

Glutathione Sepharose 4 Fast Flow

Glutathione Sepharose™ 4 Fast Flow is an affinity chromatography medium for easy, one-step purification of glutathione S-transferase (GST) fusion proteins produced using the pGEX series of expression vectors, other glutathione S-transferases and glutathione binding proteins.

GST fusion proteins can be purified directly from bacterial lysates using Glutathione Sepharose 4 Fast Flow. The fusion proteins are eluted under mild, non-denaturing conditions that preserve protein antigenicity and function.

Glutathione Sepharose 4 Fast Flow has high flow properties, which makes it excellent for scale-up. Table 1 lists the characteristics of Glutathione Sepharose 4 Fast Flow.

Detailed information about the production of GST fusion proteins can be found in the GST Gene Fusion System Handbook (1).

Description

Gel properties

Glutathione Sepharose 4 Fast Flow is designed for purification of glutathione S-transferase (GST) fusion proteins produced using the pGEX series of expression vectors (3), other glutathione S-transferases and glutathione binding proteins.

GST fusion proteins can be purified directly from bacterial lysates with a one-step method using Glutathione Sepharose 4 Fast Flow. The fusion proteins are eluted under mild, non-denaturing conditions that preserve protein antigenicity and function.

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

The total binding capacity is approximately 10 mg recombinant GST/ml gel. The dynamic binding capacity will vary depending on the flow rate and the sample.

If removal of the GST moiety (a naturally occurring protein with M_r 26 000) is required, the fusion protein can be digested with the appropriate site-specific protease while bound to Glutathione Sepharose 4 Fast Flow or, alternatively, after elution. Cleavage of GST fusion protein bound to the column/bulk matrix eliminates the extra step of separating the released protein from GST, since the GST moiety remains bound (1, 4, 15). The cleaved target protein is eluted using binding buffer.

Table 1. Characteristics of Glutathione Sepharose 4 Fast Flow.

Ligand	glutathione and 10-carbon linker arm
Ligand concentration	120–320 μmol glutathione/ml medium
Binding capacity	\approx 10 mg recombinant glutathione S-transferase/ml medium, GST, M, 26 000
Dynamic binding capacity	\approx 11 mg GST fusion protein/ml medium M, 43 000 (GSTrap™ FF 1 ml at 1 ml/min=156 cm/h)
Mean particle size	90 μm
Bead structure	highly cross-linked 4% agarose
Maximum back pressure	0.1 MPa, 1 bar (when packed in XK columns). May vary if used in other columns
Maximum flow rate*	450 cm/h 15 ml/min, using XK 16/20 column with 5 cm bed height, run at room temperature with aqueous buffer
Recommended flow rates	Sample loading: <100 cm/h (< 3 ml/min using XK 16/20 column) Washing and elution: 100–300 cm/h (3–10 ml/min using XK 16/20 column)
Chemical stability	All commonly used aqueous buffers, e.g. 1 M acetate pH 4.0 and 6 M guanidine hydrochloride for 1 hour at room temperature
pH stability	pH 3–12
Storage temperature	+ 4–8 °C
Storage buffer	20 % ethanol

* **Note:** One of the most important parameters affecting the binding of GST fusion proteins or other glutathione binding proteins to Glutathione Sepharose 4 Fast Flow is the flow rate. Due to the relatively slow binding kinetics between GST and glutathione, it is important to keep the flow rate low during sample application for maximum binding capacity.

Operation

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 before use.

Binding buffer: PBS, pH 7.3

(140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 ,
1.8 mM KH_2PO_4 , pH 7.3)

Elution Buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

Sample preparation

The sample should be centrifuged and/or filtered through a 0.45 μm filter before it is applied to the medium.

If the sample is too viscous, dilute it with binding buffer to prevent clogging the column. It is not necessary to filter the sample before performing batch purification.

Batch purification

Preparation of Glutathione Sepharose 4 Fast Flow

1. Determine the bed volume of Glutathione Sepharose 4 Fast Flow required for your purification.

Note: The 25 ml package has a slurry concentration of approx. 30% and the 100 ml and 500 ml packages have a slurry concentration of approx. 50%. To obtain the desired volume (using 100 ml or 500 ml packages), use twice the volume of 50% Glutathione Sepharose 4 Fast Flow slurry (*i.e.* 1 ml slurry will give a bed volume of 0.5 ml).

