instructions

product code:

27-4570-01
27-4570-02

Bulk and RediPack GST Purification Modules

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

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GE imagination at work

Handling

Storage
Store at 4 °C.
Do not freeze.

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Quality control

GST Purification Modules are tested for their ability to purify a GST fusion protein by Glutathione Sepharose 4B affinity chromatography.

Materials not supplied

- **Sonicator**—equipped with appropriate tips.
- **Bottle**—500–1 000 ml for dilution of PBS.
- **Reagents, equipment and gels**—for SDS-PAGE

Components of the kit

The Bulk GST Purification Module can purify up to 50 mg of GST Fusion protein; the RediPack GST Purification Module can purify a maximum 20 mg of fusion protein.

The following components require preparation before use. Refer to "Essential preliminaries" (page 12) for details.

10× PBS	1.4 M NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4 (pH 7.3).
Reduced glutathione	0.154 g. Addition of 50 ml of dilution buffer produces a solution of 10 mM glutathione, 50 mM Tris-HCl (pH 8.0).
Dilution buffer	50 mM Tris-HCl (pH 8.0).
IPTG	Isopropyl β-D-thiogalactoside, 500 mg.
Glutathione Sepharose™ 4B	10 ml of Glutathione Sepharose 4B supplied as a 75% slurry in 20% ethanol (Bulk GST Purification Module, 27-4570-01) or two RediPack columns containing 2 ml of gel in 20% ethanol as a preservative (RediPack GST Purification Module, 27-4570-02).
Disposable columns	Five disposable columns are provided in the Bulk GST Purification Module (27-4570-01).

Materials not supplied *(continued)*

- **2× YTA medium**—

Tryptone	16 g/l
Yeast extract	10 g/l
NaCl	5 g/l

  Dissolve above ingredients in 900 ml of distilled H_2O. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 liter. Sterilize by autoclaving for 20 min. Once the medium has cooled, aseptically add 1 ml of a 100 mg/ml ampicillin stock solution (final concentration 100 µg/ml). To prepare as a solid medium, add 12–15 g of agar prior to autoclaving.

- **6× SDS loading buffer (1)**—0.35 M Tris-HCl (pH 6.8), 10.28% (w/v) SDS, 36% (v/v) glycerol, 0.6 M dithiothreitol (or 5% β-mercaptoethanol), 0.012% (w/v) bromophenol blue. Store in 0.5 ml aliquots at -20 °C.

World Wide Web address

http://www.gehealthcare.com/lifesciences

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

Safety warnings and precautions

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

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

Introduction

The Glutathione S-transferase (GST) Gene Fusion System is an integrated system for the expression, purification and detection of fusion proteins produced in *E. coli*. The system consists of three major components: pGEX plasmid vectors, two GST Purification Modules and the GST Detection Module. A series of site-specific proteases complements the system. The pGEX plasmids are designed for inducible, high-level intracellular expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST (2). Fusion proteins are easily purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B contained in the GST Purification Modules. Cleavage of the desired protein from GST is achieved using a site-specific protease whose recognition sequence is located immediately upstream from the multiple cloning site on the pGEX plasmids. Fusion proteins can be detected using a colorimetric assay or immunoassay provided in the GST Detection Module. The system has been used successfully in many applications such as molecular immunology (3), vaccine production (4, 5) and studies involving protein-protein (6) and DNA-protein (7) interactions.

pGEX vectors

The pGEX vectors feature a *tac* promoter for chemically inducible, high-level expression and the *lacI^q* gene, which allows the use of any *E. coli* host. Collectively, the pGEX series of vectors cover all three translational reading frames and contain the protease-recognition sites for either thrombin, factor Xa or PreScission™ Protease. pGEX-2TK contains the recognition site for the catalytic subunit of cAMP-dependent protein kinase. This site allows direct labelling of fusion proteins

using [γ-^{32}P]ATP for autoradiographic detection (8). For detailed maps of the pGEX vectors, please refer to Appendix 2, pages 28–29. Complete DNA sequences and restriction site data are available at the GE Healthcare Web site (http://www.gehealthcare.com/lifesciences).

Purification modules

The GST Purification Modules feature the GST-glutathione affinity system for a rapid, mild procedure for the affinity purification of GST fusion proteins. Using Glutathione Sepharose 4B, fusion proteins can be purified to > 90% in a single step. Fusion proteins are recovered from the matrix under mild elution conditions (10 mM glutathione) that preserve antigenicity and functionality of the proteins.

GST fusion proteins are produced in *E. coli* cells carrying a recombinant pGEX plasmid. Protein expression from a pGEX plasmid is under the control of the *tac* promoter, which is induced by the lactose analog isopropyl β-D-thiogalactoside (IPTG). Induced cultures are allowed to express GST fusion proteins for several hours, after which time cells are harvested, and then lysed by mild sonication. The bacterial lysate is cleared of cellular debris by centrifugation, and the cleared lysate is ready to be applied directly to Glutathione Sepharose 4B.

After the fusion proteins are bound to the matrix, it is washed with buffer to remove nonspecifically bound proteins. Bound GST fusion proteins can then be eluted with the mild elution buffer and used in a variety of applications.

