instructions

product code:

27-4574-01

Glutathione Sepharose 4B

Warning
For research use only.
Not recommended or intended for the diagnosis of disease in humans or animals.
Do not use internally or externally in humans or animals.

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GE imagination at work

Handling

Storage

Store at 4 °C. Do not freeze.

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Quality control

Glutathione Sepharose 4B (27-4574-01) is tested for its ability to bind > 8 mg recombinant GST per ml of drained gel.

Materials not supplied

Reagents

- **1× PBS**—140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2 HPO_4$, 1.8 mM KH_2PO_4 (pH 7.3).
- **Glutathione elution buffer**—0.154 g of reduced glutathione dissolved in 50 ml of 50 mM Tris-HCl, pH 8.0.
- **6× SDS loading buffer**—0.35 M Tris-HCl, pH 6.8, 10.28% (w/v) SDS, 36% (v/v) glycerol, 0.6 M dithiothreitol (or 5% β-mercaptoethanol), 0.012% (w/v) bromophenol blue. Store in 0.5 ml aliquots at -70 °C.

Components of the kit

The following component is included in the kit:

Glutathione Sepharose™ 4B	100 ml supplied in an aqueous solution containing 20% ethanol

World Wide Web address

http://www.gehealthcare.com/lifesciences

Visit the GE Healthcare home page for regularly updated product information.

Materials not supplied *(continued)*

- **Thrombin**—Dissolve 500 cleavage units of lyophilized bovine thrombin (> 7500 units/mg protein) in 0.5 ml of 1× PBS pre-chilled to 4 °C. Swirl gently to dissolve the thrombin. One cleavage unit cleaves 90% of 100 µg of a test fusion protein in 16 h at 22 °C in 10 mM glutathione, 50 mM Tris-HCl, pH 8.0. In order to preserve activity, store the thrombin solution in small aliquots at -70 °C. (The approximate molecular weight of bovine thrombin is 37 kDa.)

Safety warnings and precautions

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practice should handle this product. Suitable protective clothing such as laboratory overalls, safety glasses, and gloves should be worn. Care should be taken to avoid contact with skin or eyes; if contact should occur, wash immediately with water (see Material Safety Data Sheet for specific recommendations).

Warning: This kit contains formamide. Gel reagents may contain acrylamide, a neurotoxin and suspected carcinogen. An optional step in the protocol requires the use of ethanol, a flammable liquid. Please follow the manufacturer's Material Safety Data Sheet regarding safe handling and use of these materials.

Materials not supplied *(continued)*

- **2xYTA medium**—
  Tryptone 16 g/l
  Yeast extract 10 g/l
  NaCl 5 g/l

  Dissolve above ingredients in 900 ml of distilled H_2O. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 liter. Sterilize by autoclaving for 20 min. After the medium cools, aseptically add 1 ml of a 100 mg/ml ampicillin stock solution (final concentration 100 µg/ml). To prepare as a solid medium, add 12–15 g of agar.
- **20% Triton X-100**
- **20% Triton X-100**—v/v in 1× PBS.

Equipment

- **Reagents, equipment and gels for SDS-PAGE.**

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Introduction

Glutathione Sepharose 4B is designed for the rapid, 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 (27-4574-01) is tested functionally for its ability to bind recombinant GST.

Protocols

Introduction

The following protocol can be conveniently scaled to purify as little as 80 µg or as much as 400 mg of GST fusion protein using Glutathione Sepharose 4B (27-4574-01).

Yield of fusion protein is highly variable and is affected by the nature of the fusion protein, the host cell, and the culture conditions used. Fusion protein yields can range from 1–3 mg/l up to 10 mg/l (2). Table 1 can be used to approximate culture volumes based on an average yield of 2.5 mg/l.

Note: The reagent volume requirements are based on a binding capacity of 8 mg of recombinant GST per ml of drained gel, which is the minimum binding capacity of Glutathione Sepharose 4B (27-4574-01). Refer to the label on the bottle for the exact binding capacity.