2. Gently shake the bottle of Glutathione Sepharose 4 Fast Flow to resuspend the slurry.
3. Use a pipette or measuring cylinder to remove sufficient slurry for use and transfer to an appropriate tube.
4. Sediment the matrix by centrifugation at $500 \times g$ for 5 min. Carefully decant the supernatant.
5. Wash the Glutathione Sepharose 4 Fast Flow by adding 5 ml PBS to each 1 ml slurry (= 0.5 ml gel). Invert to mix.
6. Sediment the matrix by centrifugation at $500 \times g$ for 5 min. Carefully decant the supernatant.
7. Repeat steps 5 and 6 one more time.

8. For each 1 ml of the original slurry of Glutathione Sepharose 4 Fast Flow, add 1 ml of PBS. This results in a final 50% slurry concentration.

Batch purification

1. Add the cell lysate to the prepared Glutathione Sepharose 4 Fast Flow and incubate for 20–30 min. at room temperature. Use gentle agitation such as end-over-end rotation.
2. Use a pipette or cylinder to transfer the mixture to an appropriate container/tube.
3. Sediment the matrix by centrifugation at $500 \times g$ for 5 min. Carefully decant the supernatant (= flow-through) and save it for measuring the binding efficiency to the medium *i.e.* by SDS-PAGE.
4. Wash the Glutathione Sepharose 4 Fast Flow by adding 5 ml PBS to each 1 ml slurry (= 0.5 ml gel). Invert to mix.
5. Sediment the matrix by centrifugation at $500 \times g$ for 5 min. Carefully decant the supernatant (= wash) and save it for SDS-PAGE analysis.
6. Repeat steps 4 and 5 twice for a total of three washes.
7. Elute the bound protein by adding 0.5 ml 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0 per 1 ml original slurry of Glutathione Sepharose 4 Fast Flow. Incubate at room temperature for 5–10 min. using gentle agitation such as end-over-end rotation.
8. Sediment the matrix by centrifugation at $500 \times g$ for 5 min. Carefully decant the supernatant (= eluted protein).
9. Repeat steps 7 and 8 twice for a total of three elutions. Check the three eluates separately for purified protein and pool according to the results.

Note:

- Due to the relatively slow binding kinetics between GST and glutathione, it is important to keep the time during sample binding for maximum binding capacity.
- Volumes and times used for elution may vary among fusion proteins. Additional elutions with higher concentrations of glutathione may be required. Flow-through, wash and eluted material from the gel should be monitored for GST fusion proteins using SDS-PAGE in combination with Western Blot if necessary.
- The GST Detection Module can be used to optimize conditions for elution or to trace steps in the purification of a GST fusion protein. The Module is designed to identify GST fusion proteins using either a biochemical or an immunological assay (3).
- The concentration of GST fusion protein can be estimated by measuring the absorbance at 280 nm. The GST tag can be approximated using the conversion; $A_{280} \sim 1$ corresponds to ~ 0.5 mg/ml.
- The concentration of GST fusion protein may also be determined by standard chromogenic methods (*e.g.* Lowry, BCA, and Bradford assays). If Lowry or BCA assays are to be used, the sample must first be buffer exchanged using a HiTrap Desalting column or dialysed against PBS to remove glutathione, which can interfere with the protein measurement. The Bradford method can be used in the presence of glutathione.
- The reuse of Glutathione Sepharose 4 Fast Flow depends on the nature of the sample and should only be performed with identical samples to prevent cross-contamination.
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Column purification

Columns

Amersham Biosciences offers many different kinds of columns to use for column purification (see the Biodirectory catalogue or www.chromatography.amershambiosciences.com for details).

Suggested columns are listed below:

HR 10/10 (10 mm i.d.) for bed volumes up to 8 ml at bed heights up to 10 cm.

XK 16/20 (16 mm i.d.) for bed volumes up to 30 ml at bed heights up to 15 cm.

XK 26/20 (26 mm i.d.) for bed volumes up to 80 ml at bed heights up to 15 cm.

Prepacked GSTrap FF 1 ml and GSTrap FF 5 ml columns are also available. For scaling up GSTPrep™ FF 16/10 (20 ml) prepacked columns are also available.

Column packing

1. Equilibrate all materials to the temperature at which the purification will be performed.
2. Eliminate air by flushing the column end pieces with PBS, pH 7.3. Make sure that no air has been trapped under the column net. Close the column outlet leaving 1–2 cm of PBS, pH 7.3 remaining in the column.
3. Gently shake the bottle of Glutathione Sepharose 4 Fast Flow to resuspend the slurry.
4. Estimate the amount of slurry needed (the medium slurry concentration is approximately 30%, 25 ml pack size or 50%, larger pack sizes).

