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* patent pending

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Description

GST•Bind Resin

GST•Bind Resin is an affinity chromatography support for rapid, single-step purification of recombinant glutathione S-transferase (GST) fusion proteins, native glutathione S-transferase, or proteins that bind glutathione. GST•Tag™ fusion proteins containing the 220 aa GST domain (e.g., those expressed from pET-41 and pET-42 series vectors) are quickly and easily purified to near homogeneity in a single chromatographic step. The gentle elution condition (10 mM reduced glutathione) avoids harsh treatments that may denature target proteins.

Novagen's preparation consists of a cross-linked agarose matrix to which reduced glutathione is covalently attached via a sulfide linkage using an 11-atom spacer arm (estimated 16Å). The high degree of substitution ensures a high binding capacity with yields of GST fusion proteins of 5–8 mg/ml settled resin.

GST•Bind Buffer Kit

The GST•Bind Buffer Kit contains a set of pre-tested buffers for optimized binding, washing and elution of target proteins from GST•Bind Resin and GST•Mag Agarose Beads.

BugBuster GST•Bind Purification Kit

The BugBuster GST•Bind Purification Kit combines the GST•Bind Resin, GST•Bind Buffer Kit reagents, Benzonase® Nuclease and BugBuster® Protein Extraction Reagent for convenient preparation of soluble cell extracts and affinity purification of GST•Tag fusion proteins. BugBuster is formulated for the gentle disruption of the cell wall of *E. coli*, resulting in the liberation of soluble protein. It provides a simple, rapid, low cost alternative to mechanical methods such as French Press or sonication for releasing expressed target proteins in preparation for purification. The proprietary formulation utilizes a mixture of non-ionic detergents that is capable of cell wall perforation without denaturing soluble protein. Benzonase is a genetically engineered endonuclease from *Serratia* marcescens (1–2). This promiscuous endonuclease attacks and degrades all forms of DNA and RNA (single stranded, double stranded, linear and circular) and is effective over a wide range of operating conditions (3). Cells are harvested by centrifugation followed by suspension in BugBuster reagent. During a brief incubation, soluble proteins are released. The extract is clarified by centrifugation, which removes cell debris and insoluble proteins. The clarified extract is ready to apply and fully compatible with GST•Bind Resin.

Note: *BugBuster is supplied in a Tris-HCl-based buffer. For applications requiring other buffers Novagen offers BugBuster (primary amine free), which contains a PIPPS buffer, and 10X BugBuster, which does not contain added buffer.*

GST•Mag Agarose Beads

GST•Mag Agarose Beads consist of reduced glutathione attached to 1–5 µ diameter magnetic agarose beads. The magnetic beads have a high binding capacity for purification of GST•Tag™ fusion proteins of 2 mg/ml settled resin. These magnetizable beads are ideal for rapid purification of multiple samples with miminal handling and are compatible with magnetic separation based high throughput applications. The GST•Mag Agarose Beads are compatible with the GST•Bind Buffer Kit.

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PopCulture™ GST•Mag Purification Kit

The PopCulture GST•Mag Purification Kit is ideally suited for high throughput (HT) protein extraction and purification with GST•Mag Beads in the original culture tube or multiwell plate without the need for centrifugation or columns. PopCulture Reagent is a Tris buffered mixture of concentrated detergents formulated to perforate the *E. coli* cell wall and extract proteins directly in the culture medium without denaturing soluble protein. To further enhance the PopCulture purification procedure, rLysozyme™ Solution is included in this kit. Lysozyme cleaves a peptidoglycan bond in the *E. coli* cell wall enhancing cell lysis and increasing the yield of protein (4–5).

Components

GST•Bind Buffer Kit

The GST•Bind Buffer Kit contains the following components sufficient to run a minimum of ten 2.5 ml columns:

- 2 × 100 ml 10X GST Bind/Wash Buffer (10X = 43 mM Na_2HPO_4, 14.7 mM KH_2PO_4, 1.37 M NaCl, 27 mM KCl, pH 7.3)
- 40 ml 10X Glutathione Reconstitution Buffer (10X = 500 mM Tris-HCl, pH 8.0)
- 1 g Glutathione, Reduced, Free Acid

BugBuster® GST Bind Purification Kit

- 2 × 100 ml BugBuster Protein Extraction Reagent
- 10,000 U Benzonase® Nuclease, Purity > 90%
- 10 ml GST•Bind Resin, 10 ml settled volume (20 ml of a 50% v/v suspension)
- 4 Chromatography columns, with closures
- 100 ml 10X GST Bind/Wash Buffer
- 40 ml 10X Glutathione Reconstitution Buffer
- 1 g Glutathione, Reduced, Free Acid

GST•Mag™ Agarose Beads

- 2 × 1 ml or 10 × 1 ml GST•Mag Agarose Beads, 1 ml settled volume (2 ml of a 50% slurry in 0.05 M Phosphate Buffer, pH 7.5, containing 0.15 M NaCl and 0.1% NaN_3)

PopCulture™ GST•Mag Purification Kit

- 15 ml PopCulture Reagent
- 300 KU rLysozyme™ Solution
- 1 ml rLysozyme Dilution Buffer
- 3 × 1 ml GST•Mag Agarose Beads (2 ml of a 50% slurry in 0.05 M Phosphate Buffer, pH 7.5, containing 0.15 M NaCl and 0.1% NaN_3)
- 2 × 100 ml 10X GST Bind/Wash Buffer
- 40 ml 10X Glutathione Reconstitution Buffer
- 1 g Glutathione, Reduced, Free Acid

Storage

Store 10X GST Bind/Wash Buffer, 10X Glutathione Reconstitution Buffer, Glutathione, Reduced, Free Acid, GST•Bind Resin and GST•Mag Agarose Beads at 4°C. Store rLysozyme Solution, rLysozyme Dilution Buffer and Benzonase at –20°C. Store BugBuster and PopCulture at room temperature.