Table 1. Reagent volume requirements for different protein yields.

Component	80 mg	16 mg	1.6 mg	80 µg
Culture volume	20 liters	4 liters	400 ml	20 ml
Volume sonicate	1 000 ml	200 ml	20 ml	1 ml
Glutathione Sepharose bed volume*	10 ml	2 ml	200 µl	10 µl
1× PBS**	100 ml	20 ml	2 ml	100 µl
Glutathione elution buffer	10 ml	2 ml	200 µl	10 µl

* To obtain the desired bed volume, use twice the volume of 50% Glutathione Sepharose slurry prepared in Protocol 1 (i.e. 1 ml of 50% Glutathione Sepharose slurry will give a bed volume of 0.5 ml).

**This volume is "per wash." Three washes are required.

1 Preparation of a 50% Glutathione Sepharose 4B slurry

Glutathione Sepharose 4B may be used for batch or column purification of glutathione S-transferases or recombinant GST fusion proteins produced using the pGEX series of expression vectors. Once the gel is equilibrated, it can be transferred to a suitable chromatography column, if desired [Empty Disposable Columns PD-10, GE Healthcare code number 17-0435-01, have a total volume capacity (gel and sample) of ~13 ml]. See Protocol 3 for details regarding column purification of Glutathione S-transferase proteins.

1.1 Referring to Table 1, determine the bed volume of Glutathione Sepharose 4B required for your application. Glutathione Sepharose 4B as supplied is approximately a 75% slurry. The following steps produce a 50% slurry.

1.2 Gently shake the bottle of Glutathione Sepharose 4B to resuspend the gel.

1.3 Use a pipet to remove sufficient slurry for use and transfer to an appropriate container/tube. Dispense 1.33 ml of the original Glutathione Sepharose 4B slurry per ml of bed volume required. One ml of drained gel is capable of binding at least 8 mg of recombinant GST. Refer to the label on the bottle for the exact binding capacity.

1.4 Sediment the gel by centrifugation at 500 × *g* for 5 min. Carefully decant the supernatant.

1.5 Wash the Glutathione Sepharose 4B with 10 ml of cold (4 °C) 1× PBS (see "Materials not supplied") per 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed. Invert to mix.

Note: Glutathione Sepharose 4B must be thoroughly washed with 1× PBS to remove the 20% ethanol storage solution. Residual ethanol may interfere with subsequent procedures.

1.6 Sediment the gel by centrifugation at 500 × *g* for 5 min. Decant the supernatant.

1.7 For each 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed, add 1 ml of 1× PBS. This produces a 50% slurry. Mix well prior to subsequent pipetting steps.

Note: Glutathione Sepharose 4B equilibrated with 1× PBS may be stored at 4 °C for up to 1 month.

2 Batch purification of glutathione S-transferase proteins

Binding

2.1 Add 2 ml of the 50% slurry of Glutathione Sepharose 4B equilibrated with 1× PBS (Protocol 1) to each 100 ml of bacterial sonicate (Appendix 3).

2.2 Incubate with gentle agitation at room temperature for 30 min.

Note: At this stage, the gel with adsorbed fusion protein may be packed into a disposable column to facilitate washing and elution steps. If a disposable column is used, refer to Protocol 3 for washing and elution instructions.

2.3 Centrifuge the suspension at 500 × *g* for 5 min to sediment the gel. Remove the supernatant.

Note: The majority of the supernatant can be discarded. However, retain a sample for analysis by SDS-PAGE or CDNB assay (see GST Detection Module, 27-4590-01) to measure the efficiency of binding to the gel.