The GST Purification Modules feature Glutathione Sepharose 4B packaged in different formats. The Bulk GST Purification Module (27-4570-01) provides a 10 ml bulk pack of Glutathione Sepharose 4B and five disposable columns. With this module, fusion proteins can be purified using either column chromatography or a batch method. The Bulk Module provides more flexibility than the RediPack format, because purification can be performed with 100 µl (8) to 2 ml (2) of Glutathione Sepharose 4B. The various scales in volume can be accommodated by either batch or subsequent column applications. The Bulk Module contains enough reagents for five 10-mg purifications. Protocols for using Glutathione Sepharose in this format are given in Protocol 4, and a timetable is given on pages 10–11.

The RediPack GST Purification Module (27-4570-02) provides convenient disposable columns prepacked with 2 ml of Glutathione Sepharose 4B, enough for two 10-mg purifications. A protocol for use of the prepacked columns is given in Protocol 5, and a timetable is given on pages 10–11.

If separation of the cloned protein from the GST affinity handle is desired, the fusion protein can be digested with the appropriate site-specific protease while the fusion protein is bound to Glutathione Sepharose 4B. Alternatively, the fusion protein can be digested following elution from the matrix. Cleavage of the bound fusion protein eliminates the extra step of separating the released protein from GST because the GST moiety remains bound to the matrix while the cloned protein is eluted using wash buffer (9).

Detection module

The GST Detection Module (27-4590-01) is designed to identify GST fusion proteins using either a biochemical or an immunological assay. GST fusion proteins produced using a pGEX plasmid in *E. coli* may be assayed using the GST substrate 1-chloro-2,4-dinitrobenzene (CDNB). This assay can be used to optimize conditions for expression or to trace steps in the purification of a GST fusion protein. GST from many sources, including the schistosomal form used in the pGEX system, has a high affinity for CDNB (10, 11). The enzyme catalyzes the conjugation of CDNB with glutathione and produces a CDNB-glutathione product with a strong molar absorption at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). The enzymatic conjugation is relatively insensitive to reaction conditions and, as such, the assay can be used with crude bacterial sonicates or with purified GST fusion protein. The GST Detection Module also provides a goat anti-GST polyclonal antibody suitable for use in ELISAs or Western blots. The Anti-GST Antibody (27-4577-01) is also available as a stand-alone product.

Protocols

Introduction

The following protocol can be conveniently scaled to purify as little as 50 µg or as much as 50 mg of GST fusion protein using the Bulk GST Purification Module (27-4570-01).

Yield of fusion protein is highly variable and is affected by the nature of the fusion protein, the host cell, and the culture conditions used. Fusion protein yields can range from 1–10 mg/l (12). Table 1 can be used to approximate culture volumes based on an average yield of 2.5 mg/l.

The RediPack GST Purification Module (27-4570-02) contains two prepacked columns, and is designed for convenient purification of up to 10 mg of GST fusion protein per column.

Table 1. Reagent volume requirements for different protein yields.

Component	50 mg	10 mg	1 mg	50 µg
Culture volume	20 liters	4 liters	400 ml	20 ml
Volume sonicate	1000 ml	200 ml	20 ml	1 ml
Glutathione Sepharose bed volume*	10 ml	2 ml	200 µl	10 µl
1× PBS**	100 ml	20 ml	2 ml	100 µl
Glutathione elution buffer	10 ml	2 ml	200 µl	10 µl

* To obtain the desired bed volume, use twice the volume of 50% Glutathione Sepharose slurry prepared in Protocol 2 (i.e. 1 ml of 50% Glutathione Sepharose slurry will give a bed volume of 0.5 ml).

**This volume is “per wash.” Three washes are required in Protocol 4 and 5.

Timetables

Preparation of large-scale bacterial sonicates (Protocol 1)

1. Grow an overnight culture containing the clone of interest (12–15 h).
2. Dilute the overnight culture and grow to log phase (2–4 h).
3. Induce gene expression with IPTG and continue to grow culture (2–6 h).
4. Pellet cells by centrifugation and resuspend in 1× PBS (20 min).
5. Lyse cells by sonication and pellet cell debris by centrifugation (45 min).
6. Analyze expression products using CDNB assay (20 min), SDS-PAGE (1–3 h) or Western blot (5–12 h).

Preparation of Glutathione Sepharose 4B (Protocol 2)

1. Wash matrix with 1× PBS (20 min).
2. Prepare a 50% slurry for batch purification method (2 min) (Bulk GST Purification Module only).
3. Pack column with matrix slurry (2 min) (Bulk GST Purification Module only).

Screening of pGEX recombinants for fusion protein expression (Protocol 3)

1. Grow small-scale cultures (3–5 h).
2. Induce using IPTG and continue to grow (1–2 h).

3. Pellet cells by centrifugation and resuspend pellet in 1× PBS (2 min).
4. Lyse cells by freeze/thaw and remove cell debris (30 min).
5. Add prepared affinity matrix to cleared sonicate (10 min).
6. Wash bound matrix with 1× PBS (10 min).
7. Elute fusion proteins using glutathione elution buffer (15 min). Please see page 12 for the buffer formulation.
8. Analyze expression products using CDNB assay (20 min), SDS-PAGE (1–3 h) or Western blot (5–12 h).

Affinity purification of fusion proteins using Glutathione Sepharose 4B (Protocol 4 and 5)

1. Add sonicated fusion protein supernatant to prepared affinity matrix (30 min).
2. Wash bound matrix with 1× PBS (10–30 min).
3. Elute fusion proteins using glutathione elution buffer (20–40 min).
4. Analyze expression products using CDNB assay (20 min), SDS-PAGE (1–3 h) or Western blot (5–12 h).

