

Glutathione **Sepharose** 4 Fast Flow

Instructions for Use

Glutathione Sepharose™ 4 Fast Flow is an affinity chromatography medium for easy, one-step purification of glutathione S-transferase (GST)-tagged proteins produced using the pGEX series of expression vectors (1), other glutathione S-transferases and glutathione binding proteins.

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

Glutathione Sepharose 4 Fast Flow has high flow properties, which makes it excellent for scale-up. Glutathione Sepharose 4 Fast Flow is also available in convenient prepacked GSTrap™ FF 1-mL and 5-mL columns as well as GSTprep™ FF 16/10 20-mL column.

[Table 1, on page 3](#) lists the characteristics of Glutathione Sepharose 4 Fast Flow.

Detailed information about the production of GST-tagged proteins can be found in the *System Handbook 18115758*.

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1 Description

Medium properties

The glutathione ligand is coupled via a 10-carbon linker to highly crosslinked 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 approximately 10 mg recombinant GST/mL 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 sitespecific protease while bound to Glutathione Sepharose 4 Fast Flow 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 Glutathione Sepharose 4 Fast Flow .

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|--------------------------|--|
| Ligand | glutathione and 10-carbon linker arm |
| Ligand concentration | 120 to 320 μ mol glutathione/mL medium |
| Binding capacity | \approx 10 mg recombinant glutathione S-transferase/mL medium, GST, M_r 26 000 |
| Dynamic binding capacity | \approx 11 mg GST-tagged protein/mL medium M_r 43 000 (GSTrap FF 1 mL at 1 mL/min=156 cm/h) |
| Mean particle size | 90 μ m |
| Bead structure | highly cross-linked 4% agarose |

| | |
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| Maximum flow rate ¹ | 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 ² | Sample loading: <100 cm/h (< 3 mL/min using XK 16/20 column) Washing and elution: 100 to 300 cm/h (3 to 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 to 12 |
| Storage temperature | +4°C to 30°C |
| Storage buffer | 20 % ethanol |

¹ H₂O at room temperature.

² Binding of GST to glutathione is flow dependent and lower flow rates often increase the binding capacity. This is important during sample loading and elution. Protein characteristics, pH and temperature may also affect the binding capacity.

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 ₂ HPO ₄ , 1.8 mM KH ₂ PO ₄ , pH 7.3) |
| Elution Buffer: | 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0 |

Note: 1 to 10 mM DTT can be included in the binding and elution buffer.

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 4 Fast Flow

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| 1 | Determine the medium volume of Glutathione Sepharose 4 Fast Flow required for your purification. |
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Note:

Glutathione Sepharose 4 Fast Flow is delivered in 20% ethanol. Prepare a 50% slurry for your purification.

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| 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 medium 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. Invert to mix. |

| Step | Action |
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| 6 | Sediment the medium by centrifugation at $500 \times g$ for 5 min. Carefully decant the supernatant. |
| 7 | Repeat steps 5 and 6 one more time. |

Batch purification

| Step | Action |
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| 1 | Add the cell lysate to the prepared Glutathione Sepharose 4 Fast Flow 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 medium 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. Invert to mix. |
| 5 | Sediment the medium 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. |

| Step | Action |
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| 7 | Elute the bound protein by adding 0.5 mL 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0 per 1 mL slurry of Glutathione Sepharose 4 Fast Flow. Incubate at room temperature for 5 to 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 maximum binding capacity. The binding efficiency may differ significantly between different GST-fusion proteins.*
 - *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 medium should be monitored for GST-tagged proteins using SDS-PAGE in combination with Western Blot if necessary.*

- The GST Detection Module (see [Chapter 12 Ordering information, on page 29](#)) 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 (1).
- 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; $A_{280} \sim 1$ 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 4 Fast Flow depends on the nature of the sample and should only be performed with identical samples to prevent cross-contamination.