2.4 Wash the Glutathione Sepharose 4B pellet with 10 bed volumes* of 1× PBS.

2.5 Centrifuge the suspension at 500 × *g* for 5 min to sediment the gel. Discard the wash.

2.6 Repeat the wash twice for a total of three washes.

Note: Protein bound to the gel may be eluted directly at this stage using Glutathione elution buffer (see "Materials not supplied"). If desired, GST fusion proteins may be cleaved while still bound to the gel with Thrombin, Factor Xa or PreScission™ Protease to liberate the protein of interest from the GST moiety. If the pGEX

*Bed volume is equal to 0.5× the volume of the 50% Glutathione Sepharose slurry used.

construct contains the recognition sequence for Thrombin, refer to Appendix 4.

Elution

2.7 To the sedimented gel, add 1.0 ml of glutathione elution buffer (see "Materials not supplied") per ml of bed volume* of Glutathione Sepharose 4B.

2.8 Mix gently to resuspend the gel. Incubate at room temperature (22–25 °C) for 10 min to liberate the fusion protein from the gel.

2.9 Centrifuge at 500 × *g* for 5 min to sediment the gel, and remove the supernatant. Transfer the supernatant to a fresh centrifuge tube.

2.10 Repeat elution and centrifugation steps twice more. Pool the three eluates.

Note: Following elution steps, a significant amount of protein may remain bound to the gel. Volumes and times used for elution may vary among fusion proteins. Additional elutions may be required. Eluates should be monitored for GST protein by SDS-PAGE or by CDNB assay (GST Detection Module, 27-4590-01).

Note: The yield of fusion protein can be estimated by measuring the absorbance at 280 nm. For the GST affinity tag, 1 $A_{280} \cong$ 0.5 mg/ml.

Note: 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 will be used, the sample must first be dialyzed against 2 000 volumes of 1× PBS to remove glutathione, which can interfere with protein measurement. The Bradford method can be performed in the presence of glutathione.

*Bed volume is equal to 0.5× the volume of the 50% Glutathione Sepharose slurry used.

3 Column purification of glutathione S-transferase proteins

Equilibration

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

3.2 Referring to Table 1, determine the bed volume of Glutathione Sepharose 4B required for your application. Glutathione Sepharose 4B as supplied is approximately a 75% slurry. The following steps produce a 50% slurry.

3.3 Gently shake the bottle of Glutathione Sepharose 4B to resuspend the gel.

3.4 Use a pipet to dispense 1.33 ml of the original Glutathione Sepharose 4B slurry per ml of bed volume required.

3.5 Tap the column to dislodge any trapped air bubbles in the gel bed. Allow to settle.

3.6 Remove the bottom cap and save for later use (For columns that do not have a reusable bottom cap, snap off the bottom closure. Parafilm™ may be used in place of the bottom cap in subsequent procedures). 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.

3.7 Wash the Glutathione Sepharose 4B with 10 ml of cold (4 °C) 1×PBS per 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed. Allow the column to drain. Replace the bottom cap or wrap with Parafilm to prevent the gel from drying.

Note: Glutathione Sepharose 4B must be washed thoroughly with 1× PBS to remove the 20% ethanol storage solution. Residual ethanol may interfere with subsequent procedures.

Note: Glutathione Sepharose 4B equilibrated with 1× PBS may be stored at 4 °C for up to 1 month.

Binding

3.8 Use a pipet to apply the bacterial sonicate (Appendix 3) to the gel in a drained and equilibrated Glutathione Sepharose 4B column.

Note: If needed, the sonicate may be clarified by filtration through a 0.45 µm filter before applying it to the column.

3.9 Remove the end cap or Parafilm, and allow the sonicate to flow through.

Note: The majority of the flow-through can be discarded. However, a sample should be retained for analysis by SDS-PAGE or CDNB assay (see GST Detection Module, 27-4590-01) to measure the efficiency of binding to the gel.

3.10 Wash the gel with 10 bed volumes* of 1× PBS. Allow the column to drain. Repeat twice more for a total of three washes.

Note: Protein bound to the gel may be eluted directly at this stage using Glutathione elution buffer (see "Materials not supplied"). If desired, GST fusion proteins may be cleaved while still bound to the gel with PreScission Protease, thrombin or Factor Xa to liberate the protein of interest from the GST moiety. If the pGEX construct contains the recognition sequence for Thrombin refer to Appendix 4. Detailed procedures are provided in the GST Gene Fusion System Handbook, GE Healthcare document 18-1157-58.

