

GST Purification Kit

Product Information

Catalog Number: **560137**
 Storage Temperature: **4°C** (please note individual storage temperatures)

<u>Contents</u>	<u>Size</u>	<u>Storage</u>
1x Insect Cell Lysis Buffer	50 ml	4°C
Protease Inhibitor Cocktail*	lyophilized	-20°C
Thrombin Powder*	20 mg	-20°C
Thrombin Dilution Buffer	1 ml	4°C
Glutathione Agarose Beads*	10 ml	4°C
Glutathione Powder	62 mg	4°C
GST Elution Buffer	40 ml	4°C
PBS Wash Buffer (1x)	3 x 125 ml	4°C

*Product Notices below

Description

The GST Purification Kit (Cat. No. 560137) contains the reagents necessary for purifying GST-fusion proteins. GST-fusion proteins can be produced by recombinant baculovirus generated using the BD BaculoGold™ Linearized Baculovirus DNA (Cat. No. 554739) and one of the pAcGHLT-A,B and C transfer vectors (Cat. No. 554792), which encode N-terminal GST and 6xHis tags followed by an extended MCS. The MCS is in a different reading frame in each of the vectors to simplify cloning (Appendix E). A protein kinase A site follows the 6xHis tag for convenient labeling of the recombinant fusion protein. Because the GST vectors also contain a 6xHis sequence the expressed recombinant protein will be a 6xHis-containing GST fusion protein. The recombinant fusion protein can be affinity purified using either glutathione or Ni-NTA agarose beads. The GST tag can be proteolytically removed from the recombinant protein at the thrombin site located between the affinity tag and the MCS.

The GST purification method is based on the remarkable selectivity and affinity of recombinant proteins equipped with a GST affinity tag for glutathione immobilized on a resin. The expressed GST fusion proteins may be purified without the use of detergents under completely non-denaturing conditions. Purifications to greater than 90% homogeneity are easily achieved in a single step by affinity chromatography using glutathione agarose beads. The affinity of GST for glutathione is so strong that it allows a highly efficient separation of GST fusion proteins from contaminating polypeptides even under non-denaturing conditions. GST fusion proteins may be purified using either batch or column procedures detailed below. Batch binding for an extended time may be preferable when purifying dilute proteins. We recommend including insect cell lysate from an infection using pAcGHLT-Xyle recombinant virus as a positive control in the affinity purification procedure.

Suggested Companion Products

<u>Catalog Number</u>	<u>Name</u>	<u>Size</u>
554792	pAcGHLT-A,B and C Transfer Vectors	5 ug each
560129	BD BaculoGold Transfection Kit	5 transfections
554739	BD BaculoGold Linearized Baculovirus DNA	5 transfections
554806	Transfection Buffer A & B	5 transfections

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Protocol

For complete co-transfection protocols, please refer to the protocols on our website at: <http://www.bdbiosciences.com/pharmingen/protocols/>

Amplification of Recombinant Baculoviruses:

After the co-transfection or after the plaque assay, the recombinant baculovirus is amplified to obtain a high titer stock solution.

- Seed 5×10^6 insect cells per 10 cm plate (approximately 60% confluent).
- Infect cells with 500 μ l of transfection supernatant or 100 μ l of the plaque pick-up viral supernatant in 15 ml of TNM-FH medium (should be infected at a multiplicity of infection (MOI) of >1).
- Incubate cells at 27°C for 3 days before harvesting the medium. The medium will contain at least 10^7 virus particles per ml and should be used for another round of amplification.

Lysate Preparation:

- Harvest cell infected with recombinant virus 3 day post infection (pi) or perform a timecourse study to determine optimal protein production.
- Spin down cells at 2,500 x g for 5 min.
- Resuspend cell pellet in ice-cold Insect Cell Lysis Buffer containing reconstituted Protease Inhibitor Cocktail. Use 1ml of lysis buffer per 2×10^7 cells.

The Protease Inhibitor Cocktail is provided as lyophilized powder. Before use, add 1 ml of pure ethanol to obtain a 50X Protease Inhibitor Cocktail. The reconstituted 50X protease inhibitor cocktail will have the following ingredients: 800 g/ml benzamidine HCl, 500 g/ml phenanthroline, 500 g/ml aprotinin, 500 g/ml leupeptin, 500 g/ml pepstatin A, 50 mM PMSF.

