawing it through a syringe needle several times or by adding appropriate amounts of DNase and/or RNase (5-10 µg/ml) to the lysis buffer and incubating the mix on ice for 15 mins.

4. *Should I add DTT (or β-mercaptoethanol) to the lysis buffer?*
   Concentrations less than or equal to 10 mM DTT (or 5 mM β-mercaptoethanol) can be used with the HiFLOW GST FLPC columns and may significantly increase binding of some GST-tagged proteins to the column.

5. *How can I regenerate the HiFLOW GST FPLC comulm?*
   We recommend that you wash the column with PBS and store in 20% ethanol between each run. If column performance and binding capacity becomes reduced then we recommend washing with harsher conditions. See 'column washing and regeneration conditions' for further details.

6. *Should I be concerned if the column partially dries out during the chromatographic steps?*
   The resin is robust although we recommend flushing out as much air as possible from the column before continuing. Partially dried resin rehydrates rapidly however the performance of the column (binding capacity and running pressure) may be affected.

7. *Can I load purified protein immediately on to an SDS-gel?*
   Proteins purified from the HiFLOW GST FPLC column under the recommended conditions can be loaded on to an SDS-polyacrylamide gel.

8. *Do I need to remove the GST-tag from the recombinant protein after purification?*
   Due to its size, the GST-tag can affect the activity, stability, or structure determination. If required a protease cleavage site (e.g. Factor Xa Protease, TEV, or enterokinase) can be engineered between the GST-tag and the target protein. The

tag can then be cleaved off and the protein re-purified by passing it back through the HiFLOW GST FPLC column in order to remove the digested tag and undigested GST-tagged protein.

9. *Under what circumstances can I re-use the column?*

The HiFLOW GST FPLC columns are designed for re-use. We recommend regular washing and cleaning between purifications in order to maintain performance. Should you observe a slowdown in flow rate or increase in back pressure then we recommended washing and regenerating the column prior to further use. See 'Column washing and regeneration conditions' for further details.

Troubleshooting assistance:

Bubbles or cracks appear in the resin bed:

- The resin has been stored at a cool temperature and then rapidly warmed up. The resin should be warmed slowly to room temperature before use.

The sample does not flow easily through the column:

- The resin is clogged with particulates. Pre-filter the sample just before loading it on to the column.
- If the column is not stored at 2-8°C, or it has been used more than once and stored in the absence of a bacteriostat, microbial growth may restrict flow through the column.

No binding or elution of the target protein is observed from the column:

- Extensive sonication can denature the GST-tagged protein resulting in loss of the GST-tag's enzyme activity and preventing the protein from binding to the column.
- Adding 1-10 mM DTT to the 'Lysis buffer' can significantly increase binding of the GST-tagged protein to the column.
- Check the pH of the 'Lysis buffer. If the pH is not within the range of 6-8 then binding of the GST-tag to the HiFLOW GST FPLC column will be affected. If required dialyse, titrated with a concentrated stock solution, or buffer exchanged the lysate to within the correct pH range.
- Test the binding conditions and HiFLOW GST FPLC column are working correctly using just the expressed GST-tag sample for bind-wash-elution.
- The retention time may not be sufficient for binding. Reduce the flow rate of the loading stage down to 0.2-0.5 ml/min (1 ml HiFLOW GST column) or 1-3 ml/min (5 ml HiFLOW GST column).
- The column may contain a build-up of precipitated, denatured, or hydrophobic substances and proteins which may impede binding. See 'Column washing and regenerating conditions' for details of washing and regeneration protocols or use a new HiFLOW GST FPLC column.

The recovery of target protein is low:

- Increase the elution time and volume of the 'Elution buffer'.
- The Elution buffer may not be strong enough for elution. Try increasing the pH from 7.4 to 8-9, increasing the salt concentration to 0.2 M NaCl, or increasing the amount of reduced glutathione in the 'Elution buffer'.
- Hydrophobic interactions from the fused protein maybe affecting elution. These may require disrupting using 0.1% Triton X-100 or 2% N-octylglucoside to improve elution of some GST-tagged proteins.

Poor resolution of the target protein:

- Multiple proteins maybe visible on the SDS-PAGE showing co-purification of the GST-tagged protein with another protein. This may be DnaK (70 kDa) or another chaperone protein involved in protein folding. These maybe removed by including the 'ATP wash buffer' step in the purification procedure (see 'protein purification conditions' for details).
- This maybe the result of partial degradation of the GST-tagged protein. Try including protease inhibitor tablets in the 'Lysis buffer'.
- In order to minimize degradation try reducing the expression, lysis and purification times.
- Co-express and purify the GST-tagged protein with a molecular chaperone to increase stability and reduce degradation. For example: DnaK, DnaJ, GroEL, or GroES (the vectors for which and readily available).
- The procedure may require performing at 4°C to reduce degradation and stabilise the GST-tagged recombinant protein.
- Target the GST-tagged protein to the periplasm region of the *E.coli* cell during expression using a signal peptide sequence (for example pelB, OmpA, DsbA, TolB and MalE). This may aid folding and stability whilst reducing degredation. This will however reduce the expression levels considerably.