3.11 Once the column with bound protein has been washed and drained, replace the bottom cap or wrap with Parafilm.

*Bed volume is that determined at step 3.2 of this protocol.

Elution

3.12 Elute the fusion protein by adding 1 ml of Glutathione elution buffer (see “Materials not supplied”) per ml bed volume*. Incubate the column at room temperature (22–25 °C) for 10 min to elute the fusion protein.

3.13 Remove the bottom cap or Parafilm, and collect the eluate. This contains the fusion protein.

3.14 Repeat the elution and collection steps twice more. Pool the three eluates.

Note: Following the elution steps, a significant amount of fusion protein may remain bound to the gel. Volumes and times used for elution may vary among fusion proteins. Additional elutions may be required. Eluates should be monitored for GST fusion protein by SDS-PAGE or by CDNB assay (GST Detection Module, 27-4590-01).

Note: The yield of fusion protein can be estimated by measuring the absorbance at 280 nm. For the GST affinity tag, 1 $A_{280} \cong$ 0.5 mg/ml.

Note: 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, the sample must first be dialyzed against 2 000 volumes of 1× PBS to remove glutathione, which can interfere with protein measurement. The Bradford method can be performed in the presence of glutathione.

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*Bed volume is that determined at step 3.2 of this protocol.

Appendix 1:
Characteristics of Glutathione Sepharose 4B

Ligand density: 7–15 µmol glutathione per ml gel.

Capacity: > 8 mg recombinant GST per ml drained gel.

Bead form: spherical, 45–165 µm wet bead diameter.

Spacer arm: 12 atoms (10 carbons).

Chemical stability: No significant loss of capacity is detected when Glutathione Sepharose 4B is exposed to 0.1 M citrate (pH 4.0), 0.1 M NaOH, 70% ethanol or 6 M guanidine hydrochloride for 2 hours at room temperature. No significant loss of binding capacity is observed after exposure to 1% SDS for 14 days.

Max. operating pressure: 7.845 kPa, 0.0785 bar.

Max. volumetric flow rate: 2.50 ml/min (run) in an HR 16/10 column (5 cm bed height) in aqueous buffer at room temperature.

Max. linear flow rate: 75 cm/h (running).

Appendix 2:
Regeneration of Glutathione Sepharose 4B

1. Wash the gel with two bed volumes of 0.1 M Tris HCl + 0.5 M NaCl, pH 8.5.
2. Wash the gel with two bed volumes of 0.1 M sodium acetate + 0.5 M NaCl, pH 4.5.
3. Repeat the above steps three to four times to give a total of four to five wash cycles of alternate buffers.
4. Re-equilibrate with 3–5 bed volumes of 1× PBS.

If the gel appears to be losing binding capacity, it may be due to an accumulation of precipitated, denatured or nonspecifically bound proteins.

To remove precipitated or denatured substances, wash the gel with 2 bed volumes of 6 M guanidine hydrochloride, immediately followed by a wash with 5 bed volumes of 1× PBS.

To remove hydrophobically bound substances, wash the gel with 3–4 bed volumes of 70% ethanol or with 2 bed volumes of a non-ionic detergent (conc. 0.1%), immediately followed by a wash with 5 bed volumes of 1× PBS.

For long-term storage (> 1 month) the following additional washes are recommended:

1. Wash the gel twice with 10 bed volumes of 1× PBS.
2. Repeat washes using 20% ethanol.
3. Store at +4 °C.
4. Re-equilibrate the gel with 1× PBS before reuse.