Site-specific proteolytic cleavage of fusion proteins

1. Add the appropriate site-specific protease solution (thrombin, Factor Xa or PreScission Protease) to the purified fusion protein and incubate (2–16 h).
2. Analyze cleaved expression products by SDS-PAGE (1–3 h).

Essential preliminaries

Prior to first use, prepare the following kit reagents as indicated:

1× PBS: Dilute the 10× PBS in the kit with sterile water to 1× PBS final concentration. Store at 4 °C.

Note: If a precipitate is present in the 10× PBS, let the solution stand at room temperature, then swirl to dissolve the precipitate before diluting.

Glutathione elution buffer: Pour the entire bottle of dilution buffer (50 ml) into the bottle containing the reduced glutathione. Shake gently until the powder is completely dissolved. Dispense into 1–10 ml aliquots, and store at -20 °C until needed. Avoid more than five freeze/thaw cycles. This solution will be referred to as "glutathione elution buffer" in the procedures which follow.

IPTG solution: To prepare a 100 mM solution, dissolve the contents of the vial containing the lyophilized IPTG in 20 ml of sterile water. Dispense as 1 ml aliquots and store at -20 °C.

Additional reagents and materials may be found in "Materials not supplied," pages 3–4.

Small aliquots of samples should be retained at key steps in the procedure for analysis of the purification method (Protocol 6).

1 Culture growth and lysis

pGEX vectors carry the *lacI^q* gene, so there are no specific host requirements for propagation of the plasmids or for expression of fusion proteins. However, *E. coli* BL21 [F^-, *ompT, hsdS* (r_B^-, m_B^-), *gal*] (13, 14) is provided with the pGEX vectors and this strain is recommended for expression of GST fusion proteins. BL21 does not transform well and an alternate strain (e.g., JM105) is recommended for maintenance of the plasmid.

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

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Culture growth

Recipes are provided in "Materials not supplied," pages 3–4.

2× YTA Medium
100 mM IPTG (see "Essential preliminaries," page 10)
1× PBS (see "Essential preliminaries," page 10)

1.1 For large scale cultures, inoculate a single colony containing a recombinant pGEX plasmid into 2–100 ml of 2× YTA medium. For small-scale screening, pick several colonies of *E. coli* transformed with the pGEX recombinants to be screened and transfer each into separate tubes containing up to 12 ml of 2× YTA medium.

Note: For comparison, inoculate a control tube with bacteria transformed with the parental pGEX plasmid.

1.2 For large scale cultures, grow the above inoculum for 12–15 h at 37 °C with vigorous shaking. Then dilute the culture 1:100 into the desired volume of 2× YTA medium.

1.3 Grow liquid cultures to an A_{600} of 0.6–0.8 (3–5 h) with vigorous agitation at 20–37 °C.

1.4 Induce fusion protein expression by adding 1–10 µl of 100 mM IPTG for each ml of culture volume (final concentration = 0.1–1.0 mM).

1.5 Continue incubation for an additional 1–2 h.

1.6 Transfer the liquid cultures to labelled centrifuge tubes. If screening ≤ 1.5 ml, each induced culture may be transferred to a microcentrifuge tube.

1.7 Centrifuge to pellet cells (e.g. 5 s in a microcentrifuge or 7 700 × *g* for 10 min in a larger centrifuge) and discard the supernatants. Drain pellets thoroughly and place tubes on ice.

1.8 Resuspend each pellet in 50 µl of ice-cold 1× PBS for each ml of culture that was centrifuged. Remove 10 µl of these resuspended cells into labelled tubes (for later use in SDS-PAGE analysis).

Cell lysis

For larger scale cultures, sonication is recommended for the lysis of cells. However, the process is only efficient for cell suspensions greater than 2 ml, representing culture volumes of at least 40 ml. For lysing cultures ≤ 12 ml, the freeze/thaw method is recommended and can be performed in standard 1.5 ml microcentrifuge tubes.

Option A: Freeze/thaw

1.9(a) Prepare a 10 mg/ml lysozyme solution in water. Add 1 μl of lysozyme solution for each 100 μl of cell suspension (e.g. 6 μl to each tube containing 600 μl of cell suspension). Vortex tubes gently to disperse lysozyme. Allow tubes to incubate at room temperature for 5 min.

1.10(a) In a fume hood, prepare a dry ice bath in an ice bucket by adding dry ice and isopropanol until a slushy consistency is achieved. Prepare a warm water bath in a separate ice bucket.

1.11(a) Place tubes containing lysozyme-treated cell suspensions in the dry ice bath until cells are frozen solid, for ~ 20 s. Transfer tubes to a flotation carrier and place in the warm water bath until the suspension becomes fully liquid, ~1 min. Repeat freeze/thaw cycle 10 times.

1.12(a) Spin at full speed in a microcentrifuge for 10 min to remove insoluble material. Decant the supernatants into fresh tubes. Save a 10 μl aliquot of the insoluble material for analysis by SDS-PAGE.

Note: If the lysate is too viscous for handling, add DNase I to a final concentration of 10 μg/ml in step 1, above.

Option B: Sonication

1.9(b) Lyse the cells using a sonicator equipped with an appropriate probe.

Note: Lysis is complete when the cloudy cell suspension becomes translucent. The frequency and intensity of sonication should be adjusted such that complete lysis occurs in 10 s, without frothing (which can denature proteins). The extent of lysis may be monitored by microscopic examination of culture sonicates.

Note: Crude sonicates can be screened for the relative level of expression of GST fusion proteins using the GST substrate, CDNB, which is included in the GST Detection Module (27-4590-01).

