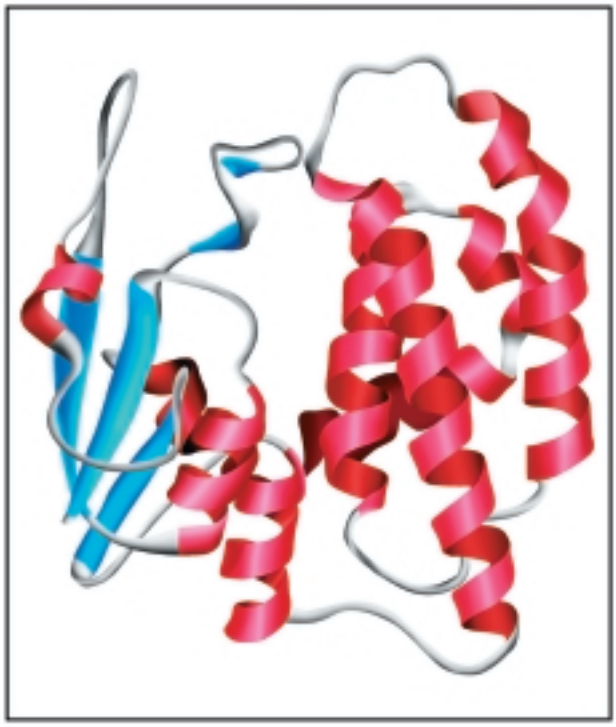
# GST Gene Fusion System

## Handbook



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Front cover shows the structure of glutathione S-transferase (human, class mu) (GSTM2-2) form A (E.C. 2.5.1.18) mutant with Trp 214 replaced by Phe (W214F). The protein was expressed in HeLa cells, as reported in Raghunathan, S. *et al.* Crystal structure of human class mu glutathione transferase GSTM2-2. Effects of lattice packing on conformational heterogeneity. *J. Mol. Biol.* **238**, 815–832 (1994).

# Contents

# Chapter 1
# Overview

The Glutathione S-transferase (GST) Gene Fusion System is a versatile system for the expression, purification, and detection of fusion proteins produced in *Eschericia coli*. The system is based on inducible, high-level expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST (1). Expression in *E. coli* yields fusion proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus. The protein accumulates within the cell's cytoplasm.

GST occurs naturally as a $M_r$ 26 000 protein that can be expressed in *E. coli* with full enzymatic activity. Fusion proteins that possess the complete amino acid sequence of GST also demonstrate GST enzymatic activity and can undergo dimerization similar to that observed in nature (2, 3, 4). The crystal structure of recombinant *S. japonicum* GST from pGEX vectors has been determined (5) and matches that of the native protein. Appendix 1 shows the characteristics of GST, as determined in pGEX-1N (6).

GST fusion proteins are purified from bacterial lysates by affinity chromatography using immobilized glutathione. GST fusion proteins are captured by the affinity medium, and impurities are removed by washing. Fusion proteins are eluted under mild, non-denaturing conditions using reduced glutathione. The purification process preserves protein antigenicity and function. If desired, cleavage of the protein from GST can be 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 colorimetric or immunological methods.

The GST Gene Fusion System has been used successfully in many applications including molecular immunology (7), the production of vaccines (8, 9), and studies involving protein-protein (10) and DNA-protein (11) interactions.

## Selecting an expression strategy

Table 1 summarizes the choices to consider when devising a strategy for fusion protein expression and purification.

**Table 1.** Criteria for devising expression and purification strategy

| Choice of | Criteria | Comments |
|---|---|---|
| Vector | Reading frame | Fusion protein must be in the same frame as the GST reading frame. |
| | Cloning sites | Must be compatible with the ends of the insert DNA. |
| | Protease cleavage site | Choose among PreScission™ Protease, Thrombin, and Factor Xa. |
| | | PreScission Protease vectors offer the most efficient method for cleavage and purification. |
| | | Cleavage site must be absent in protein to be expressed. |
| Insert | Reading frame and orientation | Must have an open reading frame in the correct orientation. |
| | Size | Must be less than 2 kb long, preferably much less. |
| | Fragment ends | Must be compatible with the vector's cloning sites such that the junctions are maintained. |

**Table 1.** Criteria for devising expression and purification strategy (continued)

| Choice of | Criteria | Comments |
|---|---|---|
| Host cells | Cloning and maintenance | Choose a strain that transforms well, such as JM105, but not one carrying the *recA1* allele. |
| | Expression | Use BL21, which is protease-deficient and designed to maximize expression of full-length fusion protein. |
| Growth conditions | Medium, temperature, induction conditions, aeration, positive selection, handling of inclusion bodies | Evaluate different parameters to optimize expression of fusion protein. Lowering the growth temperature, increasing aeration, and altering induction conditions should be investigated first. |
| Purification method | For initial screening | Batch method with Glutathione Sepharose™ 4B, for 2–3 ml culture. |
| | | MicroSpin™ GST Purification Module, with prepacked columns containing Glutathione Sepharose 4B, for cultures up to 12 ml, using either microcentrifuge or MicroPlex™ 24 Vacuum. |
| | For large-scale cultures | MicroSpin GST Purification Module provides high throughput when used with MicroPlex 24 Vacuum (up to 48 samples simultaneously). Yields up to 400 µg of fusion protein. |
| | Preparative purification | GSTrap™ FF 1 ml and 5 ml prepacked columns containing Glutathione Sepharose 4 Fast Flow. May be run in series to increase yields. Five ml columns yield 50–60 mg of fusion protein*. |
| | | GSTPrep™ FF 16/10 prepacked column containing Glutathione Sepharose 4 Fast Flow. May be run in series to increase yields. Twenty ml column for convenient preparative purification of GST fusion proteins, yielding 200–240 mg of fusion protein*. |
| | | Glutathione Sepharose 4B, for batch purification and for packing small columns. Yields 8 mg/ml*. |
| | | Glutathione Sepharose 4 Fast Flow, for packing high-performance columns for use with purification systems and scaling up. Yields 10–12 mg/ml*. |
| Detection method | Type of detection method | GST 96-Well Detection Module for ELISA. Uses 100 µl of sample/well. Ideal for screening expression systems and chromatographic fractions. Useful when amount of expressed protein is unknown or when increased sensitivity is required. Gives estimate of relative level of expression. |
| | | GST Detection Module with CDNB enzymatic assay. Uses 5–50 µl of sample. Rapid assay; ideal for screening. Gives estimate of relative level of expression. |
| | | Western blot using anti-GST antibody. Uses 5–10 µl of sample. Highly specific; detects only GST fusion protein. Little or no background detectable when using detection systems with optimized concentrations of secondary HRP-conjugated antibody. |
| | | SDS-PAGE with Coomassie™ or silver staining. Uses 5–10 µl of sample. Provides information on size and percent purity. Detects fusion protein and contaminants. |
| Cleavage option | On-column or off-column | On-column cleavage is generally recommended since many potential contaminants can be washed out and the target protein eluted with a higher level of purity. |
| | | Off-column cleavage is suggested if optimization of cleavage conditions is necessary. |
| | Choice of protease | PreScission Protease: The GST tag can be removed and the protein purified in a single step on the column. Because the protease is maximally active at 4 °C, cleavage can be performed at low temperatures, thus improving stability of the target protein. |
| | | Thrombin or Factor Xa sites can be cleaved either while the fusion protein is bound to the column or in solution after elution from the column. Either protease can be removed using Benzamidine Sepharose Fast Flow (high sub). |

** Yield is protein dependent.*

These topics are discussed in detail in the following chapters. The handbook includes procedures (Fig 1) and examples showing use of the GST system, as well as a troubleshooting guide and extensive appendices.