5. Pour out the slurry. Pouring it down a glass rod held against the wall of the column will minimize the introduction of air bubbles.
6. Immediately fill the column with PBS, pH 7.3, mount the column top-piece onto the column and connect the column to a pump.
7. Open the outlet of the column and set the pump to run at the desired flow rate. Ideally, Fast Flow media are packed at constant pressure not exceeding 1 bar (0.1 MPa) in XK columns. If the packing equipment does not include a pressure gauge, use a packing flow rate of max. 15 ml/min, 450 cm/h (XK 16/20 column) or 6 ml/min, 450 cm/h (HR 10/10 column). If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate the pump can deliver. This should also give a reasonably well-packed bed.
8. Maintain the packing flow for at least 3 bed volumes after a constant bed height is obtained. Mark the bed height on the column.
Note: Do not exceed 75% of the packing flow rate during purification.
9. Stop the pump and close the column outlet. Remove the top-piece from the column and carefully fill the rest of the column with PBS, pH 7.3 to form an upward meniscus at the top.
10. Insert the adaptor into the column at an angle, ensuring that no air is trapped under the net.
11. Slide the adaptor slowly down the column (the outlet of the adaptor is open) until the mark is reached (see step 8). Lock the adaptor in position. Connect the column to a pump or a chromatography system and start equilibration. Re-position the adaptor if necessary.

Column purification

1. Equilibrate the column with approx. 5 column volumes of PBS, pH 7.3.
2. Apply the centrifuged and/or filtered sample.
3. Wash the column with 5–10 column volumes of PBS, pH 7.3 or until no material appears in the flow-through. Save the flow-through for measuring the binding efficiency to the medium, *i.e.* by SDS-PAGE.
4. Elute the bound protein with 5–10 column volumes of 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0.

Note:

- One of the most important parameters affecting the binding of GST fusion proteins or other glutathione binding proteins to Glutathione Sepharose 4 Fast Flow is the flow rate. Due to the relatively slow binding kinetics between GST and glutathione, it is important to keep the flow rate low during sample application for maximum binding capacity.
- Volumes and times used for elution may vary among fusion proteins. Additional elutions with higher concentrations of glutathione may be required. Flow-through, wash and eluted material from the column should be monitored for GST fusion proteins using SDS-PAGE in combination with Western Blot if necessary.
- The GST Detection Module can be used to optimize conditions for elution or to trace steps in the purification of a GST fusion protein. The Module is designed to identify GST fusion proteins using either a biochemical or an immunological assay (3).

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- The concentration of GST fusion protein may also be determined by standard chromogenic methods (*e.g.* Lowry, BCA, and Bradford assays). If Lowry or BCA assays are to be used, the sample must first be buffer exchanged using a HiTrap Desalting column or dialysed against PBS to remove glutathione, which can interfere with the protein measurement. The Bradford method can be used in the presence of glutathione.
- The reuse of Glutathione Sepharose 4 Fast Flow depends on the nature of the sample and should only be performed with identical samples to prevent cross-contamination.

Cleaning Glutathione Sepharose 4 Fast Flow

If the gel appears to be losing binding capacity, it may be due to an accumulation of precipitate, denatured or non-specifically bound proteins.

Removal of precipitated or denatured substances:

- Wash with 2 column volumes of 6 M guanidine hydrochloride, immediately followed by 5 column volumes of PBS, pH 7.3.

Removal of hydrophobically bound substances:

- Wash with 3-4 column volumes of 70% ethanol or 2 column volumes of 1% Triton™ X-100, immediately followed by 5 column volumes of PBS, pH 7.3.

Storage

Store the packed column at +4–8 °C in 20% ethanol.

Cleavage of GST fusion proteins

In most cases, the fusion partner of interest retains functional activity and the functional test can be performed using intact fusion with GST. If removal of the GST tag is necessary, fusion proteins containing a PreScission™ Protease recognition site, a thrombin recognition site or a factor Xa recognition site may be cleaved either while bound to Glutathione Sepharose 4 Fast Flow or in solution after elution.

Cleavage after elution is suggested if optimization of cleavage conditions is necessary. Samples can easily be removed at various time points and analyzed by SDS-PAGE to estimate the yield, purity and extent of digestion. The amount of protease used, the temperature and the length of incubation required for complete digestion may vary depending on the fusion partner.