Appendix 3:
Preparation of large-scale bacterial sonicates

pGEX vectors contain the *lacI*^{q} gene, so there are no specific host requirements for propagation of the plasmids or for expression of fusion proteins. However, *E. coli* BL21 [F^-, *ompT*, *hsdS* (r_B^- , m_B^-), *gal*] (3, 4) is provided with the pGEX vectors and this strain is recommended for expression of GST fusion proteins. BL21 does not transform well and an alternate strain (e.g. JM105) is recommended for maintenance of the plasmid.

Before performing a large-scale purification, check protein expression in the culture or perform a small pilot experiment to establish optimal conditions for expression. Fusion protein expression can be monitored during growth and induction by SDS-PAGE or by measuring GST activity with the GST Detection Module (27-4590-01).

Small samples should be retained at key steps in the procedure for analysis of the purification method.

1. Use a single colony of *E. coli* cells containing a recombinant pGEX plasmid to inoculate 2–100 ml of 2xYTA medium (see "Materials not supplied").
2. Incubate for 12–15 hours at 37 °C with vigorous shaking.
3. Dilute the culture 1:100 into fresh prewarmed 2xYTA medium, distribute into appropriate flasks and grow at 20–30 °C with shaking until the A_{600} reaches 0.5–2.

Note: To ensure adequate aeration, fill flasks to only 20–25% capacity (e.g. 20 ml in a 100 ml flask).

Note: Optimize the growth temperature and A_{600} for induction; these will vary with each fusion protein.

4. Add 100 mM IPTG to a final concentration of 0.1 mM and continue incubation for an additional 2–6 hours.
5. Transfer the culture to appropriate centrifuge bottles or tubes and centrifuge at 7 700 × *g* (e.g. 8 000 rpm in a Beckman JA20 rotor) for 10 min at 4 °C to sediment the cells.
6. Discard the supernatant and drain the pellet. Place the pellet on ice.
7. Using a pipet, completely suspend the cell pellet in 50 µl of ice-cold 1× PBS per ml of culture.
8. Disrupt suspended cells using a sonicator equipped appropriately for the suspended volume. Sonicate on ice in short bursts. Save an aliquot of the sonicate for analysis by SDS-PAGE.

Note: Cell disruption is indicated by partial clearing of the suspension, or may be checked by microscopic examination. Avoid frothing, which may denature the fusion protein. Oversonication can also lead to co-purification of host proteins with the GST fusion protein.

Note: Detection of GST activity can be performed using the CDNB assay at this stage. This can be a useful screening technique and provide confidence that protein expression is sufficient to justify subsequent steps (see GST Detection Module, 27-4590-01).

9. Add 20% Triton X-100 to a final concentration of 1%. Mix gently for 30 min to aid in solubilization of the fusion protein.
10. Centrifuge at 12 000 × *g* (e.g. 10 000 rpm in a Beckman JA20 rotor) for 10 min at 4 °C. Transfer the supernatant to a fresh container. Save aliquots of the supernatant and the cell debris pellet for analysis by SDS-PAGE. These samples can be used to identify the fraction that contains the fusion protein.
11. Proceed with either Protocol 2 or Protocol 3.

Appendix 4:
Thrombin cleavage of GST fusion proteins

During the following procedures, samples should be removed at various time points and analyzed by SDS-PAGE to estimate the yield, purity and extent of thrombin digestion. The amount of thrombin, temperature and length of incubation required for complete digestion of a given GST fusion protein varies. Generally, cleavage should be complete following overnight treatment with 10 cleavage units/mg fusion protein. Optimal conditions for each fusion should be determined in pilot experiments. For some applications, thrombin should be subsequently removed from the sample by chromatography.

Cleavage of eluted GST fusion protein

In most cases, the fusion partner of interest retains functional activity such that functional tests can be performed using the intact fusion with GST. If removal of the GST affinity tail is necessary, fusion proteins containing a thrombin recognition site may be cleaved in solution as follows:

1. To the eluate from either batch or column purification, add 10 µl of thrombin solution (10 units) (prepared as described in "Materials not supplied") per mg fusion protein.
2. Mix gently and incubate at room temperature (22–25 °C) for 2–16 hours.
3. Once digestion is complete, GST can be removed by first removing glutathione by extensive dialysis (e.g. 2 000 volumes) against 1× PBS followed by batch or column purification on Glutathione Sepharose 4B. The purified protein of interest will be found in the flow-through.