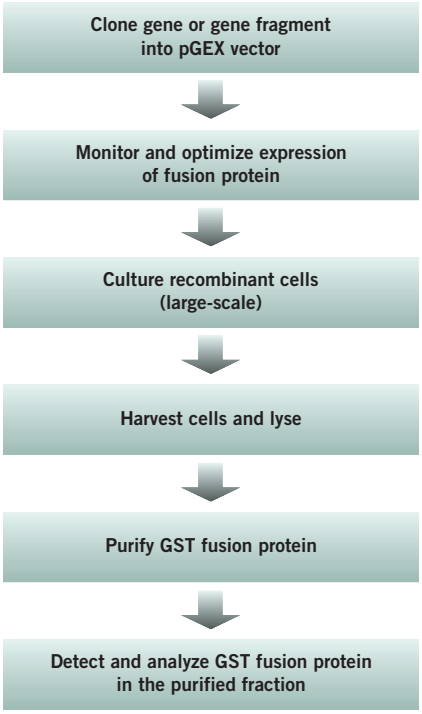


**Fig 1.** A typical protocol for expression and purification of GST fusion proteins. On- or off-column cleavage of the GST tag is an option.

## Symbols used in this handbook

This symbol indicates general advice that can improve procedures or provide recommendations for action under specific situations.

This symbol denotes advice that should be regarded as mandatory and gives a warning when special care should be taken.

chemicals, buffers, and equipment

experimental protocol

# Chapter 2
# Cloning the gene or gene fragment into a pGEX expression vector

## pGEX vectors

GST fusion proteins are constructed by inserting a gene or gene fragment into the multiple cloning site of one of the ten pGEX vectors. Expression is under the control of the *tac* promoter, which is induced by the lactose analog isopropyl β-D thiogalactoside (IPTG). All pGEX vectors are also engineered with an internal *lac*$I^q$ gene. The *lac*$I^q$ gene product is a repressor protein that binds to the operator region of the *tac* promoter, preventing expression until induction by IPTG, thus maintaining tight control over expression of the insert.

In addition to offering chemically inducible, high-level expression, the vectors allow mild elution conditions for release of fusion proteins from the affinity medium. Thus, effects on antigenicity and functional activity of the protein are minimized. The vectors have a range of protease cleavage recognition sites as shown in Table 2.

**Table 2.** Protease cleavage sites of pGEX vectors

| Vector | Cleaved by |
|---|---|
| pGEX-6P-1, pGEX-6P-2, pGEX-6P-3 | PreScission Protease |
| pGEX-4T-1, pGEX-4T-2, pGEX-4T-3 | Thrombin |
| pGEX-5X-1, pGEX-5X-2, pGEX-5X-3 | Factor Xa |
| pGEX-2TK<br>Allows detection of expressed proteins by direct labelling *in vitro* (12) | Thrombin |

Collectively, the pGEX-P, pGEX-T, and pGEX-X series vectors provide all three translational reading frames beginning with the *Eco*R I restriction site (Fig 2). The same multiple cloning sites (MCS) in each vector ensure easy transfer of inserts. pGEX-6P-1, pGEX-4T-1, and pGEX-5X-1 can directly accept and express cDNA inserts isolated from λgt11 libraries.

pGEX-2TK has a different MCS from that of the other vectors. pGEX-2TK is uniquely designed to allow the detection of expressed proteins by directly labelling the fusion products *in vitro* (12). This vector contains the recognition sequence for the catalytic subunit of cAMP-dependent protein kinase obtained from heart muscle. The protein kinase site is located between the thrombin recognition site and the MCS. Expressed proteins can be directly labelled using protein kinase and [$\gamma$–$^{32}$P]ATP and readily detected using standard radiometric or autoradiographic techniques.

Refer to Appendix 2 for a listing of the control regions of the pGEX vectors. Complete DNA sequences and restriction site data are available at the Amersham Biosciences web site (http://www.amershambiosciences.com) and from GenBank™. GenBank accession numbers are listed in Appendix 2.

**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-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

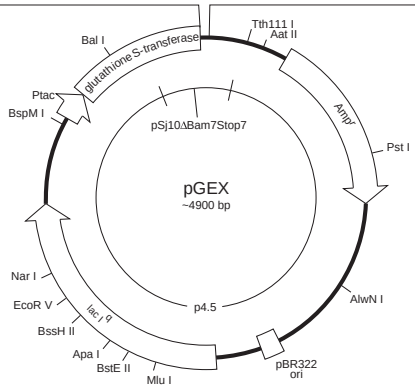


**Fig 2.** Map of the glutathione S-transferase fusion vectors showing the reading frames and main features. See Appendix 2 for the control regions of the ten vectors.

Select the proper vector to match the reading frame of the cloned insert.

pGEX-6P PreScission Protease vectors offer the most efficient method for cleavage and purification of GST fusion proteins. Site-specific cleavage is performed with simultaneous immobilization of the protease on the column. The protease has high activity at low temperature so that all steps can be performed in the cold room to protect the integrity of the target protein. Cleavage enzyme and GST tag are removed in a single step, as described in Chapter 6.

## The host

Although a wide variety of *E. coli* host strains can be used for cloning and expression with the pGEX vectors, there are specially engineered strains that are more suitable and that may maximize expression of full-length fusion proteins. Strains deficient in known cytoplasmic protease gene products, such as Lon, OmpT, DegP or HtpR, may aid in the expression of fusion proteins by minimizing the effects of proteolytic degradation by the host (13–16).

Using *E. coli* strains that are not protease-deficient may result in proteolysis of the fusion protein, seen as multiple bands on polyacrylamide gels (SDS-PAGE) or Western blots.

*E. coli* BL21, a strain defective in OmpT and Lon protease production, has been specifically selected to give high levels of expression of GST fusion proteins. It is the host of choice for GST fusion expression studies. Details on the genotype and handling of *E. coli* BL21 are found in Appendix 1.

A lyophilized (noncompetent) culture of *E. coli* BL21 is supplied with all pGEX vectors and is also available separately.

Use an alternative strain for cloning and maintenance of the vector (e.g. JM105) as BL21 does not transform well. However, do not use an *E. coli* strain carrying the *recA1* allele for propagation of pGEX plasmids. There have been reports that these strains can cause rearrangements or deletions within plasmid DNA.

## Insert DNA

Insert DNA must possess an open reading frame and should be less than 2 kb long. Whether subcloned from another vector or amplified by PCR, the insert must have ends that are compatible with the linearized vector ends. Using two different restriction enzymes will allow for directional cloning of the insert into the vector.

Directional cloning will optimize for inserts in the correct orientation.