4 Column purification

Columns

Cytiva offers many different kinds of columns to use for column purification (see the Biodirectory catalogue or [cytiva.com/protein-purification](https://www.cytiva.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™ 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 (see [Chapter 12 Ordering information, on page 29](#)).

Column packing

| Step | Action |
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| 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 to 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 60%). |
| 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. |

| Step | Action |
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| 6 | Immediately fill the column with PBS, pH 7.3, mount the column toppiece 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 (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: <i>Do not exceed 75% of the packing flow rate during purification.</i> |
| 9 | Stop the pump and close the column outlet. Remove the toppiece 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. |

| Step | Action |
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| 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. |
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Column purification

| Step | Action |
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| 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 to 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 to 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 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. 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 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 (1).*
- *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; $A_{280} \sim 1$ 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 4 Fast Flow depends on the nature of the sample and should only be performed with identical samples to prevent cross-contamination.*

5 Cleaning Glutathione Sepharose 4 Fast Flow

If the 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 to 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.

6 Storage

Store the packed column at 4°C to 30°C in 20% ethanol.

7 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 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 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.

8 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 medium

Step Action

- 1** Follow steps 1 to 6 under “Batch purification” or steps 1 to 3 under “Column purification”.
 - 2** Wash the tagged protein bound Glutathione Sepharose 4 Fast Flow with 10 bed volumes of PreScission cleavage buffer.
 - 3** Prepare the PreScission Protease mix: For each mL of Glutathione Sepharose 4 Fast Flow 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 4 Fast Flow 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 \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 tagged protein and the PreScission Protease will remain bound to the Glutathione Glutathione Sepharose 4 Fast Flow.
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PreScission Protease cleavage of eluted GST-tagged protein

Assumption: 8 mg GST fusion protein bound/mL medium

| Step | Action |
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| 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 4 Fast Flow 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 4 Fast Flow to remove the GST moiety of the tagged protein and the PreScission Protease from the protein of interest. |
| 5 | Incubate for 20 to 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 |

9 Prepare thrombin solution

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-tagged protein bound to the column/bulk medium

Assumption: 8 mg GST fusion protein bound/mL medium

| Step | Action |
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| 1 | Follow steps 1 to 6 under "Batch purification" or steps 1 to 3 under "Column 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 tagged protein bound/mL medium). |
| 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 ($+22^\circ\text{C}$ to 25°C) for 2 to 16 hours. |

| Step | Action |
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| 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 tagged protein and analyse it. If batch format is used, centrifuge the suspension at 500 x 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 tagged protein will remain bound to the Glutathione Sepharose 4 Fast Flow. |
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Thrombin cleavage of eluted GST-tagged protein

Assumption: 8 mg GST fusion protein bound/mL medium

| Step | Action |
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| 1 | Add 10 μ L (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 μ L (80 U) of thrombin solution for each mL of Glutathione Sepharose 4 Fast Flow bed volume from which the tagged protein was eluted. (8 mg fusion protein bound/mL medium). |
| 2 | Incubate at room temperature (+22°C to 25°C) for 2 to 16 hours. |

| Step | Action |
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| 3 | 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. |
| 4 | Incubate for 20 to 30 min. at room temperature. |
| 5 | 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 tagged protein will remain bound to the Glutathione Sepharose 4 Fast Flow |

10 Prepare factor Xa solution

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:

| Step | Action |
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| 1 | Dissolve 400 U factor Xa in 400 μL cold water (1 U/ μL). |
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| 2 | Swirl gently to dissolve. |
| 3 | Freeze as 80 μ L aliquots 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 medium

| Step | Action |
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| 1 | Follow steps 1 to 6 under "Batch purification" or steps 1 to 3 under "Column purification". |
| 2 | 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). |
| 3 | Prepare the factor Xa mix: For each mL of Glutathione Sepharose 4 Fast Flow bed volume, prepare a mixture of 80 μ L (80 units) of factor Xa and 920 μ L 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 4 Fast Flow pellet. Gently shake or rotate the suspension. |
| 5 | Incubate at room temperature ($+22^{\circ}\text{C}$ to 25°C) for 2 to 16 hours. |

| Step | Action |
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| 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 $500 \times g$ 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 tagged protein will remain bound to the Glutathione Sepharose 4 Fast Flow . |
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Factor Xa cleavage of eluted GST-tagged protein