Note: Large-scale sonicates may require the addition of 20% Triton™ X-100 to a final concentration of 1% to facilitate solubilization of the fusion protein.

1.10(b) Centrifuge sonicates for 5 min to remove insoluble material. Transfer the supernatants to fresh tubes. Save a 10 µl aliquot of the insoluble material for analysis by SDS-PAGE.

2 Preparation of Glutathione Sepharose 4B

Prepare kit reagents as described on page 12 before starting this procedure.

The Bulk GST Purification Module (27-4570-01) may be used for batch purification or to pack columns included with the kit with variable bed volumes of matrix.

Option A: Bulk matrix

2.1(a) Referring to Table 1 on page 9, determine the bed volume of Glutathione Sepharose 4B required for your application.

2.2(a) Gently shake the bottle of Glutathione Sepharose 4B to resuspend the matrix.

2.3(a) Use a pipet to remove sufficient slurry for use and transfer to an appropriate container/tube. (Glutathione Sepharose 4B as supplied is approximately a 75% slurry. The following procedure results in a 50% slurry. Based on the bed volume requirements from Table 1, dispense 1.33 ml of the original Glutathione Sepharose 4B slurry per ml of bed volume required.)

2.4(a) Sediment the matrix by centrifugation at 500 × *g* for 5 min. Carefully decant the supernatant.

2.5(a) Wash the Glutathione Sepharose 4B by adding 10 ml of cold (4 °C) 1× PBS (diluted from the 10× stock, see page 12) per 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed. Invert to mix.

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

2.6(a) Sediment the matrix by centrifugation at 500 × *g* for 5 min. Decant the supernatant.

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

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

Option B: Bulk pack columns

2.1(b) For each purification, remove the top cap from a disposable column included with the Bulk GST Purification Module and place upright in an appropriate rack/clamp.

2.2(b) Referring to Table 1 on page 9, determine the bed volume of Glutathione Sepharose 4B required for your application.

2.3(b) Gently shake the bottle of Glutathione Sepharose 4B to resuspend the matrix.

2.4(b) Use a pipet to remove sufficient slurry for use and transfer to the disposable column. (Glutathione Sepharose 4B as supplied is approximately a 75% slurry. Based on the bed volume requirements from Table 1, dispense 1.33 ml of the original Glutathione Sepharose 4B slurry per ml of bed volume required.)

2.5(b) Tap the column to dislodge any trapped air bubbles in the matrix bed. Allow to settle.

2.6(b) Remove the bottom cap and save for later use. Allow the column to drain.

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

2.7(b) Wash the Glutathione Sepharose 4B by adding 10 ml of cold (4 °C) 1× PBS (diluted from the 10× stock, see page 12) per 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed. Allow the column to drain.

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

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

Option C: RediPack columns

2.1(c) Remove the top cap from the RediPack column and pour off the excess liquid.

2.2(c) Remove the bottom cap and cut the tip off of the column. Save the cap for later use. Allow the column to drain.

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

2.3(c) Wash the Glutathione Sepharose 4B by adding 20 ml of cold (4 °C) 1× PBS (diluted from the 10× stock, see page 12). Allow the column to drain.

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

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

❸ Screening of pGEX recombinants for fusion protein expression

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The following steps may be used prior to large-scale purification to check clones for expression of the desired fusion protein. Due to the small scale of the screening process (~ 5 µg fusion protein), affinity purification should only be performed using the Bulk GST Purification Module in a batch method using bed volumes for the 50 µg scale (see Table 1, page 9). Disposable columns or RediPack columns should not be used at this small scale.

3.1 Grow, induce and lyse 2 ml cultures of *E. coli* transformed with pGEX recombinants as described in Protocol 1.

3.2 Add 20 µl of a 50% slurry of Glutathione Sepharose 4B (prepared as described in Protocol 2) to each lysate supernatant and mix gently for 5 min at room temperature.

3.3 Add 100 µl of 1× PBS, vortex briefly, and centrifuge for 5 s to sediment the Sepharose beads.

3.4 Discard the supernatants. Repeat this 1× PBS wash twice for a total of three washes.

3.5 Elute the fusion protein by the addition of 10 µl of glutathione elution buffer (prepared as described in "Essential preliminaries," page 12). Suspend the Sepharose beads and incubate at room temperature for 5 min.

3.6 Centrifuge in a microcentrifuge for 5 min to sediment the Sepharose beads, and transfer the supernatants to fresh tubes.

4 Batch purification of fusion proteins using bulk Glutathione Sepharose 4B

Batch binding/column wash

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

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

4.3 Use a pipet to transfer the matrix to a disposable column.

Note: If maintenance of the sample in batch format is desired, do not transfer the matrix to the column. All centrifugations for washing and elution may be performed at 500 × *g* for 5 min.

4.4 Tap the column to dislodge any trapped air bubbles in the matrix bed. Allow to settle.

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

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

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

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

Note: Fusion protein bound to the matrix may be eluted directly (see below) at this stage using glutathione elution buffer (prepared as described in "Essential preliminaries") or the protein may be cleaved while bound to the matrix with the appropriate site-specific protease to liberate the protein of interest from the GST moiety.

Elution

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

4.8 Elute the fusion protein by the addition of 1 ml of glutathione elution buffer (prepared as described in "Essential preliminaries," page 12) per ml bed volume*. Incubate the column at room temperature (22–25 °C) for 10 min to elute the fusion protein.