## Summary of procedures

In the procedures below, the gene or gene fragment is cloned into the appropriate pGEX vector, and the host cells used for the cloning steps are transformed. The presence of the insert is verified, then a stock of DNA is prepared that can be used repeatedly in various procedures such as sequencing, mutagenesis, and cloning. Table 3 lists the procedures described in this chapter.

**Table 3.** Procedures for cloning the gene or gene fragment into a pGEX expression vector

| Procedure | | Description | Comments |
|---|---|---|---|
| 1 | | Restriction digestion of pGEX vectors | If digesting with two enzymes, consider gel-purifying the DNA before proceeding. |
| 2 | | Dephosphorylation of linearized pGEX vector | Use recommended amount of enzyme so heat inactivation will be complete. |
| 3 | | Ligation of insert to pGEX DNA | Using Ready-To-Go™ T4 DNA Ligase will reduce incubation time substantially. |
| 4 | | Preparation of competent cells and transformation with pGEX DNA | Transform uncut pGEX DNA in parallel with recombinant DNA prepared above. Carry out all steps aseptically. |
| 5 | 5.1 | Screening using Ready-To-Go PCR Beads | Protocol uses the pGEX 5' and 3' Sequencing Primers. Ready-To-Go PCR Beads minimize pipetting steps. |
| | 5.2 | Screening using standard PCR | Also uses the pGEX Sequencing Primers. |
| 6 | | Small-scale isolation of pGEX DNA | Standard miniprep. |
| 7 | | Large-scale isolation of pGEX DNA | Kit-based, but standard procedures also work well. |

# 1. Restriction digestion of pGEX vectors

### Reagents required

pGEX DNA

10× One-Phor-All Buffer PLUS ($OPA^+$): 100 mM Tris acetate, 100 mM magnesium acetate, 500 mM potassium acetate, pH 7.5 (optional)

Restriction enzyme

Many restriction enzymes are compatible with $OPA^+$ (see the Amersham Biosciences catalog for details), and its recipe is provided here as a convenience. The buffer is also recommended for use in the dephosphorylation and ligation procedures that follow.

### Steps

1. Prepare the following reaction mixture. Volumes may vary depending on the amount of pGEX DNA to be digested. We recommend a final DNA concentration in the reaction mixture of 0.1 µg/µl.
   5 µg of pGEX DNA
   5–10 µl of 10× One-Phor-All Buffer PLUS ($OPA^+$) or buffer supplied with enzyme
   5–10 µl of optional components (e.g. BSA, Triton™ X-100, NaCl, etc.)
   10–25 units of restriction enzyme
   $H_2O$ to 50 µl
2. Incubate at the appropriate temperature for 2–16 h.
3. Examine a small aliquot of the reaction by agarose gel electrophoresis to verify that the pGEX DNA has been digested to completion.
4. If digestion with a second enzyme is required, adjust the concentration of $OPA^+$ and/or additional components, and the reaction volume as appropriate, add new enzyme, and continue incubation.
5. Monitor the progress of the digestion as in step 3.

Be alert for incomplete or failed double digestion. Continue digestion if necessary.

6. Dephosphorylate the pGEX DNA with an alkaline phosphatase if it is to be used following digestion with a single restriction enzyme (Procedure 2). If using $OPA^+$, dephosphorylation can be performed in the same tube immediately following digestion.

If the pGEX DNA was digested with two restriction enzymes, consider agarose-gel-purifying the linearized vector prior to dephosphorylation. This can be conveniently accomplished with Sephaglas™ BandPrep Kit.

## 2. Dephosphorylation of linearized pGEX vector

**Reagents required**

Calf intestinal alkaline phosphatase

10× One-Phor-All Buffer PLUS ($OPA^+$): 100 mM Tris acetate, 100 mM magnesium acetate, 500 mM potassium acetate, pH 7.5

Phenol: Redistilled phenol saturated with TE buffer containing 8-hydroxy quinoline (17)

Chloroform/isoamyl alcohol: Reagent-grade chloroform and isoamyl alcohol, mixed 24:1

3 M sodium acetate, pH 5.4, aqueous solution

Ethanol (70%, 95%)

TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA

**Steps**

1. Dilute sufficient calf intestinal alkaline phosphatase for all dephosphorylations to be performed. When diluted, 1–2 µl should provide 0.1 unit to the reaction. For dilution, use 10× $OPA^+$ and $H_2O$ to give a final buffer concentration of 1× $OPA^+$.
2. Add 0.1 unit (1–2 µl of diluted enzyme) of alkaline phosphatase to the digested pGEX DNA and incubate for 30 min at 37 °C.

In radiolabel and transformation studies, dephosphorylation appears complete within 5 min when using 0.5× or 1× $OPA^+$. When 2× $OPA^+$ is used, an incubation period of 15–30 min is required for complete dephosphorylation.

3. Heat inactivate the alkaline phosphatase at 85 °C for 15 min.

Heat inactivation is complete for concentrations of alkaline phosphatase of 0.1 unit or less, but is not effective for concentrations greater than 1 unit.

4. Add an equal volume of phenol to the aqueous sample. Vortex for 1 min and centrifuge for 5 min at full speed to separate the phases.
5. Transfer the upper aqueous phase to a fresh tube and add an equal volume of chloroform/isoamyl alcohol. Vortex for 1 min, then centrifuge for 5 min at full speed to separate the phases.
6. Transfer the upper aqueous phase to a fresh tube and add 0.1 volume of 3 M sodium acetate, pH 5.4 and 2.5 volumes of 95% ethanol. Mix and place at -20 °C for 15 min.
7. Centrifuge at 4 °C for 15 min, remove the supernatant, and wash the pellet with 1 ml of 70% ethanol.
8. Recentrifuge for 2 min, drain thoroughly, and either air-dry the DNA pellet or dry it under vacuum.
9. Dissolve the DNA pellet in 10–20 µl of TE buffer.

pGEX DNA can be stored at -20 °C for later use. Avoid repeated freezing and thawing.

## 3. Ligation of insert to pGEX DNA

Ready-To-Go T4 DNA Ligase can be used to achieve ligations in 30–45 min. An alternate procedure is described below.

**Reagents required**

Insert DNA

ATP, 100 mM

10× One-Phor-All Buffer PLUS ($OPA^+$): 100 mM Tris acetate, 100 mM magnesium acetate, 500 mM potassium acetate, pH 7.5

T4 DNA ligase

**Steps**

- The linearized pGEX DNA and insert DNA should be present at a vector to insert ratio of 1:5 moles of ends. The moles of ends of linear DNA can be calculated with the following formula:

  moles of ends = 2 × (g of DNA)/[(# of bp) × (649 Daltons/bp)]

  Example: 100 ng of pGEX DNA (0.06 pmol of ends) would require 100 ng of a 1 kb insert (0.3 pmol of ends).

- For ligation of cohesive ends, the final reaction mix should contain 1 mM ATP (diluted) and 0.5–5 units of T4 DNA ligase, and should be incubated for 1–4 h at 10 °C.
- For ligation of blunt ends, the final reaction mix should contain 0.1–1 mM ATP (diluted) and 10–15 units of T4 DNA ligase, and should be incubated for 2–16 h at 4–16 °C.