Cleavage of fusion protein bound to bulk matrix

1. Prepare the thrombin reaction mixture as follows. For each ml of Glutathione Sepharose bed volume*, mix 50 µl of resuspended thrombin (prepared as described in "Materials not supplied") and 950 µl of 1× PBS.
2. To the Glutathione Sepharose pellet from Protocol 2, add the thrombin reaction mixture. Gently swirl to mix and then shake or rotate the slurry at room temperature for 2–16 hours.
3. Centrifuge the suspension at 500 × *g* for 5 min to pellet the Sepharose beads.
4. Carefully remove and save the eluate in a fresh tube. This will contain the protein of interest, while GST should remain bound to the gel.

Cleavage of fusion protein bound to column matrix

1. Prepare the thrombin reaction mixture as above.
2. Replace the bottom cap (or wrap with Parafilm) on the washed column from Protocol 3 and add the thrombin reaction mixture.
3. Remove the bottom cap or Parafilm from the column and allow the thrombin reaction mixture to enter into the bed of Glutathione Sepharose 4B. Once liquid begins to flow from the bottom of the column, replace the bottom cap or wrap with Parafilm and allow the column to stand at room temperature for 2–16 hours.
4. Following incubation, remove the bottom cap or Parafilm and collect the eluate in a fresh tube. Add 0.5 ml of 1× PBS for each ml

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*Bed volume is equal to 0.5× the volume of the 50% Glutathione Sepharose slurry used.

of Glutathione Sepharose bed volume and collect into the same tube containing the eluate. This will contain the protein of interest, while GST remains bound to the gel.

Troubleshooting

Problem: High basal level of expression.

Basal level expression (i.e. expression in the absence of an inducer, such as IPTG), present with most inducible promoters, can affect the outcome of cloning experiments for toxic inserts by selecting against inserts cloned in the proper orientation. Basal level expression can be minimized by catabolite repression (e.g. growth in the presence of glucose). The *tac* promoter is not subject to catabolite repression as was suggested in previous versions of this booklet. However, there is a *lac* promoter located upstream between the 3' end of the *lacI*^{q} gene and the *tac* promoter. This lac promoter is subject to catabolite repression and may contribute to the basal level of expression of inserts cloned into the pGEX multiple cloning site.

Possible causes/solutions

1. *Upstream promoter.* If the basal level of expression is too high for your application, addition of 2% glucose to the growth medium may help. Growth in the presence of 2% glucose will decrease the basal expression level associated with the upstream *lac* promoter but will not affect basal level expression from the *tac* promoter. The presence of glucose should not significantly affect overall expression following induction with IPTG.

Problem: Protein is not detected by Coomassie staining of an SDS gel of a bacterial sonicate.

Possible causes/solutions

1. *Optimize expression conditions.* Optimizing expression conditions can dramatically improve yields. Investigate the effects of cell strain, medium composition, incubation temperature and induction conditions on fusion protein yield. Exact conditions will vary for each fusion protein.
2. *Check DNA sequences.* It is essential to clone protein-coding DNA sequences in the proper translation frame in pGEX vectors. Sequence cloning junctions using 5' pGEX Sequencing Primer (27-1410-01) and 3' pGEX Sequencing Primer (27-1411-01) to verify that inserts are in-frame with GST.
3. *Analyze a small aliquot of an overnight culture by SDS-PAGE.* Generally, a highly expressed protein will be visible by Coomassie staining when 5–10 µl of an induced culture whose A_{600} is ~ 1.0 is loaded on the gel. Run nontransformed host *E. coli* cells and cells transformed with the parental pGEX vector in parallel as negative and positive controls, respectively. The presence of the fusion protein in this total cell preparation and its absence from a clarified sonicate may indicate the presence of inclusion bodies (see below).
4. *Hidden protein.* Check for expression by immunoblotting. Some fusion proteins may be masked on an SDS-polyacrylamide gel by a bacterial protein of approximately the same molecular weight. Immunoblotting can identify fusion proteins in these cases. Run an SDS-polyacrylamide gel of induced cells as above and transfer the proteins to a nitrocellulose or PVDF membrane. Detect fusion protein using anti-GST antibody (included in the GST Detection Module, 27-4590-01, and available separately, 27-4577-01).