Optimal conditions for each fusion should be determined in pilot experiments, *e.g.* incubation time may be reduced by adding a greater amount of enzyme.

1. PreScission Protease

PreScission Protease, M_r 46 000.

PreScission cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.5.

PreScission Protease cleavage of GST fusion protein bound to the column/bulk medium

Assumption: 8 mg GST fusion proteins bound/ml medium

1. Wash the fusion protein bound Glutathione Sepharose 4 Fast Flow with 10 bed volumes of PreScission cleavage buffer.
2. Prepare the PreScission Protease mix: For each ml of Glutathione Sepharose 4 Fast Flow bed volume, prepare a mixture of 80 μ l (160 units) of PreScission Protease and 920 μ l of PreScission cleavage buffer at +5 °C. (8 mg fusion protein bound/ml medium).
3. Load the PreScission Protease mixture onto the column. Seal the column.

If batch format is used, add PreScission Protease mixture to the Glutathione Sepharose 4 Fast Flow pellet. Gently shake or rotate the suspension.

4. Incubate at +5 °C for 4 hours.
5. Following incubation, wash the column with approx. 3 bed volumes of PreScission cleavage buffer. Collect the eluate in different tubes to avoid dilution of the fusion protein and analyse it. If batch format is used, centrifuge the suspension at 500 \times g for 5 minutes to pellet the Glutathione Sepharose 4 Fast Flow and carefully transfer the eluate to a tube. The eluate will contain the protein of interest, while the GST portion of the fusion protein and the PreScission Protease will remain bound to the Glutathione Sepharose 4 Fast Flow.

PreScission Protease cleavage of eluted GST fusion protein

Assumption: 8 mg GST fusion protein bound/ml medium

1. Following elution of the GST fusion protein from either batch or column, remove the reduced glutathione from the eluate using a quick buffer exchange on HiTrap Desalting, a PD-10 column or HiPrep™ 26/10 Desalting depending on sample volume, or dialyse against PreScission cleavage buffer.
2. Add 1 μ l (2 U) of PreScission Protease for each 100 μ g of fusion protein in the eluate. If the amount of fusion protein in the eluate has not been determined, add 80 μ l (160 units) of PreScission Protease for each ml of Glutathione Sepharose 4 Fast Flow bed volume. (8 mg fusion protein bound/ml medium). (Bed volume is equal to $0.5 \times$ the volume of the 50% Glutathione Sepharose 4 Fast Flow slurry used).
3. Incubate at +5 °C for 4 hours.
4. Once digestion is complete, apply the sample to washed and equilibrated Glutathione Sepharose 4 Fast Flow to remove the GST moiety of the fusion protein and the PreScission Protease from the protein of interest.
5. Incubate for 20–30 min. at room temperature.
6. Sediment the medium by centrifugation at $500 \times g$ for 5 min. The protein of interest will be found in the supernatant.

2. Thrombin

Thrombin, M_r 37 000.

Thrombin cleavage buffer: PBS, pH 7.3.

Preparation of thrombin solution:

1. Dissolve 500 U thrombin in **cold** 500 μ l PBS, pH 7.3 (1 U/ μ l).
2. Swirl gently to dissolve.
3. Freeze as 80 μ l aliquots and keep at -80°C .

Thrombin cleavage of GST fusion protein bound to the column/ bulk medium

Assumption: 8 mg GST fusion protein bound/ml medium

1. Follow steps 1–5 under “Purification”.
2. Prepare the thrombin mix: For each ml of Glutathione Sepharose 4 Fast Flow bed volume, prepare a mixture of 80 μ l (80 units) of thrombin and 920 μ l of PBS, pH 7.3 (8 mg fusion protein bound/ml gel).
(Bed volume is equal to $0.5 \times$ the volume of the 50% Glutathione Sepharose 4 Fast Flow slurry used).
3. Load the thrombin mix onto the column. Seal the column.
If batch format is used, add the thrombin mixture to the Glutathione Sepharose 4 Fast Flow pellet.
Gently shake or rotate the suspension.
4. Incubate at room temperature ($22\text{--}25^\circ\text{C}$) for 2-16 hours.
5. Following incubation, wash the column with approx. 3 bed volumes of PBS, pH 7.3. Collect the eluate in different tubes to avoid dilution of the fusion protein and analyse it. If batch format is used, centrifuge the suspension at $500 \times g$ for 5 minutes to pellet the Glutathione Sepharose 4 Fast Flow and carefully

transfer the eluate to a tube. The eluate will contain the protein of interest and thrombin, while the GST portion of the fusion protein will remain bound to the Glutathione Sepharose 4 Fast Flow.