1. Based upon the above considerations, prepare the following reaction mixture specific for your application:
   1–5 µl of linearized pGEX DNA
   1–5 µl of insert DNA
   2 µl of 10× One-Phor-All Buffer PLUS ($OPA^+$)
   0.2 µl of 100 mM ATP
   0.5–15 units of T4 DNA ligase
   $H_2O$ to 20 µl
2. Incubate for either 1–4 h at 10 °C (cohesive ends) or 2–16 h at 4–16 °C (blunt ends).
3. Terminate the reaction by heating at 65 °C for 10 min.

The ligation reaction can be used directly to transform competent cells. Otherwise, it can be stored at -20 °C until needed.

## 4. Preparation of competent cells and transformation with pGEX DNA

In these procedures, *E. coli* host cells are made competent and then transformed with either uncut pGEX DNA or recombinant pGEX DNA.

If electroporation is used to transform the cells, see Appendix 3. Otherwise, proceed as described below.

Transform 1 ng of uncut (supercoiled) vector DNA in parallel with recombinant pGEX ligations to determine the efficiency of each competent cell preparation.

This protocol is based on the procedure of Chung *et al.* (18).

All steps in this procedure should be carried out aseptically.

**Reagents required**

Use double-distilled $H_2O$ for preparation of all solutions.

Glycerol stock of *E. coli* host strain

LB medium and LB medium plates (prepared fresh): Combine 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 900 ml $H_2O$. Stir to dissolve, and adjust volume to 1 l. Sterilize by autoclaving. To prepare as a solid medium, add 1.2–1.5% agar.

TSS (transformation and storage solution) (ice-cold): For 100 ml: combine 1.0 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 10.0 g polyethylene glycol ($M_r$ 3350), 5.0 ml dimethylsulfoxide (DMSO), and 5.0 ml $MgCl_2$ (1 M) in 70 ml of sterile distilled $H_2O$. Stir until dissolved. Adjust the pH to 6.5 with HCl or NaOH. Adjust to 100 ml with sterile distilled $H_2O$. Sterilize by filtering through a 0.2 µm filter. Store at 4 °C. Stable for up to 6 months.

LBG medium (LB + 20 mM glucose): Dissolve 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 900 ml of distilled $H_2O$. Sterilize by autoclaving. After the medium has cooled to 50–60 °C, add 10 ml of sterile 2 M glucose. Adjust to 1 l with sterile distilled $H_2O$. To prepare as a solid medium, add 1.2–1.5% agar.

LBAG medium and plates (LBG + 100 µg/ml ampicillin): See recipe for LBG medium, above. After autoclaving, cool the medium to 50 °C, then aseptically add 1 ml of a 100 mg/ml ampicillin stock solution (final concentration 100 µg/ml). To prepare as a solid medium, add 1.2–1.5 % agar.

Ampicillin stock solution: Dissolve 400 mg of the sodium salt of ampicillin in 4 ml of $H_2O$. Sterilize by filtration and store in small aliquots at -20 °C.

Glycerol: 80% in sterile distilled $H_2O$

**Steps**

**Preparation of competent cells**

1. Using sterile technique, streak an *E. coli* host strain (e.g. JM105, BL21, etc.) from a glycerol stock onto an LB medium plate. Incubate overnight at 37 °C.
2. Isolate a single colony and inoculate 50–100 ml of LB broth. Incubate at 37 °C with shaking at 250 rpm. Grow cells to an $A_{600}$ of 0.4–0.5.

It is critical that the absorbance is not more than 0.5. This will take approximately 3–6 h.

Pre-warming the broth to 37 °C will shorten the growth time.

3. Sediment the cells at approximately 2500 × g for 15 min at 4 °C, then gently resuspend in 1/10 volume (5–10 ml) of ice-cold TSS and place on ice.

Cells must be used for transformations within 2–3 h.

**Transformation of competent cells**

1. For each ligation reaction, as well as for the uncut vector control and the negative control (untransformed competent *E. coli* host cells), add 1 ml of freshly prepared competent *E. coli* host cells to separate pre-chilled 50 ml sterile disposable centrifuge tubes. Store on ice.
2. Add 20 µl of each ligation reaction or 1 ng of uncut vector to the competent cells, swirl gently to mix, and place on ice for 45 min. Do not add any DNA to the negative control but instead add 20 µl of sterile distilled $H_2O$.
3. Incubate the tubes in a 42 °C water bath for 2 min, then chill briefly on ice.
4. For each sample, immediately transfer 100 µl of the transformed cells to a 17 × 100 mm tube (Falcon) containing 900 µl of LBG medium (pre-warmed to 37 °C) and incubate for 1 h at 37 °C with shaking (250 rpm).

5. Plate 100 µl of the diluted, transformed cells from the ligated samples and 10 µl of the diluted, transformed cells from the uncut vector sample onto separate LBAG plates. Also plate 100 µl of the untransformed, competent *E. coli* host cells. Incubate the plates at 37 °C overnight, then proceed to screening using Procedure 5.
6. To prepare a frozen stock culture, add 100 µl of the diluted, transformed cells containing the pGEX DNA to 1 ml of LBAG medium and incubate for 30 min at 37 °C with shaking at 250 rpm. After incubation, add 200 µl of sterile 80% glycerol and mix with a pipet tip. Store at -70 °C.

# 5. Screening

The pGEX 5' and 3' Sequencing Primers can be used in the rapid screening of transformants by PCR, in conjunction with Ready-To-Go PCR Beads (Procedure 5.1) or in standard PCR (Procedure 5.2).

Screening is needed to verify that the insert is in the proper orientation and the correct junctions are present such that the reading frame is maintained.

## 5.1. Screening using Ready-To-Go PCR Beads

**Reagents required**

Ready-To-Go PCR Beads

pGEX 5' Sequencing Primer (5 pmol/µl)

pGEX 3' Sequencing Primer (5 pmol/µl)

**Steps**

1. Resuspend a bead in 25 µl of $H_2O$ as per standard instructions.
2. Add 10 pmol each of pGEX 5' and 3' Sequencing Primers to the resuspended bead.
3. Gently touch a sterile micropipet tip to the bacterial colony to be screened and then transfer to the resuspended PCR bead. Pipet gently to disperse bacterial cells.

Avoid transferring too much of the bacterial colony. Results are better when cell numbers are low.

Streak some of the bacteria remaining on the micropipet tip onto an LB medium grid plate as a source for Procedures 6 and 7.

4. Overlay the reaction mixture with 50 µl of mineral oil.
5. Amplify in a thermal cycler with the following cycle parameters:
   35 cycles:
   95 °C for 1 min
   58 °C for 1 min
   72 °C for 2 min
6. Transfer the aqueous phase from under the oil layer to a clean tube. Analyze 10–20 µl by agarose gel electrophoresis.