4.9 Remove the end cap and collect the eluate. This contains the fusion protein.

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

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* Bed volume is equal to 0.5× the volume of the 50% Glutathione Sepharose slurry used or 0.75× the volume of the original Glutathione Sepharose slurry.

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

Note: The yield of fusion protein can be estimated by measuring the absorbance at 280 nm. The GST affinity tag can be approximated by 1 $A_{280} \cong 0.5$ mg/ml. This calculation is based on the extinction coefficient of the GST monomer using a Bradford protein assay. Other protein determination methods may produce different extinction coefficients.

Note: The yield of protein may also be determined by standard chromogenic methods (e.g., Lowry, BCA, Bradford, etc.). If a Lowry or BCA-type method is used, the sample must first be dialyzed against 2 000 volumes of 1× PBS to remove glutathione, which can interfere with protein measurement. The Bradford method can be performed in the presence of glutathione.

5 Column purification: RediPack or disposable columns

Binding

5.1 Use a pipet to apply the bacterial lysate (Protocol 1) to the matrix in a drained and washed Glutathione Sepharose 4B RediPack or disposable column (Protocol 2).

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

5.2 Remove and save the end cap and allow the lysate to flow through.

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

5.3 Wash the matrix by the addition of 10 bed volumes* of 1× PBS. Allow the column to drain. Repeat twice more for a total of three washes.

Note: Fusion protein bound to the matrix may be eluted directly (see below) at this stage using glutathione elution buffer (prepared as described in “Essential preliminaries”) or the protein may be cleaved while bound to the matrix with the appropriate site-specific protease to liberate the protein of interest from the GST moiety.

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* Bed volume is equal to 0.5× the volume of the 50% Glutathione Sepharose slurry used or 0.75× the volume of the original Glutathione Sepharose slurry. RediPack columns contain a 2 ml bed volume.

Elution

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

5.5 Elute the fusion protein by the addition of 1 ml of glutathione elution buffer (prepared as described in “Essential preliminaries”) per ml bed volume*. Incubate the column at room temperature (22–25 °C) for 10 min to elute the fusion protein.

5.6 Remove the end cap and collect the eluate. This contains the fusion protein.

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

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

Note: The yield of fusion protein can be estimated by measuring the absorbance at 280 nm. The GST affinity tag can be approximated by 1 $A_{280} \cong 0.5$ mg/ml. This calculation is based on the extinction coefficient of the GST monomer using a Bradford protein assay. Other protein determination methods may result in different extinction coefficients.

Note: The yield of protein may also be determined by standard chromogenic methods (e.g., Lowry, BCA, Bradford, etc.).
If a Lowry or BCA-type method is to be used, the sample must first be dialyzed against 2 000 volumes of 1× PBS to remove glutathione, which can interfere with protein measurement.
The Bradford method can be performed in the presence of glutathione.

6 Analysis of Glutathione Sepharose 4B-purified fusion proteins

SDS-PAGE analysis

6.1 Transfer 10 µl aliquots of each sample to be analyzed (e.g. samples retained following cell resuspension and lysis, column flow-throughs, washes, eluates, etc.) to fresh tubes.

6.2 To each sample, add 2 µl of 6× SDS loading buffer (see “Materials not supplied,” pages 3–4). Vortex briefly and heat for 5 min at 90–100 °C.

6.3 Load the samples onto a 10–12.5% SDS-polyacrylamide gel.

6.4 Run the gel for the appropriate time and stain with Coomassie™ blue to visualize the parental GST (made in control cells carrying the parental pGEX vector) and the fusion protein.

Note: Transformants expressing the desired fusion protein will be identified by the presence of a novel fusion protein larger than the parental 29 kDa GST*.

If the above analysis indicates that the fusion protein has adsorbed to the Glutathione Sepharose 4B, you may proceed to large scale purification. If the fusion protein is absent from the purified material, it may be insoluble or expressed at very low levels; refer to the troubleshooting guide (page 33) for a discussion of this problem. Interpretation is sometimes complicated when fusion proteins break down and release the 26 kDa GST moiety. Such cases are usually recognized by the reduced level of the full-size fusion protein, and a series of proteolytic fragments down to the 26 kDa GST moiety.

* Parental pGEX vectors produce a 29 kDa GST fusion protein containing amino acids coded for by the pGEX multiple cloning site.

CDNB assay

In addition to SDS-PAGE analysis of recombinants, the relative level of expression of GST fusion protein can be estimated using the GST substrate CDNB (1-chloro-2,4-dinitrobenzene) which is included in the GST Detection Module (27-4590-01).

Additional analyses

If recombinants expressing fusion proteins cannot be identified using the methods described above, clones can also be identified by Western blot analysis using the anti-GST antibody contained in the GST Detection Module. Another alternative is to perform a functional assay, if available, specific for the protein of interest.

The yield of fusion protein can be estimated by measuring the absorbance at 280 nm. The amount of GST affinity tag can be approximated by 1 $A_{280} \cong 0.5$ mg/ml. This calculation is based on the extinction coefficient of the GST monomer using a Bradford protein assay. Other protein determination methods may generate different extinction coefficients.

The yield of protein may also be determined by standard chromogenic methods (e.g. Lowry, BCA, Bradford, etc.). If a Lowry or BCA-type method is to be used, the sample must first be dialyzed against 2 000 volumes of 1× PBS to remove glutathione, which can interfere with protein measurement. The Bradford method can be performed in the presence of glutathione.