Note: *Storage of BugBuster Reagent and PopCulture Reagent at temperatures below 4°C may cause precipitation of the detergents. Incubate BugBuster or PopCulture in a room temperature water bath with gentle swirling or inversion to redissolve.*

Storage of rLysozyme Solution at –70°C may result in precipitation and loss of activity. Dilutions of rLysozyme Solution with rLysozyme Dilution Buffer of 1:100 or less are stable at 4°C for one week. Do not store diluted rLysozyme at –20°C because it will lose activity and/or precipitate.

Overview

This technical bulletin describes methods for *E. coli* cell extract preparation (mechanical disruption, BugBuster® Protein Extraction Reagent, and PopCulture™ Reagent) and procedures for purification of protein with a GST•Tag™ sequence using the GST•Bind Resin or GST•Mag™ Agarose Beads. These procedures start with a cell culture that has been induced for target protein production. The target protein should contain the schistosomal 220 amino acid GST domain (GST•Tag sequence), which must be properly folded to allow interaction with the immobilized reduced glutathione on the GST•Bind Resin or GST•Mag Agarose Beads. A detailed discussion of the induction of target proteins using Novagen's pET System can be found in the pET System Manual (Technical Bulletin 055), which accompanies pET systems, and is also available at www.novagen.com.

While some target proteins remain soluble in the cytoplasm, other proteins form insoluble aggregates, or inclusion bodies, in *E. coli*. In addition to the target protein, inclusion bodies also contain contaminating bacterial proteins and nucleic acids. If most or all of the target protein is insoluble, it is possible to solubilize the protein from purified inclusion bodies and refold the protein prior to running the GST•Bind purification.

Cell Extract Preparation

Considerations before you begin:

- Consider using BugBuster or PopCulture Reagent with Benzonase® Nuclease and rLysozyme™ Solution to extract the soluble proteins and/or prepare inclusion bodies, which greatly simplifies the procedure by avoiding mechanical disruption.
- Additives such as 2-mercaptoethanol, dithiothreitol, and EDTA can be used with cell extracts for GST•Bind purification.
- If necessary, protease inhibitors may be added to the buffers to protect against degradative enzymes. Because inhibitors are often unnecessary, we recommend proceeding without their addition first. It should also be noted that serine protease inhibitors should be used with caution if the target protein is to be treated with thrombin, Factor Xa or enterokinase, because any active inhibitor carried through the purification may affect cleavage reactions. If proteolytic degradation of the target protein is a problem, try adding the following: *p*-APMSF (10–100 µM; Calbiochem 178281); Pepstatin A (1 µM; Calbiochem 516482); Leupeptin (10–100 µM; Calbiochem 108975); Aprotinin (2 µg/ml; Calbiochem 616398); or Protease Inhibitor Cocktail Set II (with EDTA, Calbiochem 539132) or III (without EDTA, Calbiochem 539134). Although it is likely that the inhibitors will be removed and/or inactivated during purification, we recommend including a dialysis or gel filtration step prior to fusion tag cleavage with rEK, Factor Xa or thrombin.
- The protocol can be scaled according to the expression level and amount of protein to be purified. See the section *GST•Bind Resin Chromatography* for a discussion of resin capacity and determination of target protein expression levels.
- The level of active GST fusion protein in crude or purified samples can be assayed with the GST•Tag Assay Kit. Only active GST will bind to GST•Bind Resin and GST•Mag™ Beads.

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Mechanical disruption method

Soluble fraction

This procedure isolates protein in the soluble fraction of *E. coli*. Prepare 1X GST Bind/Wash Buffer for the procedures below by diluting the 10X supplied stock with deionized water or prepare according to buffer compositions given on page 3.

1. Harvest the cells by centrifugation at 10,000 × g for 5 min. Decant the supernatant and allow the cell pellet to drain as completely as possible. Resuspend the cells in 4 ml ice-cold 1X GST Bind/Wash Buffer per 100 ml culture. If desired, NP-40 or another non-ionic detergent can be added to 0.1% to reduce non-specific binding. If resuspension is difficult, a Dounce homogenizer, a blender or sonicator can be used to break up the cell pellet.
2. With the sample in a tube on ice or in a salt-ice bath, sonicate. The conditions are not specified here because results depend on the type of sonicator probe used, the power setting, and the shape and size of the vessel holding the cells. Avoid long sonication times where the sample could heat up; instead, break up the sonication into bursts with cooling in between. Sonicate until the sample is no longer viscous. If the DNA is not sheared by sonication, the extract will be so viscous that it will clog up the column. Larger cell masses may be broken in 1X GST Bind/Wash buffer by alternative methods such as French Press. **Optional:** Add rLysozyme™ Solution to a final concentration of 45–60 KU/gram of cell paste. Mix by pipetting up and down. Incubate at 30°C for 15 min prior to sonication. rLysozyme Solution can be diluted in rLysozyme Dilution Buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1% Triton® X-100]. Dilutions should be used as soon as possible and stored on ice or at 4°C until use. Do not store diluted rLysozyme Solution at -20°C as freezing may result in loss of activity.

rLysozyme Solution (Cat. No. 71110-3) in combination with sonication causes efficient bacterial cell lysis.

