GE Healthcare

Instructions 52-2303-00 AK **Affinity media**

Glutathione Sepharose 4B

Glutathione Sepharose™ 4B is designed for the single-step purification of glutathione S-transferases, glutathione-dependent proteins and recombinant derivatives of glutathione S-transferase, including glutathione S-transferase (GST) fusion proteins produced using the pGEX series of expression vectors. GST fusion proteins can be purified directly from bacterial lysates using Glutathione Sepharose 4B. Proteins are eluted under mild, non-denaturing conditions that preserve protein antigenicity and functionality. Cleavage of the desired protein from GST is achieved using a site-specific protease whose recognition sequence is located immediately upstream from the multiple cloning site on the pGEX plasmids.

Glutathione Sepharose 4B is also available in convenient prepacked GSTrap™ 4B 1-ml and 5-ml columns as well as in pre-packed spin columns and 96-well filter plates (see Ordering information).

Table 1 lists the characteristics of Glutathione Sepharose 4B.

Detailed information about the production of GST-tagged proteins can be found in the GST Gene Fusion System Handbook (see Ordering information).

Sepharose

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1. Description

Chromatography medium properties

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

The total binding capacity is > 5 mg GST/ml chromatography medium. The dynamic binding capacity will vary depending on several factors such as target protein, flow rate etc.

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

Table 1: Characteristics of Glutathione Sepharose 4B

Ligand:	glutathione and 10-carbon linker arm
Ligand concentration:	200–400 µmol glutathione/g washed and dried medium
Binding capacity[†]:	> 5 mg glutathione-S-transferase/ml medium
Matrix:	4% agarose
Average particle size:	90 µm
Recommended flow rate*:	< 75 cm/h
Chemical stability:	Stable to all commonly used aqueous buffers. Exposure to 0.1 M NaOH, 70% ethanol, or 6 M guanidine hydrochloride for 2 h at room temperature or to 1% (w/v) SDS for 14 d causes no significant loss of activity.
pH stability:	4–13
Storage temperature:	4–30°C
Storage buffer:	20% ethanol

[†] The binding of GST-tagged proteins depends on size, conformation and concentration of the protein in the sample loaded. Binding of GST to glutathione is also flow dependent, and lower flow rates often increase the binding capacity. This is important during sample loading. Protein characteristics, pH and temperature may affect the binding capacity.

[*] H_2O at room temperature.

2. 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

Note: 1–10 mM DTT can be included in the binding and elution buffer to increase purity. However, this may result in lower yield of target protein.

Sample preparation

The sample should be centrifuged and/or filtered through a 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.

3. Batch purification

Preparation of Glutathione Sepharose 4B

1. Determine the volume of Glutathione Sepharose 4B required for your purification.

Note: Glutathione Sepharose 4B is delivered in 20% ethanol. Prepare a 50% slurry for your purification.

2. Gently shake the bottle of Glutathione Sepharose 4B 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 medium by centrifugation at 500 × g for 5 min. Carefully decant the supernatant.
5. Wash the Glutathione Sepharose 4B by adding 5 ml Binding buffer to each 1 ml slurry. Invert to mix.
6. Sediment the medium by centrifugation at 500 × g for 5 min. Carefully decant the supernatant.
7. Repeat steps 5 and 6 one more time.

Batch purification

1. Add the cell lysate to the prepared Glutathione Sepharose 4B and incubate for at least 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 chromatography medium by centrifugation at 500 × 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 4B by adding 5 ml Binding buffer to each 1 ml slurry. Invert to mix.
5. Sediment the medium by centrifugation at 500 × 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 Elution buffer per 1 ml slurry of Glutathione Sepharose 4B. Incubate at room temperature for 5–10 min. using gentle agitation such as end-over-end rotation.
8. Sediment the medium by centrifugation at 500 × 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 that sufficient time is allowed to obtain maixmum binding capacity. The binding efficiency may differ significantly between different GST-tagged proteins.
- Volumes and times used for elution may vary among proteins. Additional elutions with higher concentrations of glutathione may be required. Flow-through, wash and eluted material from the medium should be monitored for GST-tagged proteins using SDS-PAGE in combination with Western Blot if necessary.
- The GST Detection Module (see Ordering information) can be used to optimize conditions for elution or to trace steps in the purification of a GST-tagged protein. The Module is designed to identify GST-tagged proteins using either a biochemical or an immunological assay.
- The concentration of GST-tagged proteins can be estimated by measuring the absorbance at 280 nm. The GST-tag can be approximated using the conversion; A_{280} ~ 1 corresponds to ~ 0.5 mg/ml.
- The concentration of GST-tagged 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 4B depends on the nature of the sample and should only be performed with identical samples to prevent cross-contamination.