DNA sequencing

Two primers specific for the pGEX vector series are available for DNA sequencing of inserts (5' pGEX Sequencing Primer, 27-1410-01; and 3' pGEX Sequencing Primer, 27-1411-01). These may be used, particularly in the event that no protein expression is detected, to verify that the cloned protein-coding sequences are in the proper orientation and translation frame relative to GST.

Appendix 1: Characteristics of Glutathione Sepharose 4B

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

Capacity: > 8 mg recombinant glutathione S-transferase per ml drained gel.

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

Spacer arm: 12 atoms (10 carbons).

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

Molecular weight exclusion limit: 2×10^7 daltons.

Max. operating pressure: 8 kPa, 0.08 bar.

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

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

Appendix 2: Map of pGEX vectors

pGEX-1λT (27-4805-01)

```
          Thrombin
Leu Val Pro Arg↓Gly Ser Pro Glu Phe Ile Val Thr Asp
CTG GTT CCG CGT GGA TCC CCG GAA TTC ATC GTG ACT GAC TGA CGA
                BamH I      EcoR I       Stop codons
```

pGEX-2T (27-4801-01)

```
          Thrombin
Leu Val Pro Arg↓Gly Ser Pro Gly Ile His Arg Asp
CTG GTT CCG CGT GGA TCC CCG GGA ATT CAT CGT GAC TGA CTG ACG
                BamH I  Sma I  EcoR I    Stop codons
```

pGEX-2TK (27-4587-01)

```
          Thrombin              Kinase
Leu Val Pro Arg↓Gly Ser Arg Arg Ala Ser Val
CTG GTT CCG CGT GGA TCT CGT CGT GCA TCT GTT GGA TCC CCG GGA ATT CAT CGT GAC TGA
                                            BamH I  Sma I  EcoR I    Stop codons
```

pGEX-4T-1 (27-4580-01)

```
          Thrombin
Leu Val Pro Arg↓Gly Ser Pro Glu Phe Pro Gly Arg Leu Glu Arg Pro His Arg Asp
CTG GTT CCG CGT GGA TCC CCG GAA TTC CCG GGT CGA CTC GAG CGG CCG CAT CGT GAC TGA
                BamH I      EcoR I  Sma I  Sal I  Xho I  Not I     Stop codons
```

pGEX-4T-2 (27-4581-01)

```
          Thrombin
Leu Val Pro Arg↓Gly Ser Pro Gly Ile Pro Gly Ser Thr Arg Ala Ala Ala Ser
CTG GTT CCG CGT GGA TCC CCA GGA ATT CCC GGG TCG ACT CGA GCG GCC GCA TCG TGA
                BamH I      EcoR I  Sma I  Sal I  Xho I  Not I     Stop codon
```

pGEX-4T-3 (27-4583-01)

```
          Thrombin
Leu Val Pro Arg↓Gly Ser Pro Asn Ser Arg Val Asp Ser Ser Gly Arg Ile Val Thr Asp
CTG GTT CCG CGT GGA TCC CCG AAT TCC CGG GTC GAC TCG AGC GGC CGC ATC GTG ACT GAC TGA
                BamH I    EcoR I  Sma I  Sal I  Xho I  Not I       Stop codons
```

pGEX-3X (27-4803-01)

```
      Factor Xa
Ile Glu Gly Arg↓Gly Ile Pro Gly Asn Ser Ser
ATC GAA GGT CGT GGG ATC CCC GGG AAT TCA TCG TGA CTG ACT GAC
                 BamH I  Sma I  EcoR I      Stop codons
```

pGEX-5X-1 (27-4584-01)

```
      Factor Xa
Ile Glu Gly Arg↓Gly Ile Pro Glu Phe Pro Gly Arg Leu Glu Arg Pro His Arg Asp
ATC GAA GGT CGT GGG ATC CCC GAA TTC CCG GGT CGA CTC GAG CGG CCG CAT CGT GAC TGA
                 BamH I     EcoR I  Sma I  Sal I  Xho I  Not I     Stop codons
```

pGEX-5X-2 **(27-4585-01)**

Factor Xa

```
Ile Glu Gly Arg↓Gly Ile Pro Gly Ile Pro Gly Ser Thr Arg Ala Ala Ala Ser
ATC GAA GGT CGT GGG ATC CCC GGA ATT CCC GGG TCG ACT CGA GCG GCC GCA TCG TGA
```

BamH I, EcoR I, Sma I, Sal I, Xho I, Not I, Stop codon

pGEX-5X-3 **(27-4586-01)**

Factor Xa

```
Ile Glu Gly Arg↓Gly Ile Pro Arg Asn Ser Arg Val Asp Ser Ser Gly Arg Ile Val Thr Asp
ATC GAA GGT CGT GGG ATC CCC AGG AAT TCC CGG GTC GAC TCG AGC GGC CGC ATC GTG ACT GAC TGA
```

BamH I, EcoR I, Sma I, Sal I, Xho I, Not I, Stop codons

pGEX-6P-1 **(27-4597-01)**

PreScission™ Protease

```
Leu Glu Val Leu Phe Gln↓Gly Pro Leu Gly Ser Pro Glu Phe Pro Gly Arg Leu Glu Arg Pro His
CTG GAA GTT CTG TTC CAG GGG CCC CTG GGA TCC CCG GAA TTC CCG GGT CGA CTC GAG CGG CCG CAT
```