3. Centrifuge the lysate at 39,000 × g for 20 min to remove debris. Filter the post-centrifugation supernatant though a 0.45 micron membrane to prevent clogging of the resin (syringe-end filters are convenient for this purpose).

Inclusion body purification

The procedure isolates and washes inclusion bodies from *E. coli* in 1X GST Bind/Wash Buffer (diluted from the 10X supplied stock with deionized water or prepared according to buffer compositions given on page 3) to remove contaminating proteins. GST•Tag fusion proteins must be refolded to reconstitute active GST before GST•Bind affinity purification. Reagents and a protocol for a simple, effective method of refolding are available in the Protein Refolding Kit (Cat. No. 70123-3). Note that active GST may not be obtained from all proteins refolded with the Protein Refolding Kit. Alternatively, if the fusion protein also contains a His•Tag® sequence, it is possible to solubilize inclusion bodies in a 1X His•Bind Binding Buffer containing 6 M urea or 6 M guanidine-HCl and load onto His•Bind resins (see Technical Bulletin 054 and 273 for details).

1. Harvest the cells by centrifugation at 10,000 × g for 5 min. Decant the supernatant and allow the cell pellet to drain as completely as possible. Resuspend the cells in 40 ml 1X GST Bind/Wash Buffer.
2. Sonicate briefly as described above to resuspend the pellet thoroughly and to shear the DNA.
3. Centrifuge at 5,000 × g for 15 min to collect the inclusion bodies and cellular debris while leaving other proteins in solution.
4. Remove the supernatant and suspend the pellet in 20 ml 1X GST Bind/Wash Buffer per 100 ml culture volume. Repeat step 3. Sonication may be necessary to resuspend the pellet. Sometimes repeating this step several times releases more trapped contaminating proteins.
5. Solubilize the washed inclusion bodies and refold prior to GST•Bind purification.

Note: rLysozyme Solution™ may be added (although it is not required) at step 4 for processing insoluble protein fractions. Lysozyme has been shown to improve the purity of inclusion body preparations by digesting cell wall debris. Add 1 KU/ml (final concentration) to the resuspended material in 1X GST Bind/Wash Buffer. Vortex gently to mix, incubate for 5–10 min and then proceed with centrifugation.

Cell extract preparation using BugBuster® Reagent

BugBuster Protein Extraction Reagent is a simple, rapid, non-mechanical method of *E. coli* cell lysis. Induced cells are harvested by centrifugation, resuspended in BugBuster and incubated briefly at room temperature. Following clarification by centrifugation, the supernatant, containing soluble protein is ready for GST•Bind purification. BugBuster can also be used for the preparation of highly purified inclusion bodies prior to working with insoluble target proteins.

BugBuster Protein Extraction Reagent plus Benzonase® Nuclease is an efficient combination for gently releasing target proteins and reducing extract viscosity prior to downstream processing. Cells are harvested by centrifugation, followed by suspension in BugBuster reagent at room temperature and Benzonase treatment. During a brief incubation, soluble proteins are released and nucleic acids are digested. Insoluble protein and cell debris is easily removed by centrifugation. The resulting low viscosity clarified extract contains soluble protein ready for GST•Bind purification. Other formulations of BugBuster are available such as 10X BugBuster (concentrated BugBuster solution), BugBuster (primary amine-free) which consists of BugBuster in PIPPS buffer and BugBuster HT (a ready to use mixture of BugBuster and Benzonase). Review Technical Bulletin 245 for more information.

The addition of rLysozyme Solution™ in combination with BugBuster Reagent and Benzonase Nuclease enhances the efficiency of protein extraction and is highly recommended to achieve complete cell lysis and release of large proteins. Addition of rLysozyme is unnecessary when using hosts expressing T7 lysozyme (e.g. pLysS).

General Considerations for BugBuster Protein Extraction Reagent

- BugBuster and Benzonase Nuclease are most efficient when used at room temperature.
- BugBuster Reagent can be used on fresh or frozen cell pellets. For comparisons of multiple samples (e.g. extended time course analysis) all cell pellets should be processed identically (all fresh or all frozen). Superior extraction efficiencies may be obtained by freezing the cell pellet prior to resuspension in BugBuster. Multiple freeze-thaw cycles are not recommended because some target proteins may be inactivated.

Note: For optimal extraction especially of high molecular weight proteins (>70 kDa), freezing of bacterial cell pellets prior to BugBuster extraction and/or addition of rLysozyme™ Solution (Cat. No. 71110-3); for non-pLysS or pLysE hosts) is highly recommended.

- Extraction efficiency is somewhat strain-dependant and appears to be especially efficient with the BL21 strain and derivatives (BLR, Tuner™, Rosetta™, and Origami™ B strains).
- Benzonase can be diluted with 50 mM Tris-HCl pH 8, 20 mM NaCl and 2 mM $MgCl_2$ for handling small quantities and can be stored at 4°C for several days without loss of activity.
- Benzonase is not recommended for nuclease free preparations. GST•Bind Resin purification may not remove all Benzonase. Residual nuclease activity can be monitored by incubation of the purified protein with RNA or DNA markers followed by gel analysis.
- Benzonase and rLysozyme™ Solution do not interfere with FRETWorks™ S•Tag™ Assay, the S•Tag Rapid Assay or the GST•Tag™ Assay.
- Benzonase Nuclease is inhibited (approximately 50% reduction in relative activity) by monovalent cation concentrations > 50 mm, phosphate concentrations > 20 mM, and by ammonium sulfate concentrations > 25 mM.
- More information on Benzonase Nuclease is available in Technical Bulletin 261.
- For SDS-PAGE and Western blot analysis of BugBuster extracts prepared by the recommended protocols, an extract load volume of approximately 2.4 µl would give a normalized amount of protein, given a concentration factor of 25 and an OD_{600} of 3 at harvest, using a 15 well mini gel. When 1.5 culture volume of BugBuster Reagent is used, this would correspond to a load volume of approximately 12 µl (since the concentration factor is 5). Because the optimal amount of material to lead will vary with the expression level of the target protein, the efficiency of the extraction, and detection sensitivity of the Western blot method, these amounts should be used as guidelines only.