Thrombin cleavage of eluted GST fusion protein

Assumption: 8 mg GST fusion protein bound/ml medium

1. Follow steps 1–6 under “Purification”.
2. Add 10 μl (10 units) of thrombin solution for each mg of fusion protein in the eluate. If the amount of fusion protein in the eluate has not been determined, add 80 μl (80 U) of thrombin solution for each ml of Glutathione Sepharose 4 Fast Flow bed volume from which the fusion protein was eluted. (8 mg fusion protein bound/ml medium). (Bed volume is equal to $0.5 \times$ the volume of the 50% Glutathione Sepharose 4 Fast Flow slurry used).
3. Incubate at room temperature (22–25 °C) for 2–16 hours.
4. Once digestion is complete, GST can be removed by first removing glutathione using a quick buffer exchange on HiTrap Desalting, a PD-10 column or HiPrep 26/10 Desalting depending on sample volume, or by dialysis against PBS, pH 7.3. Follow this by applying the sample to washed and equilibrated Glutathione Sepharose 4 Fast Flow.
5. Incubate for 20–30 min. at room temperature.
6. Sediment the medium by centrifugation at $500 \times g$ for 5 min. The supernatant will contain the protein of interest and thrombin, while the GST portion of the fusion protein will remain bound to the Glutathione Sepharose 4 Fast Flow.

3. Factor Xa

Factor Xa, M_r 48 000.

Note: Factor Xa consists of two subunits linked by disulfide bridges. As glutathione can disrupt disulfide bridges, it should be removed from the sample prior to the cleavage reaction.

Factor Xa cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl_2 , pH 7.5

Preparation of factor Xa solution:

1. Dissolve 400 U factor Xa in 400 μl cold water (1 U/ μl).
2. Swirl gently to dissolve.
3. Freeze as 80 μl aliquots and keep at -80°C .

Factor Xa cleavage of GST fusion protein bound to the column/ bulk medium

Assumption: 8 mg GST fusion protein bound/ml medium

1. Wash the fusion protein bound Glutathione Sepharose 4 Fast Flow with 10 bed volumes of factor Xa cleavage buffer. (Bed volume is equal to $0.5 \times$ the volume of the 50% Glutathione Sepharose 4 Fast Flow slurry used).
2. Prepare the factor Xa mix: For each ml of Glutathione Sepharose 4 Fast Flow bed volume, prepare a mixture of 80 μl (80 units) of factor Xa and 920 μl of factor Xa cleavage buffer. (8 mg fusion protein bound/ml medium).
3. Load the factor Xa mixture onto the column. Seal the column. If batch format is used, add factor Xa mixture to the Glutathione Sepharose 4 Fast Flow pellet. Gently shake or rotate the suspension.
4. Incubate at room temperature ($22-25^\circ\text{C}$) for 2–16 hours.

5. Following incubation, wash the column with approx. 3 bed volumes of factor Xa cleavage buffer. Collect the eluate in different tubes to avoid dilution of the fusion protein and analyse it. If batch format is used, centrifuge the suspension at $500 \times g$ for 5 minutes to pellet the Glutathione Sepharose 4 Fast Flow and carefully transfer the eluate to a tube. The eluate will contain the protein of interest and factor Xa, while the GST portion of the fusion protein will remain bound to the Glutathione Sepharose 4 Fast Flow.

Factor Xa cleavage of eluted GST fusion protein

Assumption: 8 mg GST fusion protein bound/ml medium

1. Follow steps 1–6 under “Purification”.
2. Remove reduced glutathione from the eluate using a quick buffer exchange on HiTrap Desalting, a PD-10 column or HiPrep 26/10 Desalting depending on sample volume, or dialyse against factor Xa cleavage buffer.
3. Add 10 μl (10 units) of factor Xa solution for each mg fusion protein in the eluate. If the amount of fusion protein in the eluate has not been determined, add 80 μl (80 units) of factor Xa solution for each ml of Glutathione Sepharose 4 Fast Flow bed volume from which the fusion protein was eluted. (8 mg fusion protein bound/ml gel).
4. Incubate at room temperature (22–25 °C) for 2-16 hours.
5. Once digestion is complete, apply the sample to washed and equilibrated Glutathione Sepharose 4 Fast Flow to remove the GST moiety of the fusion protein.
6. Incubate for 20–30 min. at room temperature.