## 5.2. Screening using standard PCR

**Reagents required**

*Taq* DNA polymerase at 5 U/µl

10× *Taq* buffer as recommended by supplier

dNTP mix: For each reaction, add 0.2 µl each of 100 mM dATP, 100 mM dCTP, 100 mM dGTP, and 100 mM dTTP to 15.2 µl of $H_2O$ for a final concentration of 0.2 mM in a 100 µl reaction.

pGEX 5' Sequencing Primer (5 pmol/µl)

pGEX 3' Sequencing Primer (5 pmol/µl)

**Steps**

1. Mix the following components in a 0.65 ml tube:
   10 µl of 10× *Taq* polymerase buffer
   16 µl of dNTP mix
   5 µl of pGEX 5' Sequencing Primer
   5 µl of pGEX 3' Sequencing Primer
   $H_2O$ to 99.5 µl
2. Gently touch a sterile micropipet tip to the bacterial colony to be screened and transfer to the above PCR mixture. Pipet gently to disperse bacterial cells.

Avoid transferring too much of the bacterial colony. Results are better when cell numbers are low.

Streak some of the bacteria remaining on the micropipet tip onto an LB medium grid plate as a source for Procedures 6 and 7.

3. Add 0.5 µl of 5 U/µl *Taq* DNA polymerase.
4. Overlay the reaction mixture with 50 µl of mineral oil.
5. Amplify in a thermal cycler with the following cycle parameters:
   25–35 cycles:
   94 °C for 1 min
   55 °C for 1 min
   72 °C for 2 min
6. Transfer the aqueous phase from under the oil layer to a clean tube. Analyze 20–40 µl by agarose gel electrophoresis.

## 6. Small-scale isolation of pGEX DNA

Rapid and phenol-free isolation of plasmid DNA is greatly simplified by the use of FlexiPrep™ Kit or GFX™ *Micro* Plasmid Prep Kit. An alternate procedure is described below.

**Reagents required**

Solution I: 100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 400 µg of heat-treated RNase I per ml of Solution I

Solution II: 0.2 M NaOH, 1% (w/v) SDS

Solution III: 3 M potassium, 5 M acetate. To prepare 100 ml, mix 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, and 28.5 ml of distilled $H_2O$.

Isopropanol

Phenol: Redistilled phenol saturated with TE buffer containing 8-hydroxy quinoline (17)

Chloroform/isoamyl alcohol: Reagent-grade chloroform and isoamyl alcohol, mixed 24:1

Phenol/chloroform: Equal parts of redistilled phenol and chloroform/isoamyl alcohol (24:1), each prepared as described above

3 M sodium acetate, pH 5.4, aqueous solution

Ethanol (70%, 95%)

TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA

**Steps**

1. Transfer 1.5 ml of an overnight culture of *E. coli* to a microcentrifuge tube and centrifuge at full speed for 30 s to pellet the cells.
2. Remove the supernatant by aspiration without disturbing the cell pellet, leaving the pellet as dry as possible.
3. Resuspend the pellet in 200 µl of solution I by vigorously vortexing.
4. Add 200 µl of solution II and mix by inverting the tube several times. Incubate at room temperature for 5 min.
5. Add 200 µl of solution III and mix by inverting the tube several times. Place on ice for 5 min.
6. Centrifuge at full speed for 5 min at room temperature.
7. Carefully decant the supernatant into a clean centrifuge tube.
8. Add 420 µl (0.7 volume) of ambient-temperature isopropanol to the supernatant and vortex to mix. Incubate for 5 min at room temperature.
9. Centrifuge at full speed for 10 min. Decant the supernatant and invert the tube to drain.
10. Resuspend the DNA pellet in 200 µl of TE buffer by vortexing.
11. Add 200 µl of phenol to the aqueous sample. Vortex for 1 min and centrifuge for 5 min at full speed to separate the phases.
12. Transfer the upper aqueous phase to a fresh tube and add 200 µl of chloroform/isoamyl alcohol. Vortex for 1 min, then centrifuge for 5 min at full speed to separate the phases.
13. Transfer the upper aqueous phase to a fresh tube and add 20 µl of 3 M sodium acetate and 500 µl of 95% ethanol. Mix and place at -20 °C for 15 min.
14. Centrifuge at 4 °C for 15 min, remove the supernatant, and wash the pellet with 1 ml of 70% ethanol.
15. Recentrifuge for 2 min, drain thoroughly, and air-dry the DNA pellet or dry it under vacuum.
16. Dissolve the DNA pellet in 20 µl of TE buffer.

pGEX DNA can be stored at -20 °C for later use. Avoid repeated freezing and thawing.

# 7. Large-scale isolation of pGEX DNA

Rapid, large-scale isolation of plasmid DNA from cultures up to 500 ml is greatly simplified by the use of FlexiPrep Kit.

**Reagents required**

2× YTA medium (2× YT + 100 µg/ml ampicillin): Prepare 2× YT medium by dissolving 16 g tryptone, 10 g yeast extract, and 5 g NaCl in 900 ml of distilled $H_2O$. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 l with distilled $H_2O$. Sterilize by autoclaving for 20 min. After autoclaving, cool the medium to 50 °C, then aseptically add 1 ml of a 100 mg/ml ampicillin stock solution (final concentration 100 µg/ml).

**Steps**

1. Grow an appropriate volume of pGEX-containing *E. coli* in 2× YTA medium overnight.
2. Dilute an inoculum of the overnight culture at least 1:100 into the desired volume of the same medium pre-warmed to the growth temperature.
3. Grow with aeration to an $A_{600}$ of 1–2.
4. Isolate plasmid DNA using FlexiPrep Kit, or protocols from reference 17.

# Chapter 3
# Monitoring expression, optimizing growth, and preparing large-scale cultures

pGEX vectors carry the *lac*I$^q$ gene, so there are no specific host requirements for propagation of the plasmids or for expression of fusion proteins. As previously noted, *E. coli* BL21 does not transform well, and an alternate strain (e.g. JM105) is recommended for maintenance of the plasmid. For all expression studies, however, BL21 is the strain of choice.

Once it has been established that the insert is in the proper orientation and the correct junctions are present (Chapter 2), the next step is to optimize fusion protein expression. Key to this step is the capability to screen crude lysates from many clones so that optimal expression levels and growth conditions can be readily determined. Once conditions are established, the researcher is ready to prepare large-scale bacterial sonicates of the desired clones.

Various methods for the purification of fusion proteins are available. In this chapter, the focus is on obtaining relatively small samples quickly, to permit the screening of many putative clones simultaneously. To this end, we recommend two purification methods for initial screening. In the first method, a crude lysate suitable for screening from 2–3 ml of culture is prepared, using a batch purification method with Glutathione Sepharose 4B. The second method uses the GST MicroSpin Purification Module, which can isolate protein from up to 12 ml of culture. Procedures using the MicroSpin Purification Module can be performed using a standard microcentrifuge or in conjunction with MicroPlex 24 Vacuum. With this latter method, fusion protein can be purified from up to 48 samples in less than 1 h.

The batch method with Glutathione Sepharose 4B is presented in this chapter. Procedures for use of the GST MicroSpin Purification Module are presented in Chapter 4.

Various detection methods are also available for screening lysates for expression of GST fusion proteins. SDS-PAGE is described below. More information and detailed procedures for several other methods can be found in Chapter 5.

After clones expressing the fusion protein have been selected, growth conditions should be evaluated for optimal expression. Media, growth temperature, culture density, induction conditions, and other variables should be evaluated. It is important to assure sufficient aeration and to minimize the time spent in each stage of growth, as well as to use positive selection for the plasmid (antibiotic resistance). The presence of inclusion bodies may affect optimization of expression, and is discussed in detail below.