Problem: Majority of protein is found in post-sonicate pellet.

Possible causes/solutions

SDS-PAGE analysis of samples collected during the preparation of the bacterial sonicate may indicate that the majority of the GST fusion protein is located in the post-sonicate pellet. Possible causes and solutions are discussed below.

1. *Sonication may be insufficient.* Cell disruption is indicated by partial clearing of the suspension or may be checked by microscopic examination. Addition of 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—this may denature the fusion protein. Oversonication can also lead to co-purification of host proteins with the GST fusion protein.

2. *Fusion protein may be insoluble (inclusion bodies).* If insufficient protein is found in the soluble fraction following centrifugation of the sonicate, it may be necessary to alter 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 level of induction by decreasing IPTG concentration to < 0.1 mM.

   – Alter timing of induction.

   – Induce for a shorter period of time.

   – Induce at a higher cell density for a short period of time.

   – Increase aeration. High oxygen transport can help prevent the formation of inclusion bodies (7).

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

If the above techniques do not significantly improve expression of soluble fusion protein, protein can be solubilized from inclusion bodies using common denaturants such as 4–8 M guanidine hydrochloride, 4–8 M urea, detergents, alkaline pH (> 9), organic solvents (8, 9), and N-lauroylsarcosine (Sarkosyl) (10, 11). Other variables that affect solubilization include time, temperature, ionic strength, ratio of denaturant to protein and the presence of thiol reagents (8, 9). For reviews, see references5, 8, 9, and 12.

Following solubilization, proteins must be properly refolded to regain function. Denaturant can be removed by dialysis, dilution, or gel filtration to refold the protein and form the correct intramolecular associations. Critical parameters during refolding include pH, 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 where GST fusion proteins formed inclusion bodies, solubilization and binding to Glutathione Sepharose 4B was achieved in the presence of 2–3 M guanidine hydrochloride or urea, and up to 2% Tween™ 20. Binding was also observed in the presence of 1% CTAB, 10 mM DTT or 0.03% SDS (2). Success of affinity purification in the presence of these agents will depend on the nature of the fusion protein.

Problem: Fusion protein does not bind to Glutathione Sepharose 4B.

Possible causes/solutions

1. *Altered Structure.* Test the binding of GST from parental pGEX. Prepare a sonicate of cells harboring the parental pGEX plasmid and check binding to the gel. If GST produced from the parental plasmid binds with high affinity, then 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 the number of washes.
2. *Fusion protein denatured by sonication.* Oversonication can denature the fusion protein and prevent its binding to Glutathione Sepharose 4B (11). Use mild sonication conditions during cell lysis.
3. *Add DTT prior to cell lysis.* The addition of DTT to a final concentration of 5 mM prior to cell lysis can significantly increase binding of some GST fusion proteins to Glutathione Sepharose 4B (11).
4. *Use fresh Glutathione Sepharose 4B.* If the Glutathione Sepharose 4B has already been used several times, it may be necessary to use fresh gel. Refer also to Appendix 2 for details.

Problem: Fusion protein is not efficiently eluted from Glutathione Sepharose 4B.