BamH I, EcoR I, Sma I, Sal I, Xho I, Not I

pGEX-6P-2 **(27-4598-01)**

PreScission™ Protease

```
Leu Glu Val Leu Phe Gln↓Gly Pro Leu Gly Ser Pro Gly Ile Pro Gly Ser Thr Arg Ala Ala Ala Ser
CTG GAA GTT CTG TTC CAG GGG CCC CTG GGA TCC CCA GGA ATT CCC GGG TCG ACT CGA GCG GCC GCA TCG
```

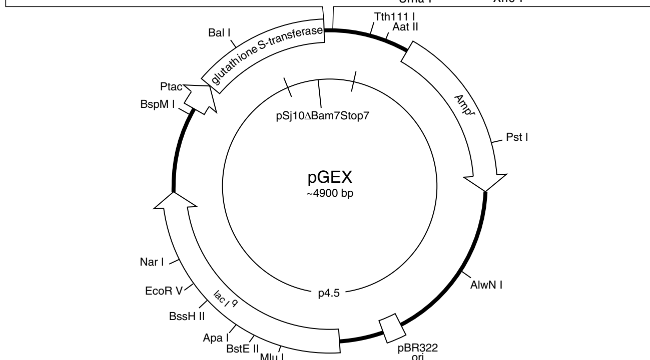
BamH I, EcoR I, Sma I, Sal I, Xho I, Not I

pGEX-6P-3 **(27-4599-01)**

PreScission™ Protease

```
Leu Glu Val Leu Phe Gln↓Gly Pro Leu Gly Ser Pro Asn Ser Arg Val Asp Ser Ser Gly Arg
CTG GAA GTT CTG TTC CAG GGG CCC CTG GGA TCC CCG AAT TCC CGG GTC GAC TCG AGC GGC CGC
```

BamH I, EcoR I, Sma I, Sal I, Xho I, Not I



Appendix 3:
Regeneration of Glutathione Sepharose 4B

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

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

   To remove precipitated or denatured substances, wash the matrix with 2 bed volumes of 6M guanidine hydrochloride and then wash immediately with 5 bed volumes of 1× PBS.

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

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

5. Wash the gel twice with 10 bed volumes of 1× PBS.

---

* Bed volume is equal to 0.5× the volume of the 50% Glutathione Sepharose slurry used or 0.75× the volume of the original Glutathione Sepharose slurry. RediPack columns contain a 2 ml bed volume.

6. Repeat washes using 20% ethanol.
7. Store at +4 °C.
8. Re-equilibrate the gel with 1× PBS before re-use.

Troubleshooting

Problem: High basal level of expression.

Possible causes/solutions

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

   Add 2% glucose to the growth medium.This will decrease the basal expression level associated with the upstream *lac* promoter but will not affect basal level expression from the *tac* promoter. The presence of glucose should not significantly affect overall expression following induction with IPTG.

Problem: No protein is detected by Coomassie-stained SDS gel of bacterial sonicate.

Possible causes/solutions

1. *Optimize expression conditions.* Optimization of expression conditions can dramatically improve yields. Investigate the effects of cell strain, medium composition, incubation temperature and induction conditions on fusion protein yield. Exact conditions will vary for each fusion protein.
2. *Check DNA sequences.* It is essential that protein-coding DNA sequences be cloned in the proper translation frame in pGEX vectors. Cloning junctions should be sequenced using 5' pGEX Sequencing Primer (27-1410-01) and 3' pGEX Sequencing Primer (27-1411-01) to verify that inserts are in-frame with GST. The reading frame of the multiple cloning site for each pGEX vector is shown in Appendix 2, pages 30–31.
3. *Analyze a small aliquot of an overnight culture by SDS-PAGE.* Generally, a highly expressed protein will be visible by Coomassie staining when 5–10 µl of an induced culture whose A_{600} is ~ 1.0 is loaded on the gel. Nontransformed host *E. coli* cells and cells transformed with the parental pGEX vector should be run in parallel as negative and positive controls, respectively. The presence of the fusion protein in this total cell preparation and its absence from a clarified lysate may indicate the presence of inclusion bodies (see below).
4. *Check for expression by immunoblotting.* Some fusion proteins may be masked on an SDS-polyacrylamide gel by a bacterial protein of approximately the same molecular weight. Immunoblotting can identify fusion proteins in these cases. Run an SDS-polyacrylamide gel of

induced cells as above and transfer the proteins to a nitrocellulose or PVDF membrane. Detect fusion protein using anti-GST antibody (included in the GST Detection Module, 27-4590-01).

5. Select a new, independently transformed clone and check for expression.

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

Possible causes/solutions

SDS-PAGE analysis of samples collected during the preparation of the bacterial lysate may indicate that the majority of the GST fusion protein is located in the post-lysate pellet (Protocol 1, page 13). Possible causes and solutions are discussed below.

1. *Sonication may be insufficient.* Cell disruption is evidenced by partial clearing of the suspension or may be checked by microscopic examination. Addition of lysozyme (0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris-HCl, pH 8.0) prior to sonication may improve results. Avoid frothing—this may denature the fusion protein. Over-sonication can also lead to co-purification of host proteins with the GST fusion protein.

2. *Fusion protein may be insoluble (inclusion bodies).* If insufficient protein is found in the soluble fraction following centrifugation of the lysate, it may be necessary to alter growth conditions:

   - Fusion protein solubility can be increased dramatically by lowering the growth temperature during induction. Experiment with growth temperatures of 20–30 °C (15, 16).