Monitor both cell density ($A_{600}$) and protein expression at each step.

Yield of fusion protein is highly variable, depending on the nature of the fusion protein, the host cell, and the culture conditions. Fusion protein yields can range up to 10 mg/l (19). Table 4 can be used to approximate culture volumes based on an average yield of 2.5 mg/l.

**Table 4.** Estimate of culture volume based on average yield

| Fusion protein yield | 12.5 µg | 50 µg | 1 mg | 10 mg | 50 mg |
|---|---|---|---|---|---|
| Culture volume | 5 ml | 20 ml | 400 ml | 4 l | 20 l |
| Volume of sonicate | 0.5 ml | 1 ml | 20 ml | 200 ml | 1000 ml |

## Summary of procedures

This chapter includes a simple procedure for preparing crude lysates for initial screening and a procedure for preparing a large-scale bacterial sonicate (see Table 5). Refer to Chapter 4 for additional purification options and to Chapter 5 for additional detection options.

**Table 5.** Procedures for screening and preparing cultures of fusion proteins

| Procedure | Description | Comments |
|---|---|---|
| 8 | Screening pGEX recombinants for fusion protein expression | Prepare sonicate from 2 to 3 ml of culture; use SDS-PAGE for detection of fusion protein. |
| 9 | Preparation of large-scale bacterial sonicates | Prepare sonicate from 0.2 to 10 l of culture, then proceed to a purification method in Chapter 4. |

## Optimizing for soluble expression versus working with inclusion bodies

High-level expression of foreign fusion proteins in *E. coli* often results in formation of inclusion bodies. Inclusion bodies comprise dense, insoluble aggregates that are failed folding intermediates (20, 21).

Formation of inclusion bodies can be advantageous in purifying an active form of an expressed fusion protein that otherwise may be unstable in the soluble fraction. However, the steps needed to solubilize and refold the fusion protein can be highly variable and may not always result in high yields of active protein. The advantages and disadvantages of inclusion bodies are summarized in Table 6.

**Table 6.** Advantages and disadvantages of inclusion bodies

| Advantages | Disadvantages |
|---|---|
| High expression levels can reduce fermentation costs. | Steps to refold the protein shift difficulties and costs downstream. |
| Expression is easily monitored by SDS-PAGE or immunoblotting. | Expression cannot be monitored directly by functional assays. |
| Inclusion bodies can be isolated to high purity and used directly as antigen. | Minor contaminants are often hydrophobic, poorly soluble membrane proteins and cell wall fragments. |
| Fusion proteins are generally protected from proteolytic breakdown. | Major contaminants are oligomers and misfolded or proteolyzed forms of the protein that can be difficult to separate. |
| Small fusions present in inclusion bodies refold with good efficiency. | If the protein does not refold well, another expression system will be needed. |

If the presence of inclusion bodies is not deemed a deterrent at this point, the researcher can proceed to optimizing growth conditions and preparing large-scale bacterial sonicates of the fusion-producing cells (Procedure 9 below). If, on the other hand, the presence of inclusion bodies is deemed a deterrent, there are two options to consider:

- Optimize as much as possible for soluble expression.
- Accept the formation of inclusion bodies but develop strategies to solubilize and refold the protein.

A variety of growth parameters can be investigated, either solely or in combination, that may provide a good yield of non-degraded fusion protein in the soluble fraction. Steps to investigate include:

- Lowering the growth temperature to between 20 °C and 30 °C.
- Increasing aeration.
- Altering induction conditions.

In general, induction at lower cell densities ($A_{600}$ = 0.5) usually results in greater yields of the fusion protein in a soluble form. However, in some cases it may be beneficial to grow the cells to a higher cell density (> 1 $A_{600}$ unit) for a shorter period of time, or simply to induce for a shorter period of time. Growing the cells to a higher cell density and either omitting induction by IPTG or reducing the IPTG concentration to 0.1 mM leads to lower yields, but more of the fusion protein is likely to be obtained in an intact form.

Although not limited to discussion of the GST protein fusion system, the chapter on prokaryotic expression in reference 22 provides an excellent discussion of many aspects of working with expression systems.

If the plasmid has been propagated in a host other than *E. coli* strain BL21, it should be transferred into BL21 for the expression of fusion protein, using Procedure 4 from Chapter 2.

Retain small samples at key steps, including before induction, at various times after induction, and during the purification steps, to analyze growth and purification methods. Once analysis is complete, prepare glycerol stocks of the positive clones and store at -80 °C.

## 8. Screening 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 of fusion protein), affinity purification should only be performed in a batch method using a Glutathione Sepharose 4B bed volume of 10 µl.

### Reagents required

**Preparation of the medium:**
Bulk Glutathione Sepharose 4B prepared to 50% slurry as described below in the procedural steps.

1× PBS (ice-cold): Dilute 10× PBS with sterile $H_2O$. Store at 4 °C. 10× PBS is 1.4 M NaCl, 27 mM KCl, 100 mM $Na_2HPO_4$, 18 mM $KH_2PO_4$, pH 7.3.

**Preparation of lysate:**
2× YTA medium (2× YT + 100 µg/ml ampicillin): Prepare 2× YT medium by dissolving 16 g tryptone, 10 g yeast extract, and 5 g NaCl in 900 ml of distilled $H_2O$. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 l with distilled $H_2O$. Sterilize by autoclaving for 20 min. After autoclaving, cool the medium to 50 °C, then aseptically add 1 ml of a 100 mg/ml ampicillin stock solution (final concentration 100 µg/ml).

100 mM IPTG: Dissolve 500 mg of isopropyl-β-D-thiogalactoside (IPTG) in 20 ml of distilled $H_2O$. Filter-sterilize and store in small aliquots at -20 °C.

Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0. Dispense in 1–10 ml aliquots and store at -20 °C until needed. Avoid more than five freeze/thaw cycles.

**SDS-PAGE analysis:**
6× loading buffer: 0.35 M Tris-HCl (pH 6.8), 10.28% (w/v) SDS, 36% (v/v) glycerol, 0.6 M dithiothreitol (or 5% β-mercaptoethanol), 0.012% (w/v) bromophenol blue. Store in 0.5 ml aliquots at -70 °C.

### Steps

#### Preparation of the medium

Glutathione Sepharose 4B as supplied is approximately a 75% slurry. The following procedure results in a 50% slurry.

Based on the bed volume requirements, dispense 1.33 ml of the original Glutathione Sepharose 4B slurry per ml of bed volume required.

1. Determine the amount of Glutathione Sepharose 4B required.

Although only 10 µl of prepared slurry is needed for each screening analysis, additional slurry should be prepared if it will also be used for larger-scale purification procedures (see Procedures 13.1 and 13.2 in Chapter 4).

2. Gently shake the bottle of Glutathione Sepharose 4B to resuspend the medium.
3. Use a pipet with a wide-bore tip to remove sufficient slurry for use and transfer the slurry to an appropriate container/tube.
4. Sediment the medium by centrifuging at 500 × g for 5 min. Carefully decant the supernatant.
5. Wash the Glutathione Sepharose 4B by adding 10 ml of cold (4 °C) 1× PBS per 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed. Invert to mix.