Possible causes/solutions

1. *Insufficient elution period.* In some instances, overnight elution at room temperature or 4 °C is most effective.
2. *Insufficient elution buffer.* Increase the volume of elution buffer. Glutathione Sepharose 4B also functions as a gel filtration medium with an approximate molecular weight exclusion limit of 2×10^7 Daltons. Small proteins (especially those liberated following cleavage with a site-specific protease) may require large elution volumes. In these cases, the batch purification method may be preferred (Protocol 2). Proteins eluted in a large volume may require concentration by ultrafiltration.
3. *Insufficient glutathione.* Increase the concentration of glutathione in the elution buffer. General protocols use 5 mM glutathione for elution. Note that when dissolved according to instructions, the concentration of glutathione used in the elution buffer is 10 mM, which should be sufficient for most applications. Additional reduced glutathione must be obtained separately. If the glutathione concentration is increased significantly above 15 mM, the buffer concentration will have to be increased to maintain proper pH (14).
4. *Ionic concentration to low.* Increase the ionic strength of the elution buffer. The addition of 0.1–0.2 M NaCl to the elution buffer also-may improve results. Keep in mind that very hydrophobic proteins may precipitate under high salt conditions. If this is the case, addition of a non-ionic detergent may improve results (see page 24).

   One researcher obtained satisfactory results using an alternative elution buffer comprised of 20 mM glutathione, 100 mM Tris-HCl (pH 8.0), and 120 mM NaCl (14).

5. *Nonspecific hydrophobic interactions may prevent solubilization and elution of fusion proteins from Glutathione Sepharose 4B (11).* Add a non-ionic detergent to the elution buffer. Addition of a non-ionic detergent can improve results. The addition of 0.1% Triton X-100 or 2% N-octyl glucoside can significantly improve elution of some GST fusion proteins (11).

Problem: Thrombin cleavage is incomplete.

Possible causes/solutions

1. *Thrombin:fusion protein ratio is incorrect.* Check the amount of fusion protein in the digestion. Adjust the amount of thrombin added to 10 cleavage units/mg of fusion protein.

2. *Insufficient incubation period.* If the fusion protein is not degraded by extensive incubation with thrombin, increase the reaction time to 20 hours and increase the amount of thrombin used in the reaction.

3. *Altered thrombin site.* Check the construct's DNA sequence. Compare with known sequence, and verify that the thrombin recognition site has not been altered during the course of cloning your fusion protein.

Problem: Multiple bands are observed on an SDS gel following thrombin cleavage.

Possible causes/solutions

1. *Proteolysis.* Investigate if additional bands are present prior to thrombin cleavage. If they are, the host cell might be degrading the fusion protein. In this case, the use of a protease-deficient strain such as BL21 might solve the problem (e.g. *lon*$^-$ or *ompT*).

2. *Fusion partner may contain recognition sequences for thrombin.* The optimum cleavage site for thrombin can have the following two structures (15):

1) P4-P3-Pro-Arg/Lys•P1'-P2' where P3 and P4 are hydrophobic amino acids and P1' and P2' are nonacidic amino acids. The Arg/Lys•P1' bond is cleaved. Examples:

	P4	P3	Pro	R/K	•	P1'	P2'
A)	Met	Tyr	Pro	Arg	•	Gly	Asn
B)	Ile	Arg	Pro	Lys	•	Leu	Lys
C)	Leu	Val	Pro	Arg	•	Gly	Ser

In A, the Arg•Gly bond is cleaved very fast by thrombin. In B, the Lys•Leu bond is cleaved. C is the recognition sequence found on the thrombin series of pGEX plasmids and the Arg•Gly bond is cleaved.

2) P2-Arg/Lys•P1', where P2 or P1' are Gly. The Arg/Lys•P1' bond is cleaved. Examples:

	P2	R/K	•	P1'
A)	Ala	Arg	•	Gly
B)	Gly	Lys	•	Ala

In A, the Arg•Gly bond is cleaved efficiently. In B, the Lys•Ala bond is cleaved.

Check the sequence of the fusion partner for the presence of thrombin recognition sites. Adjusting time and temperature of digestion can produce selective scission at the desired thrombin site. If adjustment of conditions does not correct the problem, reclone the insert into a pGEX Factor Xa-based expression vector.

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