Glossary:

affinity chromatography - chromatographic separation based on a specific interaction between an immobilized ligand and a binding site on a macromolecule.

chaperone protein– protein which assists with the folding or stability of another.

chaotropic agent - a molecule which interferes with hydro-phobic interactions by disrupting the ordered structure of water molecules. Examples include urea and guanidine hydrochloride.

chelating agent – a compound such as EDTA or EGTA that is able to combine with a metal ion to form a structure with one or more rings.

cleared lysate – the soluble cell extract after the cell debris and other particulates have been removed by centrifugation.

column bed volume (CV) - the total volume occupied by the chromatographic packed bed. It is also referred to as the column volume or CV.

DL-Dithiothreitol (DTT) – reducing agent used to break disulphide bonds.

expression vector – a cloning vector intended for the foreign gene to be expressed in the host organism.

french pressure cell – a device that uses high shear forces to rupture microbial cells. The suspension is poured into a chamber, which is closed at one end by a needle valve and at the other end by a piston. Pressures of up to 16,000 lb/in2 are applied by a hydraulic press against a closed needle valve. When the desired pressure is attained, the needle valve is fractionally opened to marginally relieve the pressure. The cells subsequently expand and rupture, thereby releasing the cellular components through the fractionally open valve.

freeze-thawing – a method that is sometimes used to break open cells by successive periods of slow freezing and thawing. Ice crystals are generated during the freezing stage, which disrupt the cells when they melt during thawing. The method, however, is slow and releases a limited amount of subcellular components.

Glutathione S-transferase (GST) - 26kDa protein whose DNA sequence is frequently integrated into expression vectors as a terminal tag for the production of recombinant proteins.

immobilized - bound to a surface, usually through covalent linkages.

inclusion bodies – quite a lot of proteins form insoluble crystalline aggregates known as inclusion bodies when they are expressed at high levels inside bacteria. The proteins can be solubilized using denaturants such as 8 M urea or 6 M guanidine hydrochloride.

ion exchange chromatography - chromatographic separation based on different charge properties of macromolecules.

isoelectric point - the pH at which the protein has no net charge.

lysozyme – an enzyme than hydrolyzes β-1,4-linkages between N-acetylmuramic acid and 2-acetamido-2-deoxy-D-glucone in peptidoglycan heteroploymers of prokaryotic cell walls. An example is egg white lysozyme and this enzyme is used to disrupt cells in order to liberate expressed proteins. 1 mg/ml lysozyme is normally added to *E.coli* cells in lysis buffer and incubated for 30 min to aid cell disruption. The pH optimum for lysozyme is pH 9.2 (Davies *et al* 1969).

recombinant protein – a protein coded for by a cloned gene which has often been modified *to increase the expression of that protein or to alter the properties of the protein.*

sonication – this technique uses ultrasonic energy to generate high transient pressures that are believed to disrupt the cells.

tris(2-chloroethyl) phosphate (TCEP) – strong irreversible reducing agent used to break disulphide bonds.

truncate - terminate prematurely or to shorten by cutting.

Literature:

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Davies, A., Neuberger, A. and Wilson, B. (1969). *Biochim. Biophys. Acta* 178, 294.

Protein Purification Applications: A Practical Approach 1995 (E.L.V. Harris and S. Angal Eds.) IRL PRESS at Oxford University Press. ISBN: 0-19-963023-2.

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Ordering information:

Product	Volume	Order Code
1 ml HiFLOW GST FPLC column (1 x 1 ml)	1 x 1ml	SLS3242
1 ml HiFLOW GST FPLC columns (5 x 1 ml)	5 x 1ml	SLS3244
5 ml HiFLOW GST FPLC column (1 x 5 ml)	1 x 5 ml	SLS3246
5 ml HiFLOW GST FPLC columns (5 x 5 ml)	5 x 5 ml	SLS3248

Technical support:

Contact our technical support and sales centre for assistance:

Telephone
FAX:
Email:
Web:

Disclaimer:

- This product is for research use only and is not intended for use in clinical diagnosis. No claims beyond replacement of unacceptable material or refund of purchase price shall be allowed.