4. Gravity flow column purification

Equilibration

For each purification, remove the top cap from a disposable column, and place the column upright in an appropriate rack/clamp.

1. Determine the medium volume of Glutathione Sepharose 4B required for your purification.

Note: Glutathione Sepharose 4B is delivered in 20% ethanol. Prepare a 50% slurry for your purification.

2. Gently shake the bottle of Glutathione Sepharose 4B to resuspend the slurry.

3. Use a pipette or measuring cylinder to remove sufficient slurry for use and transfer to the column. Tap the column to dislodge any trapped air bubbles in the chromatography medium bed. Allow to settle.

4. Remove the bottom cap and save for later use. Allow the column to drain.

Note: Gentle pressure with a gloved thumb over the top of the column may be required to start the flow of liquid.

5. Wash the Glutathione Sepharose 4B with at least 5 column volumes of Binding buffer. Allow the column to drain. Replace the bottom cap.

Note: Glutathione Sepharose 4B must be washed thoroughly with binding buffer to remove the 20% ethanol storage solution. Residual ethanol may interfere with subsequent procedures.

Binding

6. Use a pipette to apply the clarified bacterial sonicate to the medium in the drained and equilibrated Glutathione Sepharose 4B column

7. Remove the end cap and allow the sonicate to flow through.

Note: Collect the flow-through and save it for analysis by SDS-PAGE or CDNB assay using GST Detection Module (see Ordering information) to measure the efficiency of binding to the medium.

8. Wash the medium with 5 column volumes of binding buffer. Allow the column to drain.

9. Repeat twice more for a total of three washes.

Note: Protein bound to the medium may be eluted directly at this stage using Elution buffer. If desired, GST tagged proteins may be cleaved while still bound to the medium with PreScission™ Protease, thrombin or Factor Xa to liberate the protein of interest from the GST moiety. Detailed procedures are provided in Section 8.

10. Once the column with bound protein has been washed and drained, replace the bottom cap.

Elution

11. Elute the bound protein by adding 0.5 ml of Elution buffer per ml bed volume of Glutathione Sepharose 4B. Incubate the column at room temperature (22–25°C) for 10 min to elute the GST-tagged protein.

12. Remove the bottom cap, and collect the eluate. This contains the GST-tagged protein.

13. Repeat steps 11 and 12 twice for a total of three elutions.

14. Check the three eluates separately for purified protein and pool those eluates containing protein.

Note: Following the elution steps, a significant amount of tagged protein may remain bound to the medium. Volumes and times used for elution may vary among proteins. Additional elutions may be required. Eluates should be monitored for GST tagged protein by SDS-PAGE or by CDNB assay using GST Detection Module (see Ordering information)

Note: The yield of tagged protein can be estimated by measuring the absorbance at 280 nm. For the GST affinity tag, A_{280} ~ 1 corresponds to ~ 0.5 mg/ml. The yield of protein may also be determined by standard chromogenic methods (e.g. Lowry, BCA, Bradford, etc.). If a Lowry or BCA type method is to be used, glutathione has to be removed by for example buffer exchange using desalting columns such as HiTrap Desalting or PD-10 Disposable columns depending on sample volume. The Bradford method can be performed in the presence of glutathione.

5. Column purification

Columns

GE Healthcare's offers many different kinds of columns to use for column purification (see the "Products for life sciences catalogue" or www.gelifesciences.com/protein-purification for details).

Suggested columns are listed below:

Tricorn™ 10/100 (10 mm i.d.) for bed volumes up to 8.5 ml at bed heights up to 10.8 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 4B 1 ml and GSTrap 4B 5 ml columns are also available. (see Ordering information).

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 Binding buffer. Make sure that no air has been trapped under the column net. Close the column outlet leaving 1–2 cm of Binding buffer remaining in the column.
3. Gently shake the bottle of Glutathione Sepharose 4B to resuspend the slurry.
4. Estimate the amount of slurry needed.
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 Binding buffer, 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, Sepharose 4B 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. 2.5 ml/min, 75 cm/h (XK 16/20 column) or 1 ml/min, 75 cm/h (Tricorn 10/100 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 toppiece from the column and carefully fill the rest of the column with Binding buffer 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 Binding buffer.
2. Apply the centrifuged and/or filtered sample.
3. Wash the column with 5–10 column volumes of Binding buffer 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 Elution buffer.