- Lyse cells on ice for 45 min.
- Clear cellular debris from lysate by centrifuging at 40,000 x g for 45 min (or filter through a 0.22 μ m filter).
- Harvest clarified lysate which should contain your recombinant GST-fusion protein.

Protein Purification Protocols

Batch Purification

1. Bead preparation for batch purification.

- Determine the amount of glutathione agarose beads needed. One ml of glutathione beads will bind approximately 5 mg of GST fusion protein. 1–2 mg of GST fusion protein is routinely obtained per liter of insect cell culture.
- Gently resuspend the glutathione agarose beads (Cat. No.51-21427B).
- Place the glutathione agarose beads as a slurry in a sterile tube.
- Centrifuge the slurry at 500x_g for 3–5 min to sediment the matrix.
- Carefully remove the supernatant by pouring off or aspirating.
- Wash the beads two times with 5–10 bead volumes of PBS Wash Buffer (Cat. No. 51-21428A) to remove the 20% ethanol preservative.
- After each wash, centrifuge the slurry at 500xg for 3–5 min to sediment the matrix.
- Draw off the supernatant.

2. Add lysate to equilibrated glutathione beads for batch purification.

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- Add the clarified lysate to the beads.
- Mix 10 volumes of insect cell lysate containing the recombinant GST fusion protein of choice with one volume glutathione agarose beads.
- Incubate the slurry for 30 min at 4°C on a rocking platform.
- Centrifuge the slurry at 500xg for 3–5 min to sediment the matrix.
- Draw off the supernatant. Keep the supernatant fractions to run on SDS-PAGE to determine whether the binding capacity of the glutathione beads was exceeded and whether all of the GST fusion protein bound to the matrix.

Note: The 10:1 ratio mentioned above is an approximation. Expression levels should be empirically determined by the researcher.

3. Wash.

- Wash the slurry beads twice with 5 bead volumes of PBS Wash Buffer (Cat. No. 51-21428A).
- Centrifuge the slurry at 500x_g for 3–5 min to sediment the matrix.

- Discard the washes.

4. Elute the fusion protein with reduced glutathione.

- Add 1 bead volume of the reconstituted GST Elution Buffer (Cat. No. 51-21429Z and Cat. No. 51-21455A) to the bead matrix.

- Gently mix the slurry.

- Incubate the slurry for 2 min at RT.

- Centrifuge the slurry at 500_x_g for 3–5 min to sediment the matrix.

- Collect the eluted fraction.

- Repeat the elution steps two more times.

- Pool the three eluted fractions.

Column Purification**1. Bead preparation for column purification.**

- Determine the amount of glutathione agarose beads needed. One ml of glutathione beads will bind approximately 5 mg of GST fusion protein. 1–2 mg of GST fusion protein is routinely obtained per liter of insect cell culture.

- Gently resuspend the glutathione agarose beads (Cat. No. 51-21427B).

- Repeat the elution steps two more times.

- Place the glutathione beads as a slurry in a suitable chromatography column.

- Allow the beads to settle and the column to drain.

- Wash the beads two times with 3–5 bead volumes of PBS Wash Buffer (Cat. No. 51-21428A) to remove the 20% ethanol preservative.

- Allow the column to drain but not to dry out.

2. Add lysate to equilibrated glutathione beads for column purification.

- Apply the clarified lysate to the column.

- Adjust the column flow rate to a maximal 5 ml/min per ml of beads. Keep the flow-through fraction to run on SDS-PAGE to determine whether the binding capacity of the glutathione beads was exceeded.

3. Wash.

- Wash the column twice with 5 bead volumes of PBS Wash Buffer (Cat. No. 51-21428A).

- Allow the column to drain.

- Discard the washes.

4. Elute the fusion protein with reduced glutathione.**BD Biosciences**

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- Add 3 bead volumes of the GST Elution Buffer (Cat. No. 51-21429Z and Cat. No. 51-21455A) to the column.
- Adjust the column flow rate to a maximal 1 ml/min per ml of beads.
- Allow the column to drain completely.
- Collect the eluted fraction.

Note: The addition of 150 mM NaCl, 5 mM CaCl₂ (or for some proteins 5 mM MgCl₂) or 0.1% β-mercaptoethanol to the GST Elution Buffer is optional but may be required for the solubility of some proteins.