7. Sediment the medium by centrifugation at $500 \times g$ for 5 min. The protein of interest will be found in the supernatant together with factor Xa.

Trouble-shooting guide

Consult the GST Gene Fusion System Handbook (3) regarding trouble-shooting for the expression and fermentation of fusion proteins.

- **Majority of fusion protein is found in the post-lysis pellet:** SDS-PAGE analysis of samples collected during the cell disruption may indicate that the majority of the GST fusion protein is located in the pellet after centrifugation.
- **Cell disruption may be insufficient:** Cell disruption is apparent by partial clearing of the suspension and can be checked by microscopic examination. Adding 0.1 volume of 10 mg/ml lysozyme in 25 mM Tris-HCl, pH 8.0 followed by 30 min. incubation prior to sonication may improve results. Avoid frothing as this may denature the fusion protein.
- **Fusion protein may be insoluble (inclusion bodies)**

Fermentation

It may be necessary to alter the growth conditions.

- Fusion protein solubility can be dramatically increased by lowering the growth temperature during induction. Experiment with growth temperatures in the range of 20–30 °C (5, 6).
- Alter the level of induction by decreasing the IPTG concentration to <0.1 mM.
- Alter the timing of the induction.
- Induce for a shorter time.

- Induce at a higher cell density for a shorter time.
- Increase aeration. High oxygen transport can help prevent the formation of inclusion bodies.

It may be necessary to combine the above approaches. The exact conditions must be determined empirically for each fusion protein.

Solubilization

If the above techniques do not significantly improve the expression of soluble fusion protein, protein can sometimes be solubilized from inclusion bodies using common denaturants such as 4–6 M guanidine hydrochloride, 4–8 M urea, alkaline pH >9, organic solvents (8,9), 0.5–2% Triton X-100, 0.5–2% N-lauroyl-sarcosine (Sarcosyl) (10,11) or other detergents. Other variables that affect solubilization include time, temperature, ionic strength, the ratio of denaturants to protein and the presence of thiol reagents (8,9). For reviews, see references (6,8,9,12).

Following solubilization by denaturants, proteins must be correctly refolded to regain function. Denaturants can be removed by gel filtration on HiLoad™ 16/60 Superdex™ 75, for example, or by dilution or dialysis to allow refolding of the protein and formation of the correct intramolecular associations. Detergent-solubilized proteins may have retained their native structure.

Critical parameters during refolding include pH, the presence of thiol reagents and the speed of denaturant removal (8,9,13). Once refolded, protein may be purified by ion exchange, gel filtration or affinity chromatography.

Fusion proteins can be purified to some extent while denatured. In some instances when GST fusion proteins formed inclusion bodies, solubilization and binding to Glutathione Sepharose was achieved in the presence of 2-3 M guanidine hydrochloride. Success has

also been achieved using up to 2 % Tween™ 20 for solubilization and binding. Binding to Glutathione Sepharose can also be achieved in the presence of 1% CTAB, 10 mM DTT or 0.03 % SDS. Successful purification in the presence of these agents may depend on the nature of the fusion protein.

- **Fusion protein does not bind to Glutathione Sepharose 4 Fast Flow**

Fusion protein denatured by sonication: Too extensive sonication can denature the fusion protein and prevent it binding to Glutathione Sepharose 4 Fast Flow. Use mild sonication conditions during cell lysis.

Add DTT prior to cell lysis: Adding DTT to a final concentration of 1–10 mM prior to cell lysis may significantly increase binding of some GST fusion proteins to Glutathione Sepharose 4 Fast Flow.

Test the binding of GST from parental pGEX: Prepare a sonicate of cells harboring the parental pGEX plasmid and check binding to the medium. If GST produced from the parental plasmid binds with high affinity, the fusion partner may have altered the conformation of GST, thereby reducing its affinity. Adequate results may be obtained by reducing the temperature used for binding to +4°C, and by limiting column washing.

Equilibrate Glutathione Sepharose 4 Fast Flow before use: Binding of GST fusion proteins to Glutathione Sepharose 4 Fast Flow is not efficient at pH less than 6.5 or greater than 8. Check that the Glutathione Sepharose 4 Fast Flow has been equilibrated with a buffer $6.5 < \text{pH} < 8.0$ (e.g. PBS) before the cell lysate is applied.