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Soluble fraction

Note: This fraction will consist of soluble protein present in both the periplasm and cytoplasm. If a separate periplasmic fraction is desired, first follow the osmotic shock procedure given in the pET System Manual (Technical Bulletin 055) or other suitable method. Then the pellet from the osmotic shock procedure can be used in this protocol.

1. Harvest cells from liquid culture by centrifugation at 10,000 × g for 10 min using a pre-weighed centrifuge tube. For small scale extractions, (1.5 ml or less) centrifugation can be performed in a 1.5 ml tube at 14,000–16,000 × g. Decant and allow the pellet to drain, removing as much liquid as possible. Determine the wet weight of the pellet.
2. Resuspend the cell pellet in room temperature BugBuster Reagent by pipetting or gentle vortexing, using 5 ml reagent per gram of wet cell paste For small cultures use up to 1/5 culture volume for resuspension (e.g. use 300 µl BugBuster for 1.5 ml cultures). There are no adverse effects to using larger volumes of BugBuster Reagent.
   Optional:
   a) Add 1 µl (25 units) Benzonase® Nuclease per 1 ml of BugBuster reagent used for resuspension. Although Benzonase requires Mg^{2+} for activation, no addition of Mg^{2+} is required for viscosity reduction and nucleic acid digestion under the conditions described here.
   b) Add 1 KU rLysozyme™ Solution per 1 ml BugBuster Reagent (5 KU/g cell paste). rLysoyzme Solution can be diluted using rLysozyme Dilution Buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1% Triton® X-100]. Dilutions should be used as soon as possible and stored on ice or at 4°C until use. Do not store diluted rLysozyme Solution at -20°C as freezing may result in loss of activity.
   c) Add protease inhibitors. Protease inhibitors are compatible with BugBuster, rLysozyme and Benzonase Nuclease. Serine protease inhibitors should be avoided if the target protein is to be treated with Thrombin (Cat. No. 69671-3), Factor Xa (Cat. No. 69036-3), or Recombinant Enterokinase (Cat. No. 69066-3). Although purification may remove active inhibitors, dialysis and gel filtration are recommended prior to cleavage.
3. Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 10–20 min at room temperature. If Benzonase was added, the extract should not be viscous at the end of the incubation.
4. Remove insoluble cell debris by centrifugation at 16,000 × g for 20 min at 4°C. If desired, save the pellet for inclusion body purification as described below.
5. Transfer the supernatant to a fresh tube. The soluble extract can be loaded directly onto GST•Bind Resin or GST•Mag Agarose Beads. Maintain clarified extracts on ice for short term storage (2–3 h) or store at 4°C. Freezing of GST•Tag proteins is not recommended.

Inclusion body purification

1. Process the induced culture according to steps 1–4 above for the soluble protein fraction.
2. Resuspend the pellet from step 4 above in the same volume of BugBuster Reagent that was used to resuspend the original cell pellet. Pipet up and down and vortex to obtain an even suspension Complete resuspension of the pellet is critical to obtaining a high purity preparation in order to solubilize and remove contaminating proteins.
3. Add rLysozyme Solution to a final concentration of 1 KU/ml. Vortex gently to mix and incubate at room temperature for 5–10 min.
4. Add 6 volumes of 1:10 diluted BugBuster reagent (in deionized water) to the suspension and vortex for 1 min.
5. Centrifuge the suspension at 5,000 × g for 15 min at 4°C to collect the inclusion bodies. Remove the supernatant with a pipet.
6. Resuspend the inclusion bodies in 0.5 the original culture volume of 1:10 diluted BugBuster, mix by vortexing, and centrifuge as in step 5 twice. Resuspend once more but centrifuge at 16,000 × g for 15 min at 4°C and remove the supernatant.
7. Resuspend the final pellet of purified inclusion bodies in the buffer of your choice. GST•Tag™ fusion proteins must be refolded to reconstitute active GST before affinity purification. The final pellet of inclusion bodies is compatible with resuspension in 1X Solubilization Buffer provided in Novagen's Protein Refolding Kit (Cat. No.70123-3; see

Technical Bulletin 234). Alternatively if the protein contains a His•Tag® sequence, it is possible to dissolve inclusion bodies in 1X His•Bind Binding Buffer containing 6M Urea or 6M guanidine-HCl and load onto His•Bind Resins (see Technical Bulletin 054 and 273).

Cell extract preparation using PopCulture™ Reagent

PopCulture Reagent efficiently extracts protein from *E. coli* directly in the culture medium without cell harvest. An induced culture of *E. coli* is treated with PopCulture Reagent for 10 min at room temperature. Proteins in this prepared extract may be assayed directly or purified by adding equilibrated GST•Bind Resin or GST•Mag Agarose Beads for purification. To further enhance the effectiveness of PopCulture-mediated cell lysis and protein extract preparation rLysozyme™ Solution (Cat. No. 71110-3) and/or Benzonase® Nuclease (Cat. No. 70746-3) may be added prior to use. rLysozyme Solution increases the efficiency of cell lysis and protein extraction and Benzonase Nuclease may be added to reduce lysate viscosity for increased flow rates during purification.