Dialyzing GST-Fusion Protein

- Remove the free glutathione by dialysis against 100 volumes of 50 mM Tris-HCl (pH 8.0) at 4°C.
- Dialyze a minimum of 4 h. Change dialysis buffer after 2 h.

Checking Purity and Recovery of Recombinant Protein

- Add 2 volumes of 3X SDS sample buffer to 1 volume of the clarified lysates.
- Run the samples on a 5–15% SDS-PAGE.
- Stain the gel with Coomassie blue.
- Check for the amount of recombinant fusion protein in the sample.

Note: Monoclonal antibodies to GST (Cat. No. 554805 and 554824) may be used to detect recombinant GST-fusion proteins by Western blot analysis.

Alternative GST Purification Procedure

In the aforementioned purification protocols, a second purification step is used to dissociate the GST from the protein of interest. In an alternate approach, only one affinity step is required.^{32,33} Briefly, the affinity resin-bound GST fusion protein is equilibrated in thrombin cleavage buffer (1 wash) followed by the addition of 2 μg thrombin per mg fusion protein. The reaction mixture is gently shaken on a rocking platform at RT for 20 min. In this reaction, the protein of interest is cleaved from the GST carrier protein and can be recovered in the supernatant after brief centrifugation. The entire procedure can be completed within a few hours and results in highly purified protein. Recovered protein should be stored at –80°C.

Cleave Fusion Protein using Site-specific Protease

Thrombin Cleavage

The GST vectors, pAcGHLT-A,B, and C, contain a thrombin cleavage site. This site enables the proteolytic cleavage of the recombinant protein from the fusion partner. Removal of the fusion partner is optional for many applications.

- Mix 1 mg of purified GST or 6xHis fusion protein containing a thrombin cleavage site with 10 thrombin units of bovine thrombin (Cat. No. 51-21430Z and Cat. No. 51-21454A).
- Incubate at RT for up to 12 h (in many cases a 20-60 min incubation will be sufficient).
- GST and uncleaved GST fusion protein may be removed by directly adding 2 volumes of glutathione-coupled resin (50% v/v) at the end of the cleavage reaction. Similarly, the 6xHis and uncleaved 6xHis fusion protein may be removed by directly adding Ni-NTA Agarose at the end of the cleavage reaction. The sample is then incubated for 30 min at 4°C and centrifuged for 10 min at 5,000 x g. The supernatant will contain the purified protein as well as thrombin and can be stored frozen at –80°C. Some proteins may require the addition of BSA or glycerol (final concentration 50%) for stability.

Note: Thrombin cleaves in 50 mM Tris-HCl buffer and does not require specific metal ions for its activity. However, we recommend using a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2.5 mM CaCl₂ and 0.1% β-mercaptoethanol for efficient cleavage. An efficient thrombin

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cleavage primarily depends on the sequence of the thrombin consensus site and the three-dimensional structure surrounding that site .

Design of the Thrombin Cleavage Site

The thrombin cleavage consensus site is XXP(K/R)*BB7, where X stands for hydrophobic apolar amino acids, P stands for proline, (K/R) symbolizes that either lysine or arginine works in this position, and B stands for non-acidic amino acids. The star symbol (*) represents the cleavage position which is at the carboxy-terminal side of the arginine or the lysine residue. The thrombin site used in the pAcGHLT and pAcHLT vectors is LVPR*GS. The desired gene is inserted at the BamHI site (at the amino acids GS), thrombin coding sequence. Cleavage by thrombin will then release the nearly authentic protein.

Troubleshooting

Note: We recommend using the pAcGHLT-XylE control vector (Cat. No. 51-21470P) as a positive control for co-transfection and subsequent protein expression in this system.