Use fresh Glutathione Sepharose 4 Fast Flow: If the Glutathione Sepharose 4 Fast Flow has already been used several times, it may be necessary to use new medium. See also “Cleaning Glutathione Sepharose 4 Fast Flow”.

- **Fusion protein is not eluted efficiently from Glutathione Sepharose 4 Fast Flow**

Increase the time used for elution: Decrease the flow during elution.

Increase the volume of elution buffer: Sometimes, especially after on-column cleavage of fusion protein, a larger volume of buffer may be necessary to elute the fusion protein.

Increase the concentration of glutathione in the elution buffer: The 10 mM recommended in this protocol should be sufficient for most applications, but exceptions exist. Try 50 mM Tris-HCl, 20–40 mM reduced glutathione, pH 8.0 as elution buffer.

Increase the pH of the elution buffer: A low pH may limit elution from Glutathione Sepharose 4 Fast Flow. Increasing the pH of the elution buffer to pH 8–9 may improve elution without requiring an increase in the concentration of glutathione used for elution.

Increase the ionic strength of the elution buffer: Adding 0.1–0.2 M NaCl to the elution buffer may also improve results.

Add a non-ionic detergent to the elution buffer: Non-specific hydrophobic interactions may prevent solubilization and elution of fusion proteins from Glutathione Sepharose 4 Fast Flow. Adding a non-ionic detergent may improve results. Adding 0.1% Triton X-100 or 2% N-octylglucosid can significantly improve elution of some GST fusion proteins.

- **M_r 70 000 protein co-purifies with the GST fusion protein**

Disrupt the association using ATP and Mg^{2+} : The M_r 70 000 protein is probably a protein product of the *E. coli* gene *dnaK*. This protein is involved in protein folding in *E. coli*. It has been reported that this association can be disrupted by incubating the fusion protein in 50 mM Tris-HCl, 2 mM ATP, 10 mM $MgSO_4$, pH 7.4 for 10 min. at +37 °C prior to loading on Glutathione Sepharose 4 Fast Flow.

Alternatively, remove the DnaK protein by passing the fusion protein solution through ATP-agarose or by ion exchange.

- **Multiple bands are observed on gels/Western Blots following elution from Glutathione Sepharose 4 Fast Flow**

Add a protease inhibitor: Multiple bands may be a result of partial degradation of fusion proteins by proteases. Adding 1 mM PMSF to the lysis solution may improve results. A non-toxic, water-soluble alternative to PMSF is 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), commercially available as Pefabloc™ SC from Boehringer Mannheim.

Note: Serine protease inhibitors must be removed prior to cleavage by thrombin or factor Xa. PreScission Protease is not a consensus serine protease and is insensitive to many of the protease inhibitors tested at Amersham Biosciences.

Use a protease-deficient host: Multiple bands may be the result of proteolysis in the host bacteria. If this is the case, the use of a host-deficient strain may be required (*e.g. lon⁻* or *ompT⁻*). *E. coli* BL21 is provided with the pGEX vectors. This strain is *ompT⁻*.

Decrease sonication: Cell disruption is apparent by partial clearing of the suspension and can be checked by microscopic examination. Adding lysozyme (0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris-HCl, pH 8.0) prior to sonication

may improve results. Avoid frothing as this may denature the fusion protein. Over-sonication can also lead to the co-purification of host proteins with the GST fusion protein.

Include an additional purification step: Additional bands may be caused by the co-purification of a variety of proteins known as chaperonins, which are involved in the correct folding of nascent proteins in *E. coli*. These include, but may not be limited to:

DnaK ($M_r \sim 70\ 000$), DnaJ ($M_r \sim 37\ 000$), GrpE ($M_r \sim 40\ 000$), GroEL ($M_r \sim 57\ 000$) and GroES ($M_r \sim 10\ 000$). Several methods for purifying GST fusion proteins from these co-purifying proteins have been described.

Cross-adsorb antibody with *E. coli* proteins: Depending on the source of the anti-GST antibody, it may contain antibodies that react with various *E. coli* proteins that may be present in your fusion protein sample. Cross-adsorb the antibody with an *E. coli* sonicate to remove anti-*E. coli* antibodies from the preparation. Anti-GST antibody from Amersham Biosciences has been cross-adsorbed against *E. coli* proteins and tested for its lack of non-specific background binding in Western Blots.