1. Culture *E. coli* transformed with an expression plasmid under standard conditions for target protein expression. See *"Growth and Induction"* below for more information.
2. Add 0.1 culture volume of PopCulture Reagent.
   Optional:
   a) Add 40 U (1 µl of a 1:750 dilution) rLysozyme Solution per 1 ml of original culture volume. Additional lysozyme is not necessary if the host strain contains the pLysS or pLysE plasmid. rLysozyme Solution can be diluted with rLysozyme Dilution Buffer. If the rLysozyme Solution is diluted, it should be used as soon as possible. Store the diluted rLysozyme Solution on ice or at 4°C until use. Do not store diluted rLysozyme Solution at –20°C because freezing may result in loss of activity.
   b) Add 1 µl (25 U) Benzonase per 1 ml of the original culture volume.
   c) rLysozyme and Benzonase can be pre-mixed with PopCulture. This allows addition of PopCulture plus the processing enzymes in a single step and facilitates rapid sample processing. Pre-mixed PopCulture, rLysozyme and Benzonase should be used the same day. and stored at 4°C.
3. Pipet up and down to mix and incubate for 10–15 min at room temperature.
4. After the PopCulture incubation is complete, this prepared extract can be assayed directly and/or combined with equilibrated GST•Bind Resin or GST•Mag Agarose Beads. Follow the standard protein purification procedures according to the type of resin used in the following pages of this technical bulletin.

Note: *Direct analysis of the target protein expression by SDS-PAGE with Coomassie blue staining is possible with highly expressed proteins. Use a maximum load volume for the well with 4X SDS Sample Buffer (Cat. No. 70607-3).*

Growth and induction

Bacterial strain: Protein extraction is efficient for both B (e.g. BL21) and K-12 (e.g. NovaBlue) strains. Other B and K-12 strains should be compatible; however, it is possible that strain-dependent differences could occur. It is important to consider the growth rate of the bacteria if performing the entire experiment in one day. In general, BL21 and its derivatives have a vigorous growth rate and are ideal for this extraction method. The use of lysozyme expressing hosts containing pLysS or pLysE plasmids increase the protein extraction efficiency without the addition of exogenous lysozyme.

Cell Culture: The entire procedure from culturing through purification can be done in one day. Therefore it is ideal for small culture volumes ($\leq$ 50 ml) or multiwell plate cultures. For example, take a single colony or several colonies of the strain from a freshly streaked plate (with appropriate antibiotics) and inoculate 3 ml of pre-warmed medium. After the OD_{600} reaches 0.8–1, inoculate 50 ml medium with the entire 3 ml or let the culture grow until the OD_{600} reaches 0.8–1.5 and then induce. Use pre-warmed medium to avoid a lag phase in bacterial growth. Note that bacterial strains with a slower growth rate may be difficult to use in a one day procedure. Alternatively, grow a culture overnight at a reduced temperature (24–26°C) and induce the following day after raising the temperature to 37°C and growing to an appropriate OD_{600} for induction.

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Additional guidelines for PopCulture Reagent optimization and compatibility

Lysozyme: Experiments with PopCulture show that the addition of lysozyme increases the efficiency of cell lysis and protein extraction (5-6). rLysozyme Solution can be added separately or T7 lysozyme can be present in the cell prior to PopCulture treatment by incorporating the pLysS or pLysE plasmid in the expression host strain.

Inclusion of the pLysS or pLysE plasmid reduces the number of manipulations during protein extraction and purification. The pLysS plasmid is based on the pACYC184 backbone and has the p15A origin of replication, making it compatible with the pBR322 and pUC derived expression plasmids such as pET and pTriEx™. The pLysS and pLysE plasmid is maintained during culturing with a final concentration of 34 µg/ml chloramphenicol.

rLysozyme™ Solution is effective using 40 U (1 µl of a 1:750 dilution of rLysozyme Solution) per 1 ml of initial culture volume. rLysoyzme Solution can be diluted using rLysozyme Dilution Buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1% Triton® X-100]. Dilutions should be used as soon as possible and stored on ice or at 4°C until use. Do not store diluted rLysozyme Solution at –20°C as freezing may result in loss of activity.

Benzonase Nuclease: Cell extracts may become viscous from nucleic acids released during cell lysis from a high density culture and/or when lysozyme is used. These nucleic acids can interfere with effective protein purification especially with conventional purification resins. The addition of Benzonase Nuclease (Cat. No. 70746-3) will degrade all forms of DNA and RNA (single stranded, double stranded, linear and circular) to 5'-monophosphate terminated oligonucleotides 2–5 bases in length (2-7). Although Benzonase Nuclease requires Mg^{2+} for activation, it does not appear to require additional Mg^{2+} under the conditions described here. The activity is sufficient for effective viscosity reduction and nucleic acid digestion. Benzonase Nuclease treatment is not generally recommended for purification of proteins that must be nuclease free. However, depending on the processing methods, Benzonase Nuclease may be removed during purification by anion exchange chromatography. Residual nuclease activity can be checked by incubation of the purified protein with RNA or DNA markers followed by gel analysis. For further details on Benzonase Nuclease see Technical Bulletin 261.

Volume of PopCulture Reagent: There are no adverse effects to using higher ratios of PopCulture Reagent. If the cells need to be concentrated prior to extraction, the volume of PopCulture can be adjusted accordingly.

Temperature of extraction: PopCulture extraction along with lysozyme and Benzonase Nuclease can be performed at room temperature or at 4°C. However, incubation times may need to be increased because rLysozyme or Benzonase activities are decreased at lower temperatures.

pH of extraction: Acidic pH (< 5.0) can degrade components of PopCulture.