GST Fusion Protein Expression is Low or Absent

- Analyze the recombinant baculovirus genome by Southern hybridization or by sequencing the DNA across cloning junctions to verify that the foreign protein coding sequences have been inserted correctly.
- Check whether the protein coding sequence of the insert is cloned in the proper translational frame by DNA sequence analysis.
- Conduct a concurrent time course of mRNA expression levels by Northern hybridization and of protein expression to determine the correlation between the mRNA and protein expression.
- A point mutation in the polyhedrin promoter may reduce mRNA levels.
- Amplify your recombinant baculovirus infection stock to at least 10^8 viruses/ml.
- Optimize the infection period to maximize protein expression.
- Conduct a time course to determine the optimal time for harvesting your recombinant protein.
- Optimize the multiplicity of infection (MOI). For protein expression, the MOI should generally be between 3–10. MOIs that are too high or too low may affect protein expression.
- Grow and infect insect cells on plates instead of spinner bottles.
- Seed cells at a different cell density.
- Try a different insect cell line for protein expression.
- Use a different insect cell culture medium.
- Maintain a constant temp of 27°C during protein expression.
- Multiple passages of viral stock may cause a loss of your gene of interest. Always keep a stock of low passage virus to use as an infection source.
- Sf9 cells in continuous culture for more than a year may lose the ability to express foreign proteins efficiently.
- Your protein may be mildly toxic to the insect cell. Harvest the infected cells earlier in the infection cycle.
- Some proteins may not be stable in virus-infected cells. Compare mRNA and protein levels.
- Membrane-bound glycoproteins and secreted proteins may be produced at lower levels than proteins that remain in the cytoplasm or are targeted to the nucleus.
- Check for your protein in both the cell pellet and supernatant.

GST Fusion Protein is Insoluble or Non-functional

- Harvest infected insect cells earlier in the infection cycle.

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- Infect insect cells with a lower MOI.
- Try a different insect cell line for protein expression.
- Review the biochemical properties of your protein.
- Co-express a dimerization partner which may keep your protein in solution.

GST Fusion Protein does not bind to Glutathione Agarose Beads

- Check binding of the unfused GST protein and the GST-XylE protein.
- In case of weak binding due to altered conformation of the GST fusion protein, lower binding temperature to 4°C and limit the number of washes.

Inefficient Elution of GST Fusion Protein

- Make fresh elution buffer from dry reduced glutathione.
- Increase incubation time of beads in elution buffer.
- Increase volume of elution.
- Increase glutathione concentration in elution buffer.
- Add increasing amounts of NaCl to elution buffer (100-500 mM).
- Try an overnight elution at RT or at 4°C.

Thrombin Cleavage

Inefficient thrombin cleavage.

- Increase the amount of thrombin (up to 100 cleavage units/mg of fusion protein). We recommend 50 cleavage units/mg of fusion protein.
- Add heparin (1–20 mM) to your thrombin cleavage buffer. Heparin has been shown to enhance thrombin cleavage.⁴²
- Increase the incubation temperature. If your protein is stable at 37°C and the preparation is low in proteases, try a 4-16 h incubation at 37°C.
- Increase the incubation time. If your protein is not degraded by extensive incubation in presence of thrombin, increase the reaction time (up to 24 h).
- Verify the existence of a functional thrombin site by sequencing. Ensure that your cloning strategy has not altered the consensus thrombin site. If the cloning of your desired protein destroyed the thrombin site, you will need to reclone your gene using a different cloning site or strategy.

***Product Notices**

Protease Inhibitor Cocktail (51-21426Z) contains 75.8% phenylmethylsulfonylfluoride which is a toxic solution.

R23/25 Toxic by inhalation and if swallowed

R34 Causes burns.

S4 Keep away from living quarters

4 Keep container in a well-ventilated place.

26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

36/37/39 Wear suitable protective clothing, gloves and eye/face protection.

45 In case of accident or if you feel unwell, seek medical advice immediately.

60 This material and its container must be disposed of as hazardous waste.

Cont.

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Glutathione Agarose Beads (51-21427B) contains 50% ethanol which is a hazardous material.

11 Highly flammable.

40 Limited evidence of a carcinogenic effect.

7/9 Keep container tightly closed and in a well-ventilated place.

16 Keep away from sources of ignition - No smoking.

23 Do not breathe gas/fumes/vapour/spray.

36/37 Wear suitable protective clothing and gloves.

60 This material and its container must be disposed of as hazardous waste.

Thrombin Powder (51-21430Z) is a harmful substance.

R 36/37/38 Irritating to eyes, respiratory system and skin.

R 42/43 May cause sensitization by inhalation and skin contact.

22 Do not breathe dust.

24 Avoid contact with skin.

37 Wear suitable gloves.

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