Assumption: 8 mg GST-tagged protein bound/mL medium

| Step | Action |
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| 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 μ L (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 μ L (80 units) of factor Xa solution for each mL of Glutathione Sepharose 4 Fast Flow bed volume from which the tagged protein was eluted. (8 mg tagged protein bound/mL medium). |

| Step | Action |
|------|---|
| 3 | Incubate at room temperature (+22°C to 25°C) for 2 to 16 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 tagged protein. |
| 5 | Incubate for 20 to 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. |

11 Troubleshooting

Consult the *System Handbook 18115758* for more detailed information and pGEX instructions regarding troubleshooting recommendations for expression, fermentation and solubilization.

GST-tagged protein does not bind to Glutathione Sepharose 4 Fast Flow

- **GST-tagged protein denatured by sonication:** Too extensive sonication can denature the tagged protein and prevent it binding to Glutathione Sepharose 4 Fast Flow. 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 to 10 mM may significantly increase binding of some GST-tagged 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 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 4 Fast Flow before use:** Binding of GST-tagged 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 to 8.0 (e.g. PBS) before the clarified 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 fresh Glutathione Sepharose 4 Fast Flow. See also [Chapter 5 Cleaning Glutathione Sepharose 4 Fast Flow, on page 13](#)
- **Decrease flow rate during sample load.** See note within [Column purification, on page 11](#).

GST-tagged 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 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 to 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 to 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 to 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 4 Fast Flow. Adding a nonionic 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 MgSO₄, 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 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 Cytiva.*
- **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 ($M_r \sim 70\,000$), 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-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 Cytiva 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 4 Fast Flow for GST is ≈ 10 mg/mL medium. In most purifications, however, the medium is not saturated with tagged protein.

Note: Ratios:

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

*Thrombin, at least 10 units/mg tagged protein.
One cleavage unit of thrombin from Cytiva digests $\geq 90\%$ of 100 μ g 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 $\leq 0.5\%$ 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. p

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 CaCl₂, 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 CaCl₂, pH 8.0. Incubate at +37 °C for 5 min. Factor Xa from Cytiva 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 *System Handbook 18115758* for details.

Reference

GST Gene Fusion System Handbook, Cytiva, Code No. 18115758.

12 Ordering information

| Product | Quantity | Product code |
|-----------------------------------|---------------------|--------------|
| Glutathione Sepharose 4 Fast Flow | 25 mL | 17513201 |
| | 100 mL | 17513202 |
| | 500 mL ¹ | 17513203 |
| GSTrap FF | 5×1 mL | 17513001 |
| GSTrap FF | 2×1 mL | 17513002 |
| GSTrap FF | 1×5 mL | 17513101 |
| GSTprep FF 16/10 | 1×20 mL | 17523401 |
| HiTrap Desalting | 5×5 mL | 17140801 |
| PD-10 Disposable column | 30 | 17085101 |
| HiPrep 26/10 Desalting | 1×53 mL | 17508701 |

¹ Larger quantities are available. Please contact Cytiva for more information.

Site-Specific Proteases

| Product | Quantity | Product code |
|----------------------|-----------|--------------|
| PreScission Protease | 500 units | 27084301 |
| Thrombin | 500 units | 27084601 |
| Factor Xa | 400 units | 27084901 |

Related Products

| Product | Quantity | Product code |
|---|--------------|--------------|
| GST Detection Module | 50 reactions | 27459001 |
| Anti-GST Antibody | 0.5 mL | 27457701 |
| Recombinant Protein Handbook | 1 | 18110502 |
| GST Gene Fusion System Handbook | 1 | 18115758 |
| Affinity Chromatography Handbook, Principles and Methods | 1 | 18102229 |

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