Note:

- One of the most important parameters affecting the binding of GST–tagged proteins or other glutathione binding proteins to Glutathione Sepharose 4B 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. Protein characteristics, pH and temperature are other factors that may affect the binding capacity.
- Volumes and times used for elution may vary among tagged 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–tagged proteins using SDS-PAGE in combination with Western Blot if necessary.
- The GST Detection Module (see Ordering information) can be used to optimize conditions for elution or to trace steps in the purification of a GST–tagged protein. The Module is designed to identify GST–tagged proteins using either a biochemical or an immunological assay.
- The concentration of GST–tagged proteins can be estimated by measuring the absorbance at 280 nm. The GST–tag can be approximated using the conversion; A_{280} ~ 1 corresponds to ~ 0.5 mg/ml.
- The concentration of GST–tagged proteins 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 4B depends on the nature of the sample and should only be performed with identical samples to prevent cross-contamination.

6. Cleaning Glutathione Sepharose 4B

If the chromatography medium appears to be losing binding capacity, it may be due to an accumulation of precipitate, denatured or nonspecifically 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 volumns of 70% ethanol or 2 column volumes of 1% Triton™ X-100, immediately followed by 5 column volumes of PBS, pH 7.3.

7. Storage

Store Glutathone Sepharose 4B at +4–30°C in 20% ethanol.

8. Cleavage of GST-tagged 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, tagged 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 4B 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 protein.

Optimal conditions for each tagged protein 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-tagged protein bound to the column/bulk medium

Assumption: 8 mg GST-tagged proteins bound/ml chromatography medium

1. Follow steps 1–6 under "Batch purification" or steps 1–3 under "Column purification".
2. Wash the tagged protein bound Glutathione Sepharose 4B with 10 bed volumes of PreScission cleavage buffer.
3. Prepare the PreScission Protease mix: For each ml of Glutathione Sepharose 4B bed volume, prepare a mixture of 80 µl (160 units) of PreScission Protease and 920 µl of PreScission cleavage buffer at +4°C. (8 mg tagged protein bound/ml medium).
4. Load the PreScission Protease mixture onto the column. Seal the column.

   If batch format is used, add PreScission Protease mixture to the Glutathione Sepharose 4B pellet. Gently shake or rotate the suspension.
5. Incubate at +4°C for 4 hours.
6. 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 × g for 5 minutes to pellet the Glutathione Sepharose 4B and carefully transfer the eluate to a tube. The eluate will contain the protein of interest, while the GST portion of the tagged protein and the PreScission Protease will remain bound to the Glutathione Sepharose 4B.

PreScission Protease cleavage of eluted GST-tagged protein

Assumption: 8 mg GST fusion protein bound/ml chromatography medium

1. 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 µl (2 U) of PreScission Protease for each 100 µg of tagged protein in the eluate. If the amount of tagged protein in the eluate has not been determined, add 80 µl (160 units) of PreScission Protease for each ml of Glutathione Sepharose 4B bed volume. (8 mg tagged protein bound/ml medium).
3. Incubate at +4°C for 4 hours.
4. Once digestion is complete, apply the sample to washed and equilibrated Glutathione Sepharose 4B to remove the GST moiety of the tagged 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 × 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 µl PBS, pH 7.3 (1 U/µl).
2. Swirl gently to dissolve.
3. Freeze as 80 µl aliqouts and keep at -80°C.

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

Assumption: 8 mg GST fusion protein bound/ml chromatography medium

1. Follow steps 1–6 under "Batch purification" or steps 1–3 under "Column purification".
2. Wash the tagged protein bound Glutathione Sepharose 4B with 10 bed volumes of Thrombin cleavage buffer.
3. Prepare the thrombin mix: For each ml of Glutathione Sepharose 4B bed volume, prepare a mixture of 80 µl (80 units) of thrombin and 920 µl of PBS, pH 7.3 (8 mg tagged protein bound/ml medium).
4. Load the thrombin mix onto the column. Seal the column.

   If batch format is used, add the thrombin mixture to the Glutathione Sepharose 4B pellet. Gently shake or rotate the suspension.
5. Incubate at room temperature (+22–25°C) for 2–16 hours.
6. 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 tagged protein and analyse it. If batch format is used, centrifuge the suspension at 500 × g for 5 minutes to pellet the Glutathione Sepharose 4B and carefully transfer the eluate to a tube. The eluate will contain the protein of interest and thrombin, while the GST portion of the tagged protein will remain bound to the Glutathione Sepharose 4B.