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

6. Sediment the medium by centrifuging at 500 × g for 5 min. Decant the supernatant.
7. For each 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed, add 1 ml of 1× PBS. This results in a 50% slurry. Mix well prior to subsequent pipetting steps.

#### Preparation of lysate

1. Pick and transfer several colonies of *E. coli* transformed with the pGEX recombinants into separate tubes containing 2 ml of 2× YTA medium.

For comparison, it is advisable to inoculate a control tube with bacteria transformed with the parental pGEX plasmid.

2. Grow liquid cultures to an $A_{600}$ of 0.6–0.8 (3–5 h) with vigorous agitation at 30–37 °C.

Lower temperatures, even as low as 20 °C, may be used if inclusion bodies are problematic (see Troubleshooting, page 84).

3. Induce fusion protein expression by adding 2 µl of 100 mM IPTG (final concentration 0.1 mM).

A higher concentration (up to 1 mM IPTG) may be used at this screening stage.

4. Continue incubation for an additional 1–2 h.
5. Transfer 1.5 ml of the liquid cultures to labelled microcentrifuge tubes.
6. Centrifuge in a microcentrifuge for 5 s and discard the supernatants.
7. Resuspend each pellet in 300 µl of ice-cold 1× PBS. Transfer 10 µl of each cell suspension into separate labelled tubes (for later use in SDS-PAGE analysis).

Except where noted, keep all samples and tubes on ice.

8. Lyse the cells using a sonicator equipped with an appropriate probe.

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 (frothing may denature proteins). Keep on ice.

Crude sonicates can be screened for the relative level of expression of GST fusion proteins using the GST substrate CDNB (1-chloro-2,4-dinitrobenzene). See Procedure 16, Chapter 5.

9. Centrifuge the lysate in a microcentrifuge for 5 min to remove insoluble material. Save a 10 µl aliquot of the insoluble material for analysis by SDS-PAGE. Transfer the supernatants to fresh tubes.
10. Add 20 µl of a 50% slurry of Glutathione Sepharose 4B (prepared as described above) to each supernatant and mix gently for 5 min at room temperature.
11. Add 100 µl of 1× PBS, vortex briefly, and centrifuge for 5 s to sediment the Glutathione Sepharose 4B beads.
12. Discard the supernatants. Repeat this 1× PBS wash twice for a total of three washes.
13. Elute the fusion protein by adding 10 µl of elution buffer. Suspend the Glutathione Sepharose 4B beads and incubate at room temperature for 5 min.
14. Centrifuge in a microcentrifuge for 5 min to sediment the Glutathione Sepharose 4B beads, then transfer the supernatants to fresh tubes.

**SDS-PAGE analysis**

1. Transfer 10 µl of each supernatant from step 14 (above) to fresh tubes.
2. To these aliquots, and to the 10 µl samples retained following steps 7 and 9 (above), add 2 µl of 6× SDS loading buffer.
3. Vortex briefly and heat for 5 min at 90–100 °C.
4. Load the samples onto a 10% or 12.5% SDS-polyacrylamide gel.
5. Run the gel for the appropriate length of time and stain with Coomassie blue or silver stain to visualize the parental GST (made in control cells carrying the parental pGEX vector) and the fusion protein.

Transformants expressing the desired fusion protein will be identified by the absence from total cellular proteins of the parental GST and by the presence of a novel, larger fusion protein. Parental pGEX vectors produce a $M_r$ 29 000 GST fusion protein containing amino acids coded for by the pGEX multiple cloning site.

## 9. Preparation of large-scale bacterial sonicates

**Reagents required**

2× YTA medium (2× YT + 100 µg/ml ampicillin): Prepare 2× YT medium by dissolving 16 g tryptone, 10 g yeast extract, and 5 g NaCl in 900 ml of distilled $H_2O$. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 l with distilled $H_2O$. Sterilize by autoclaving for 20 min. After autoclaving, cool the medium to 50 °C, then aseptically add 1 ml of a 100 mg/ml ampicillin stock solution (final concentration 100 µg/ml).

100 mM IPTG: Dissolve 500 mg of isopropyl-β-D-thiogalactoside (IPTG) in 20 ml of distilled $H_2O$. Filter-sterilize and store in small aliquots at -20 °C.

1× PBS (ice-cold): Dilute 10× PBS with sterile $H_2O$. Store at 4 °C. 10× PBS is 1.4 M NaCl, 27 mM KCl, 100 mM $Na_2HPO_4$, 18 mM $KH_2PO_4$, pH 7.3.

20% Triton X-100

**Steps**

1. Use a single colony of *E. coli* cells containing a recombinant pGEX plasmid to inoculate 2–100 ml of 2× YTA medium.
2. Incubate for 12–15 h at 37 °C with vigorous shaking.
3. Dilute the culture 1:100 into fresh pre-warmed 2× YTA medium, and grow at 30–37 °C with shaking until the $A_{600}$ reaches 0.5–2.

Lower temperatures, even as low as 20 °C, may be used if inclusion bodies are problematic (see Troubleshooting, page 84).

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

Optimize the growth temperature and $A_{600}$ for induction as these will vary with each fusion protein.

4. Add 100 mM IPTG to a final concentration of 0.1–1.0 mM and continue incubation for an additional 2–6 h. The optimal concentration can only be determined empirically.
5. Transfer the culture to appropriate centrifuge containers and centrifuge at 7700 × g (e.g. 8000 rpm in a Beckman JA20 rotor) for 10 min at 4 °C to sediment the cells.
6. Discard the supernatant and drain the pellet. Place on ice.
7. Using a pipet, completely suspend the cell pellet by adding 50 µl of ice-cold 1× PBS per ml of culture.
8. Disrupt the suspended cells using an appropriately equipped sonicator for the suspended volume. Sonicate on ice in short bursts.

Save an aliquot of the sonicate for analysis by SDS-PAGE as described in Procedure 8 above.

Cell disruption is evidenced by partial clearing of the suspension or may be checked by microscopic examination. Avoid frothing as this may denature the fusion protein. Over-sonication can also lead to co-purification of host proteins with the GST fusion protein.

Detection of GST activity can be performed at this stage using one of the methods described in Chapter 5.

9. Add 20% Triton X-100 to a final concentration of 1%. Mix gently for 30 min to aid in solubilization of the fusion protein.
10. Centrifuge at 12 000 × g (e.g. 10 000 rpm in a Beckman JA20 rotor) for 10 min at 4 °C. Transfer the supernatant to a fresh container. Save aliquots of the supernatant and the cell debris pellet for analysis by SDS-PAGE as described in Procedure 8. These samples can be used to identify the fraction in which the fusion protein is located.

Analyze the aliquots as soon as possible; the longer they remain at 4 °C, the greater the risk of proteolysis.

11. Proceed with one of the purification procedures detailed in Chapter 4.

# Chapter 4
# Purification of GST fusion proteins

GST fusion proteins are easily purified from bacterial lysates by affinity chromatography using glutathione immobilized to a matrix such as Sepharose (Fig 3). When applied to the affinity medium, fusion proteins bind to the ligand, and impurities are removed by washing with binding buffer. Fusion proteins are then eluted from the Glutathione Sepharose under mild, non-denaturing conditions that preserve both protein antigenicity and function.