Protein assays: Because proteins generally retain their activities and conformation, protein specific activity and immunoassays are likely to be compatible with PopCulture extraction. PopCulture is compatible with the GST•Tag™ Assay, S•Tag™ Rapid Assay and FRETWorks™ S•Tag Assay Kits. Protein Assays including BCA and Coomassie blue are also fully compatible with PopCulture extracts.

Protease inhibitors: Protease inhibitors may be added with the PopCulture Reagent to the culture medium. Serine protease inhibitors should be avoided if the target protein is to be treated with the serine proteases thrombin, Factor Xa or enterokinase. Active inhibitor may be carried through the purification process and affect cleavage reactions. Note that protease inhibitor cocktails which include EDTA would not be compatible with GST•Bind Resin or GST•Mag Agarose Beads.

GST Bind Resin Chromatography

Optimal results with GST•Bind Resin are usually obtained by the use of an amount of resin such that the binding capacity is 10–20% excess over the amount of target protein in the extract sample. Thus, it is usually beneficial to obtain an estimate of the mass of target protein in the extract, and then prepare a corresponding amount of resin. As a guideline, the capacity of the GST•Bind Resin is estimated at 5–8 mg/ml settled resin. A yield of 20 mg of target protein per 100 ml culture is not unusual in the pET system. However, the expression level of individual target proteins varies considerably depending on the gene sequence, vector construct, host strain and conditions of growth and induction. SDS-PAGE or Western blotting can roughly estimate the mass of target protein in crude extracts. More accurate quantification can be obtained using the S•Tag™ Rapid Assay or FRETWorks™ S•Tag Assay for S•Tag fusion proteins, or other protein-specific assays. The most reliable data are obtained using the GST•Tag Assay, which measures the amount of functional GST in the extract. Since only functional GST is able to bind to the resin and there may be a significant fraction of the fusion protein which is improperly folded or otherwise inactive, this method accurately determines the amount of target protein that could be expected to be purified by glutathione affinity.

GST•Bind buffer preparation

1. Dilute an appropriate amount of the supplied stock of 10X GST Bind/Wash Buffer to 1X with sterile deionized water before use. You will need approximately 15 volumes of GST Bind/Wash Buffer for column chromatography, 26 volumes for batch purification from extracts without medium (mechanical or BugBuster method) or 51 volumes for batch purification from extracts with medium (PopCulture method). One volume is equivalent to the settled bed volume (e.g. 100 µl of slurry yields 50 µl of resin for a settled bed volume of 50 µl)
2. Prepare 10X GST Elution Buffer containing 100mM reduced glutathione by dissolving 1 g reduced glutathione in 32.5 ml 10X Glutathione Reconstitution Buffer. After the 10X GST Elution Buffer is prepared, it must be divided into working volumes and stored at –20°C to prevent oxidation of the glutathione. 10X GST Elution Buffer is stable at –20°C for 6 months with no more than 5 freeze/thaw cycles. Immediately before use, dilute the 10X GST Elution Buffer to 1X with sterile deionized water. You will need approximately 3 volumes of 1X GST Elution Buffer for column chromatography or batch purification from extracts without medium (mechanical or BugBuster method) or 2–6 volumes for batch purification from extracts with medium (PopCulture method).

Note: The 1X GST Elution Buffer must be prepared fresh immediately before use to prevent oxidation of the glutathione.

Column chromatography

Notes: Purification is performed at room temperature. Allow the resin and buffer components to equilibrate to room temperature before use. In addition to a column format, GST•Bind Resin can also be used in a batchwise fashion (see page 9).

1. Gently mix the bottle of GST•Bind Resin by inversion until completely suspended. Using a wide-mouth pipet, transfer the desired amount of slurry to a column. Allow the resin to pack under gravity flow. When pipetting the resin, remember that it is supplied as a 50% slurry. Small polypropylene columns (Cat. No. 69673-3) hold 2.5 ml of settled resin and can be conveniently used to purify up to 12–20 mg of target protein.
2. When the level of storage buffer (20% ethanol) drops to the top of the column bed, wash the resin with 5 volumes of GST Bind/Wash Buffer.

Note: Immediately before loading the column, bring the extract rapidly to room temperature using a room temperature water bath.

3. Allow the GST Bind/Wash Buffer to drain to the top of the column bed and load the column with the prepared extract. A flow rate of about 10 column volumes per hour is optimal for efficient purification. If the flow rate is too fast, more impurities will contaminate the eluted fraction. Collect the flow-through fraction and store on ice.

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4. Wash the column with 10 volumes of 1X GST Bind/Wash Buffer. Collect the flow-through fraction and store on ice.
5. Elute the bound protein with 3 volumes of 1X GST Elution Buffer.
6. Analyze the flow through fractions for the presence of the target protein. Target protein that does not have functional GST will not bind to the resin. Inactive GST•Tag protein may be purified using another method or solubilization and refolding may reconstitute GST activity

Small scale purification -batch method

Extract prepared without medium – mechanical or BugBuster Reagent

Notes: *Settled bed volumes of 50 µl to 200 µl can be handled in 1.5 ml microcentrifuge tubes; for larger volumes use 15 ml or 50 ml sterile disposable centrifuge tubes. For each wash, add the buffer, invert the tube several times to mix, and spin for 1–5 min at 400–1000 × g.*