   - Alter level of induction by decreasing IPTG concentration to < 0.1 mM.

   - Alter timing of induction.

– Induce for a shorter period of time.

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

– Increase aeration. High oxygen transport can help prevent the formation of inclusion bodies (17).

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

If the above techniques do not significantly improve expression of soluble fusion protein, protein can be solubilized from inclusion bodies using common denaturants such as 4–8 M guanidine hydrochloride, 4–8 M urea, detergents, alkaline pH (> 9), organic solvents (18, 19), N-lauroyl-sarcosine (Sarkosyl) (20, 21). Other variables that affect solubilization include time, temperature, ionic strength, ratio of denaturant to protein and the presence of thiol reagents (18, 19). For reviews see references 15, 18, 19, 22 and 23.

Following solubilization, proteins must be refolded properly to regain function. Denaturant can be removed by dialysis, dilution, or gel filtration to allow refolding of the protein and formation of the correct intramolecular associations. Critical parameters during refolding include pH, presence of thiol reagents and the speed of denaturant removal (18, 19, 24). Once refolded, protein may be purified by ion exchange, gel filtration or affinity chromatography.

Fusion proteins can be purified to some extent while denatured. In some instances where GST fusion proteins formed inclusion bodies,

solubilization and binding to Glutathione Sepharose 4B was achieved in the presence of 2–3 M guanidine HCl or urea. Success has also been achieved using up to 2% Tween™ 20 for solubilization and binding. Binding to Glutathione Sepharose 4B can also be achieved in the presence of 1% CTAB, 10 mM DTT or 0.03% SDS (11). Success of affinity purification in the presence of these agents will depend on the nature of the fusion protein.

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

Possible causes/solutions

1. *Excessive flow rate.* Decrease the flow rate to improve binding.
2. *Altered binding site.* Test 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, then the fusion partner may have altered the conformation of GST, thereby reducing its affinity. Adequate results may be obtained by reducing the temperature used for binding to 4 °C, and by limiting the number of washes.
3. *Over-sonication may have denatured the fusion protein.* Check the lysate microscopically to monitor cell breakage. Use mild sonication conditions during cell lysis.
4. *Sonication may be insufficient.* Check microscopically or monitor nucleic acid release by measuring the A_{260}. Addition of lysozyme (0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris-HCl, pH 8.0) prior to sonication may improve results. Avoid frothing—this may denature the fusion protein.

5. *Masked binding site*. Add 1–10 mM DTT prior to cell lysis. This can significantly increse binding of some GST fusion protteins to Glutathione Sepharose. The optimal concentration must be determined empirically for eac fusion protein.

6. *Use fresh Glutathione Sepharose 4B*. If the Glutathione Sepharose 4B has already been used several times, it may be necessary to use fresh matrix. Refer also to Appendix 3, “Regeneration of Glutathione Sepharose 4B.”

7. *Check that the column has been equilibrated with a buffer $6.5 < pH < 8.0$ (e.g. 1× PBS) before applying fusion protein*. The correct pH range is critical for efficient binding.

8. *Column capacity may have been exceeded*. If using GSTrap FF columns (1 ml or 5 ml), either link two or three columns in series to increase capacity, use a GSTPrep FF 16/10 column, or pack a larger column.

9. *Fusion protein may be in inclusion bodies*. Please refer to troubleshooting discussions regarding insoluble protein complexes.

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

Possible causes/solutions

1. *Increase the duration of elution*. In some instances, overnight elution at room temperature or 4°C is most effective.

2. *Increase the volume of elution buffer.* Glutathione Sepharose 4B will also function as a gel filtration medium with an approximate molecular weight exclusion limit of 2×10^7 Daltons. Small proteins (especially those liberated following cleavage with a site-specific protease)

may require large elution volumes and concentration by ultrafiltration. In these cases, the batch purification method may be preferred (Protocol 4).

3. *Increase the concentration of glutathione in the elution buffer.* General protocols use 5 mM glutathione for elution. Note that when dissolved according to instructions, the concentration of glutathione used in the elution buffer is 10 mM, which should be sufficient for most applications. Additional reduced glutathione must be obtained separately. If the glutathione concentration is increased above 15 mM, the buffer concentration will have to be increased to maintain proper pH (24). For example, try 50 mm Tris-HCl, 20–40 mm reduced glutathione, pH 8.0, as the elution buffer.

4. *Increase the ionic strength of the elution buffer.* The addition of 0.1–0.2 M NaCl to the elution buffer may also improve results. Keep in mind that very hydrophobic proteins may precipitate under high salt conditions. If this is the case, addition of a non-ionic detergent may improve results (see below).

   One researcher obtained satisfactory results using the following elution buffer. 20 mM glutathione, 100 mM Tris-HCl (pH 8.0), 120 mM NaCl (24).

5. *Add a non-ionic detergent to the elution buffer.* Nonspecific hydrophobic interactions may prevent solubilization and elution of fusion proteins from Glutathione Sepharose 4B (21). Addition of a non-ionic detergent such as 0.1% Triton X-100 or 2% N-octyl glucoside can significantly improve elution of some GST fusion proteins (21).

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	1 × 5 ml	17-5131-01
GSTPrep FF 16/10	1 × 20 ml	17-5234-01
Glutathione Sepharose FF	25 ml	17-5132-01
	100 ml	17-5132-02
	500 ml	17-5132-03
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* U.S. Patent No. 5,603,899 has been issued to GE Healthcare for multiple column chromatography assembly.

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