Thrombin cleavage of eluted GST-tagged protein

Assumption: 8 mg GST fusion protein bound/ml chromatography medium

1. 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 Thrombin cleavage buffer.
2. Add 10 µl (10 units) of thrombin solution for each mg of tagged protein in the eluate. If the amount of tagged protein in the eluate has not been determined, add 80 µl (80 U) of thrombin solution for each ml of Glutathione Sepharose 4B bed volume from which the tagged protein was eluted. (8 mg fusion protein bound/ml medium).
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 4B.
5. Incubate for 20–30 min. at room temperature.
6. Sediment the medium by centrifugation at 500 × g for 5 min. The supernatant will contain the protein of interest and thrombin, while the GST portion of the tagged protein will remain bound to the Glutathione Sepharose 4B.

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 aliqouts and keep at -80°C.

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

Assumption: 8 mg GST fusion protein bound/ml chromatography medium

1. Follow steps 1–6 under “Batch purification” or steps 1–3 under “Column purification”.
2. Wash the tagged protein bound Glutathione Sepharose 4B with 10 bed volumes of factor Xa cleavage buffer.
3. Prepare the factor Xa mix: For each ml of Glutathione Sepharose 4B bed volume, prepare a mixture of 80 µl (80 units) of factor Xa and 920 µl of factor Xa cleavage buffer. (8 mg tagged protein bound/ml medium).

4. Load the factor Xa mixture onto the column. Seal the column. If batch format is used, add factor Xa mixture to the Glutathione Sepharose 4B pellet. Gently shake or rotate the suspension.
5. Incubate at room temperature (+22–25°C) for 2–16 hours.
6. 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 tagged protein and analyse it. If batch format is used, centrifuge the suspension at 500 × g for 5 minutes to pellet the Glutathione Sepharose 4B 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 tagged protein will remain bound to the Glutathione Sepharose 4B.

Factor Xa cleavage of eluted GST-tagged protein

Assumption: 8 mg GST-tagged protein bound/ml chromatography medium

1. 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.
2. Add 10 µl (10 units) of factor Xa solution for each mg tagged protein in the eluate. If the amount of tagged protein in the eluate has not been determined, add 80 µl (80 units) of factor Xa solution for each ml of Glutathione Sepharose 4B bed volume from which the tagged protein was eluted. (8 mg tagged protein bound/ml medium).
3. Incubate at room temperature (+22–25°C) for 2–16 hours.
4. Once digestion is complete, apply the sample to washed and equilibrated Glutathione Sepharose 4B to remove the GST moiety of the tagged protein.
5. Incubate for 20–30 min. at room temperature.
6. Sediment the medium by centrifugation at 500 × g for 5 min. The protein of interest will be found in the supernatant together with factor Xa.

9. Trouble shooting guide

Consult the GST Gene Fusion System Handbook for more detailed information and pGEX instructions regarding troubleshooting recommendations for expression, fermentation and solubilization.

GST-tagged protein does not bind to Glutathione Sepharose 4B

- **GST-tagged protein denatured by sonication:** Too extensive sonication can denature the tagged protein and prevent it binding to Glutathione Sepharose 4B. Use mild sonication conditions during cell lysis. Conditions for sonication must be empirically determined.
- **Add DTT prior to cell lysis and to buffers:** Adding DTT to a final concentration of 1–10 mM may significantly increase binding of some GST-tagged proteins to Glutathione Sepharose 4B.
- **Test the binding of GST from parental pGEX:** Prepare a sonicate of cells harboring the parental pGEX plasmid and check binding to the matrix. If GST produced from the parental plasmid binds with high affinity, the fusion protein 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 4B before use:** Binding of GST-tagged proteins to Glutathione Sepharose 4B is not efficient at pH less than 6.5 or greater than 8. Check that the Glutathione Sepharose 4B has been equilibrated with a buffer 6.5 to 8.0 (e.g. PBS) before the clarified cell lysate is applied.
- **Use fresh Glutathione Sepharose 4B:** If the Glutathione Sepharose 4B has already been used several times, it may be necessary to use fresh Glutathione Sepharose 4B. See also ”Cleaning Glutathione Sepharose 4B”.
- **Decrease flow rate during sample load.** See note p. 12.