1. Gently mix the bottle of GST•Bind Resin by inversion until completely suspended. Using a wide-mouth pipet, transfer the desired amount of slurry to a centrifuge tube.
2. Pellet the resin by centrifugation, carefully remove the supernatant and discard. Add 5 volumes of 1X GST Bind/Wash Buffer, invert to mix and spin as before.
3. Discard the final supernatant and add 1 volume of 1X GST Bind/Wash Buffer. Resuspend the resin (now a 50% slurry) by inversion.
4. Add the protein sample and incubate the mixture at room temperature for 30 min with gentle agitation.
5. Centrifuge and transfer the supernatant, which contains unbound proteins, to a fresh tube and store on ice. Resuspend the resin in 10 volumes of 1X GST Bind/Wash Buffer, spin, remove the supernatant, store on ice and repeat.
6. Elute the bound protein from the pellet resin by adding 1 volume of 1X GST Elution Buffer. Incubate at room temperature with gentle agitation for 10 min. Centrifuge as in step 6 and transfer the supernatant, which contains the purified fusion protein, to a fresh tube. Repeat the elution twice more, each time with 1 volume of 1X GST Elution Buffer and pool the supernatants.
7. Analyze the eluted fraction and the supernatant from step 5 for the presence of the target protein. Target protein that does not have functional GST will not bind to the GST•Mag Agarose Beads. Inactive GST•Tag protein may be purified using another method or solubilization and refolding may reconstitute GST activity.

Extracts prepared with medium – PopCulture™ Reagent

A settled bed volume of 50 µl is recommended per 1 ml of original culture volume. For each wash step add the appropriate buffer and remove the supernatant by centrifuging for 1–5 min. at 400–1000 × g. Transfer the entire mixture to a 0.5 cm diameter column or use a vacuum filter plate system. In the following protocol, one volume is equivalent to the settled bed volume (e.g. 100 µl of slurry yields 50µl of resin for a settled bed volume of 50 µl).

1. Gently mix the bottle of GST•Bind Resin by inversion until completely suspended. Using a wide-mouth pipet, transfer the desired amount of slurry to a centrifuge tube.
2. Pellet the resin by centrifugation, carefully remove the supernatant and discard. Add 5 volumes of 1X GST Bind/Wash Buffer, invert to mix and spin as before.
3. Discard the final supernatant and add 1 volume of 1X GST Bind/Wash Buffer. Resuspend the resin (now a 50% slurry) by inversion.
4. Add the equilibrated resin to the prepared extract and mix gently by pipetting. Incubate for 5 min with mixing.

Note: *The sample can be processed using a vacuum filter plate system, transferred to a 0.5 cm diameter column or a microcentrifuge tube. As required, centrifuge at 400–1000 × g prior to removing the supernatant in the following wash steps.*

5. Centrifuge and transfer the supernatant, which contains unbound proteins, to a fresh tube and store on ice. Resuspend resin in 40 volumes of 1X GST•Bind/Wash buffer, spin, remove the supernatant, store on ice and repeat.

6. Elute the bound protein by adding 1–3 volumes of 1X GST Elution Buffer. Incubate at room temperature with gentle agitation for 5 min. Transfer the supernatant, which contains the purified fusion protein, to a fresh tube.
   Optional: Repeat the elution again with 1–3 volumes of 1X GST Elution Buffer and pool the supernatants. Larger elution volumes may recover more target protein.
7. Analyze the supernatant from step 5 for the presence of the target protein. Target protein that does not have functional GST will not bind to the resin. Inactive GST•Tag protein may be purified using another method or solubilization and refolding may reconstitute GST activity

Regeneration and storage

GST•Bind Resin can be reused several times without regeneration. However, if flow rates or protein capacity decline, the cause is usually the accumulation of non-specifically bound proteins or protein aggregates. To remove bound proteins, the resin should be washed with 10 volumes 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, followed by 10 volumes of 100 mM sodium acetate, pH 4.5, 0.5 M NaCl. Other buffers that may remove column contaminants include 6 M urea, 6 M guanidine-HCl, or low polarity solvents such as 50–70% ethanol or 50% ethylene glycol. Any of these treatments should be immediately followed by a full equilibration with 10 column volumes of 1X GST Bind/Wash Buffer. For long-term storage, the resin should be equilibrated in 20% ethanol/80% water and stored at 4 C.

GST•Mag™ Agarose Beads

It is usually beneficial to obtain an estimate of the quantity of target protein in the extract, and then prepare a corresponding amount of GST•Mag Agarose Beads. As a guideline, the capacity of the magnetic beads is 2 mg/ml settled resin. SDS-PAGE or Western blotting can roughly estimate the mass of target protein in crude extracts. More accurate quantification can be obtained using the S•Tag™ Rapid Assay or FRETWorks™ S•Tag Assay for S•Tag fusion proteins, or other protein-specific assays. The most reliable data are obtained using the GST•Tag Assay, which measures the amount of functional GST in the extract. Since only functional GST is able to bind to the magnetic beads and there may be a significant fraction of the fusion protein which is improperly folded or otherwise inactive, this method accurately determines the amount of target protein that could be expected to be purified by glutathione affinity.

GST•Bind Buffer preparation

1. Dilute an appropriate amount of the supplied stock of GST Bind/Wash Buffer to 1X with sterile deionized water before use. Approximately 42 volumes of GST•Bind/Wash Buffer is needed for batch purification from extracts without medium (mechanical or BugBuster method) or 73–93 volumes for batch purification from extracts with medium (PopCulture method). One volume is equivalent to the settled bed volume (e.g. 100 µl of slurry yields 50 µl of resin for a settled bed volume of 50 µl).
2. Prepare 10X GST Elution Buffer containing 100 mM reduced glutathione by dissolving 1 g reduced glutathione in 32.5 ml 10X Glutathione Reconstitution Buffer. Once the 10X GST Elution Buffer is prepared, it must be divided into working volumes and stored at –20°C to prevent oxidation of the glutathione. 10X GST Elution Buffer is stable at –20°C for 6 months with no more than 5 freeze/thaws. Immediately before use, dilute the 10X GST Elution Buffer to 1X with sterile deionized water. You will need between 2–8 volumes of 1X GST Elution Buffer for purification from extracts without medium (mechanical or BugBuster Reagent) or 4–8 volumes from extracts with medium (PopCulture Reagent).