- **PreScission protease, thrombin or factor Xa cleavage are incomplete**

The PreScission protease, thrombin or factor Xa to fusion protein ratios are incorrect: Check the amount of fusion protein in the digestion. Note that the capacity of Glutathione Sepharose 4 Fast Flow for GST is approximately 10 mg/ml medium. In most purifications, however, the matrix is not saturated with fusion protein.

Ratios: PreScission protease, at least 10 units/mg fusion protein.

Thrombin, at least 10 units/mg fusion protein. One cleavage unit of thrombin from Amersham Biosciences digests 90 % of 100 μ g of a test fusion protein in 16 hours at +22 °C.

Factor Xa, at least 1% (w/w) fusion protein. For some fusion proteins, up to 5% factor Xa can be used. The optimum amount must be determined empirically.

In some cases, a fusion protein concentration of 1 mg/ml has been found to give optimal results. Adding 0.5% (w/v) to the reaction buffer can significantly improve factor Xa cleavage with some fusion proteins. Various concentrations of SDS should be tested to find the optimum concentration.

Increase incubation time and enzyme concentration: For PreScission Protease, thrombin or factor Xa, increase the reaction time to 20 hours or more if the fusion protein is not degraded by extensive incubation. The amount of enzymes can also be increased.

Verify the presence of specific cleavage sites: Check the DNA sequence of the construct. Compare it with a known sequence and verify that the different specific cleavage sites for the enzyme used have not been altered during the cloning of your fusion protein.

- **Ensure that cleavage enzyme inhibitors are absent**

PreScission Protease: Buffer exchange on HiTrap Desalting, a PD-10 column or HiPrep 26/10 Desalting depending on the sample volume, or dialyse the fusion protein against 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5 before cleavage.

Factor Xa: Buffer exchange on HiTrap Desalting, a PD-10 column or HiPrep 26/10 Desalting depending on the sample volume, or dialyse against 50 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl₂, pH 7.5 before cleavage.

Factor Xa is not properly activated: Functional factor Xa requires activation of factor X with Russell's viper venom. Activation conditions are a ratio of Russell's viper venom to factor Xa of 1% in 8 mM Tris-HCl, 70 mM NaCl, 8 mM CaCl₂, pH 8.0. Incubate

at +37 °C for 5 min. Factor Xa from Amersham Biosciences has been preactivated by this procedure.

- **The first amino acid after the factor Xa recognition sequence is Arg or Pro**

Check the sequence of the fusion partner to be sure that the first three nucleotides after the factor Xa recognition sequence do not code for Arg or Pro.

- **Multiple bands are observed on SDS gels following enzyme cleavage**

Determine when the bands appear: Test to be certain that additional bands are not present prior to PreScission Protease, thrombin or factor Xa cleavage. Such bands may be the result of proteolysis in the host bacteria.

- **Fusion partner may contain recognition sequences for PreScission Protease, thrombin or factor Xa:**

Check the sequences. See the GST Gene Fusion System Handbook (3) for details.

References

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Ordering information

Designation	No. Supplied	Code No.
Glutathione Sepharose 4 Fast Flow	25 ml	17-5132-01
	100 ml	17-5132-02
	500 ml*	17-5132-03
GSTrap FF	5×1 ml	17-5130-01
GSTrap FF	2×1 ml	17-5130-02
GSTrap FF	1×5 ml	17-5131-01
GSTPrep FF 16/10	1×20 ml	17-5234-01
HiTrap Desalting	5×5 ml	17-1408-01
PD-10 Disposable column	30	17-0851-01
HiPrep 26/10 Desalting	1×53 ml	17-5087-01
HiLoad 16/60 Superdex 75	1×120 ml	17-1068-01

* Larger quantities are available. Please contact Amersham Biosciences for more information.

Site-Specific Proteases

Designation	No. Supplied	Code No.
PreScission Protease	500 units	27-0843-01
Thrombin	500 units	27-0846-01
Factor Xa	400 units	27-0849-01

Related Products

Designation	No. Supplied	Code No.
Benzamidine Sepharose 4 Fast Flow	25 ml	17-5123-10
GST Detection Module	50 reactions	27-4590-01
GST 96-well Detection Module	5 plates	27-4592-01
pGEX Vectors (GST Gene Fusion)	Different vectors are available	
Anti-GST Antibody	5 ml	27-4577-01
<i>E. coli</i> BL21	1 vial	27-1542-01
Recombinant Protein Handbook	1	18-1105-02
GST Gene Fusion System Handbook	1	18-1157-58

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