GST-tagged protein is not eluted efficiently from Glutathione Sepharose 4B

- **Increase the time used for elution:** Decrease the flow during elution.
- **Increase the volume of elution buffer:** Sometimes, especially after on-column cleavage of tagged protein, a larger volume of buffer may be necessary to elute the tagged 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 4B. 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.
- **Use fresh elution buffer** (reduced glutathione).
- **Add a non-ionic detergent to the elution buffer:** Non-specific hydrophobic interactions may prevent solubilization and elution of tagged proteins from Glutathione Sepharose 4B. Adding a non-ionic detergent may improve results. Adding 0.1% Triton X-100 or 2% N-octylglucoside can significantly improve elution of some GST-tagged proteins.

Multiple bands are observed after electrophoresis/ Western Blotting analysis of eluted target protein.

- **M_r 70 000 protein co-purifies with the GST-tagged protein:** 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 tagged 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 4B.

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

- **Add a protease inhibitor:** Multiple bands may be a result of partial degradation of tagged 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 GE Healthcare.

- **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-tagged 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 ~ 70 000), DnaJ (M_r ~ 37 000), GrpE (M_r ~ 40 000), GroEL (M_r ~ 57 000) and GroES (Mr ~ 10 000). Several methods for purifying GST-tagged 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 tagged protein sample. Cross-adsorb the antibody with an *E. coli* sonicate to remove anti-*E. coli* antibodies from the preparation. Anti-GST antibody from GE Healthcare has been cross-adsorbed against *E. coli* proteins and tested for its lack of non-specific background binding in Western Blots.

Incomplete cleavage of GST-tagged proteins

- **The PreScission Protease, thrombin or factor Xa to fusion protein ratios are incorrect:** Check the amount of tagged protein in the digestion. Note that the capacity of Glutathione Sepharose 4B for GST is at least 5 mg/ml chromatography medium. In most purifications, however, the medium is not saturated with tagged protein.

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

Thrombin, at least 10 units/mg tagged protein. One cleavage unit of thrombin from GE Healthcare digests ≥ 90% of 100 µg of a test tagged protein in 16 hours at +22°C.

Factor Xa, at least 1% (w/w) tagged protein. For some tagged proteins, up to 5% factor Xa can be used. The optimum amount must be determined empirically.
In some cases, a tagged protein concentration of 1 mg/ml has been found to give optimal results. Adding ≤ 0.5% SDS (w/v) to the reaction buffer can significantly improve factor Xa cleavage with some tagged 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 tagged 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 tagged protein.

Ensure that cleavage enzyme inhibitors are absent:

- **PreScission Protease:** Buffer exchange or dialyse the tagged 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 $CaCl_2$, pH 7.5.
- **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 $CaCl_2$, pH 8.0. Incubate at +37°C for 5 min. Factor Xa from GE Healthcare 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 tagged 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 after electrophoresis analysis of cleaved target protein:

- **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.
- **Tagged partner may contain recognition sequences for PreScission Protease, thrombin or factor Xa:** Check the sequences. See the GST Gene Fusion System Handbook (see Ordering information) for details.

10. Ordering information

Product	Quantity	Code No.
Glutathione Sepharose 4B	10 ml	17-0756-01
	100 ml	17-0756-05
	300 ml	17-0756-04

Related Products

Product	Quantity	Code No.
GSTrap 4B	5 × 1 ml	28-4017-45
GSTrap 4B	100 × 1 ml*	28-4017-46
GSTrap 4B	1 × 5 ml	28-4017-47
GSTrap 4B	5 × 5 ml	28-4017-48
GSTrap 4B	100 × 5 ml*	28-4017-49
GST SpinTrap Purificaton Module	50 × 50 µl	27-4570-03
GST MultiTrap 4B	4 × 96-well plates	28-4055-00
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
GST Detection Module	50 reactions	27-4590-01
GST 96-well Detection module	5 plates	27-4592-01
Anti-GST Antibody	0.5 ml	27-4577-01

Site-Specific Proteases

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

* Available by specific customer order

Literature

Product	Quantity	Code No.
GST Gene Fusion System Handbook	1	18-1157-58
Recombinant Protein Purification Handbook	1	18-1142-05
Glutathione Sepharose- Total solutions for preparation of GST-tagged proteins Selection Guide	1	28-9168-33
Pure simplicity for tagged proteins Brochure	1	28-9353-64

www.gelifesciences.com/protein-purification
www.gelifesciences.com

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

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