Note: The 1X GST Elution Buffer must be prepared fresh immediately before use to prevent oxidation of the glutathione.

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GST•Mag™ Agarose Beads purification

Extracts prepared without medium – mechanical and BugBuster Reagent

Purification is performed at room temperature. Allow the magnetic beads and buffer components to equilibrate to room temperature before use.

1. Gently mix the bottle of GST•Mag Agarose Beads by inversion until completely suspended. Transfer the desired amount of slurry to a 1.5 ml, 15 ml or 50 ml tube. Place the tube in a Magnetight™ rack or stand (Cat. No. 69964-3 or 70747-3) to collect the beads.
2. Remove the supernatant and wash the beads 3 times with 4 volumes 1X GST Bind/Wash Buffer For each wash, remove the tube from the magnetic rack to resuspend the beads with buffer and then replace in magnetic rack to remove the buffer.

Note: Immediately before adding the extract to the beads bring the extract rapidly to room temperature using a room temperature water bath.

3. After the final wash, resuspend the beads in 1 volume of 1X GST Bind/Wash Buffer to make a 50% slurry.
4. Combine the equilibrated beads with the prepared cell extract. Mix well by inverting several times. Incubate on a shaking platform for 10–20 min.
5. Place tube in a magnetic rack to collect the beads and remove the supernatant.

Note: The supernatant should be retained for analysis of any target protein with inactive GST that cannot bind to the magnetic beads.

6. Wash the beads 3 times with 8 volumes of 1X GST Bind/Wash Buffer. For each wash, remove the tube from the magnetic rack to resuspend the beads with buffer and then replace in the magnetic rack to remove the buffer.
7. Elute the bound protein with 2–8 volumes of 1X GST Elution Buffer. After removing the tube from the magnetic rack, resuspend the beads and incubate for 10 min with occasional mixing by tapping the bottom of the tube. Do not invert the tube.

Note: Typically 3 volumes of 1X Elution Buffer are sufficient for elution but the volume may be adjusted as needed.

8. Place the tube in a magnetic rack to collect the beads. Remove eluted protein to a fresh tube.
9. Analyze the eluted fraction and the supernatant from step 5 for the presence of the target protein. Target protein that does not have functional GST will not bind to the GST•Mag Agarose beads. Inactive GST•Tag protein may be purified using another method or solubilization and refolding may reconstitute GST activity.

Extracts prepared with medium – PopCulture Reagent

For each 1 ml of original culture prepared with PopCulture Reagent, use 50 µl of a 50% slurry of GST•Mag Beads. Bead volume may be adjusted according to yield estimates. Purification is performed at room temperature. Allow the magnetic beads and buffer components to equilibrate to room temperature before use.

1. Gently mix the bottle of GST•Mag Agarose Beads by inversion until completely suspended. Transfer the appropriate amount of resuspended GST•Mag Agarose Beads to a tube or multiwell plate. Place the tube in a magnetic rack (Cat. No. 69964 or 70747) or other magnetic device to collect the beads.
2. Remove the supernatant and wash the beads 3 times with 4 volumes 1X GST Bind/Wash Buffer For each wash, remove the tube from the magnetic rack to resuspend the beads with buffer and then replace in magnetic rack to remove the buffer.

Note: Immediately before adding the extract to the beads bring the extract rapidly to room temperature using a room temperature water bath.

3. After the final wash, resuspend the beads in 1 volume of 1X GST Bind/Wash Buffer.
4. Combine the equilibrated beads with the prepared cell extract. Incubate for 5 min with mixing.
5. Remove the supernatant and store on ice.

6. Wash the beads 3 times with 20–30 volumes of 1X GST Bind/Wash Buffer. For each wash, remove the sample from the magnetic field to resuspend the beads and then apply the magnetic field to remove the buffer.
7. Elute the bound protein with 2–4 volumes of 1X GST Elution Buffer. Incubate for 5 min and remove the supernatant.
   Optional: Repeat with an additional 2–4 volumes of 1X GST Elution Buffer and pool the supernatants.
8. Analyze the eluted fraction and the supernatant from step 5 for the presence of the target protein. Target protein that does not have functional GST will not bind to the GST•Mag Agarose beads. Inactive GST•Tag protein may be purified using another method or solubilization and refolding may reconstitute GST activity.

Processing the Sample after Elution

After eluting the purified protein from the GST•Bind Resin or GST•Mag™ Agarose Beads, the sample can be concentrated or the buffer changed by one of several methods. The storage buffer for the purified protein is often determined through an empirical process. Inappropriate storage buffer may lead to precipitation of the protein.

1. Dialyze into the buffer of choice. After dialysis, the sample may be concentrated by sprinkling solid polyethylene glycol (15,000–20,000 molecular weight) or Sephadex G-50 (Pharmacia) on the dialysis tubing. Use dialysis tubing with an exclusion limit of 6,000 MW or less, and leave the solid in contact with the tubing until the desired volume is reached, replacing it with fresh solid as necessary.
2. Use plastic disposable microconcentrator units (e.g., Centricon; Amicon) as directed by the manufacturer to concentrate the sample or exchange the buffer with ultrafiltration.

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