If separation of the cloned protein from the GST affinity tag is desired, the fusion protein can be digested with an appropriate site-specific protease while the fusion protein is bound to Glutathione Sepharose. Alternatively, the fusion protein can be digested following elution from the medium (see Chapter 6 for both of these alternatives). 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 medium while the cloned protein is eluted using wash buffer (23).

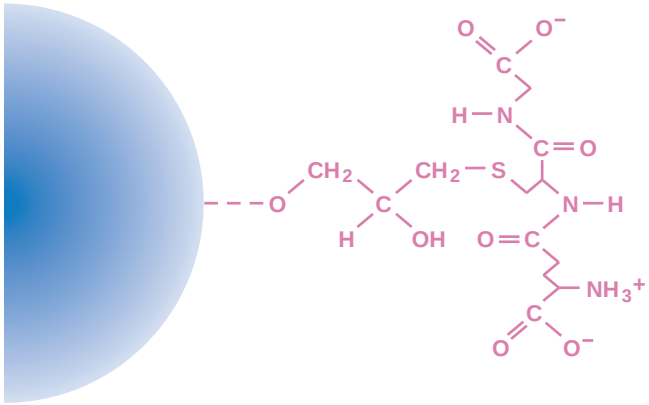



**Fig 3.** Terminal structure of Glutathione Sepharose. Glutathione is attached to Sepharose by coupling to the oxirane group using epoxy-activation. The structure of glutathione is complementary to the glutathione S-transferase binding site.

## Selecting an affinity chromatography product

Products designed to meet specific purification needs are available for purification of GST fusion proteins, as shown in the selection guide in Table 7. All of these products rely on affinity chromatography using gravity flow, centrifugation, vacuum, syringe, or pump action to purify the protein. A comparison of the physical characteristics of Glutathione Sepharose 4B and Glutathione Sepharose 4 Fast Flow is given in Table 8.

**Table 7.** Selection guide summarizing purification options for GST fusion proteins

| Prepacked column or bulk media | Amount of fusion protein for a single purification | Comments |
|---|---|---|
| MicroSpin GST Purification Module | Up to 400 µg | Prepacked columns, buffers, and chemicals that are ready to use.<br>High throughput when used with MicroPlex 24 Vacuum (up to 48 samples simultaneously). |
| GSTrap FF 1 ml and 5 ml columns | 1 ml columns: 10–12 mg<br>5 ml columns: 50–60 mg | Prepacked columns ready to use with either a syringe, a pump, or a chromatographic system. May be run in series to increase yields.<br>Packed with Glutathione Sepharose 4 Fast Flow. |
| GSTPrep FF 16/10 column | 200–240 mg | Prepacked column, ready to use. For convenient preparative purifications.<br>Packed with Glutathione Sepharose 4 Fast Flow. |
| Glutathione Sepharose 4B | 8 mg/ml | For batch purification and for packing small columns. |
| Glutathione Sepharose 4 Fast Flow | 10–12 mg/ml | For packing high-performance columns for use with purification systems and scaling up. |

**Table 8.** Comparison of Glutathione Sepharose 4B and Glutathione Sepharose 4 Fast Flow

| Physical characteristics | Glutathione Sepharose 4B | Glutathione Sepharose 4 Fast Flow |
|---|---|---|
| Ligand | Glutathione and 10-carbon linker arm | Glutathione and 10-carbon linker arm |
| Ligand concentration | 200–400 µmol glutathione/g washed and dried resin | 120–320 µmol glutathione/ml medium |
| Binding capacity | > 8 mg recombinant glutathione S-transferase/ml medium | 10–12 mg recombinant glutathione S-transferase/ml medium |
| Dynamic binding | NA | 11 mg GST fusion protein/ml medium<br>$M_r$: 43 kDa (GSTrap FF 1 ml at 1 ml/min) |
| Mean particle size | 90 µm | 90 µm |
| Bead structure | 4% agarose | Highly cross-linked 4% agarose |
| Chemical stability | Stable to all commonly used aqueous buffers. Exposure to 0.1 M NaOH, 70% ethanol, or 6 M guanidine hydrochloride for 2 h at room temperature or to 1% (w/v) SDS for 14 d causes no significant loss of activity. | Stable to all commonly used aqueous buffers and 6 M guanidine hydrochloride for 1 h at room temperature |
| pH stability | 4–13 | 6–9 |
| Storage temperature | 4–8 °C | 4–8 °C |
| Storage buffer | 20% ethanol | 20% ethanol |

*Note:* Binding capacity of GST fusion proteins to Glutathione Sepharose 4B and Glutathione Sepharose 4 Fast Flow is protein dependent and may therefore vary between different proteins.

Decreasing the flow rate during purification may increase yield.

## General considerations for purification of GST fusion proteins

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 mg/l up to 10 mg/l (19). Table 9 can be used to approximate culture volumes based on an average yield of 2.5 mg/l.

**Table 9.** Reagent volume requirements for different protein yields

| Fusion protein yield | 50 mg | 10 mg | 1 mg | 50 µg |
|---|---|---|---|---|
| Culture volume | 20 l | 4 l | 400 ml | 20 ml |
| Volume sonicate | 1 l | 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 the procedures that follow (i.e. 1 ml of 50% Glutathione Sepharose slurry will give a bed volume of 0.5 ml). The bed volume is always 1/100 of the sonicate volume.*
*†This volume is per wash. Three washes are required per sample in the following procedures.*

Use high-quality water and chemicals for sample and buffer preparation. Samples should be centrifuged immediately before use and/or filtered through a 0.45 µm filter. If the sample is too viscous, dilute with binding buffer to prevent column clogging.

One of the most important parameters affecting the binding of GST fusion proteins to Glutathione Sepharose is the flow rate. Since the binding kinetics between glutathione and GST are relatively slow, it is important to keep the flow rate low during sample application to achieve maximum binding capacity.

The binding properties of the target protein can be improved by adjusting the sample to the composition of the binding buffer. Dilute in binding buffer or perform a buffer exchange using a desalting column such as HiTrap Desalting 5 ml or HiPrep 26/10 Desalting.

Volumes and times used for elution may vary among fusion proteins. Further elution with higher concentrations of glutathione (20–50 mM) may improve yield. At concentrations above 15 mM glutathione, the buffer concentration should also be increased to maintain the pH within the range 8–9. Flow-through, wash, and eluted material from the column should be monitored for GST fusion proteins using SDS-PAGE in combination with Western blot if necessary.

Following the elution steps, a significant amount of fusion protein may remain bound to the medium. 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 (see Chapter 5).

Dimer formation is inevitable with GST fusion proteins since GST itself is a homodimer when folded. The presence of dimers should not interfere with purification, but if desired, a prepacked column with the gel filtration medium Superdex™ can be used to purify monomers from dimers and other aggregates (see Table 10).

**Table 10.** Prepacked columns for purifying monomeric proteins from dimers and other aggregates

| Sample volume | Separation range | |
|---|---|---|
| | $M_r$ 3000–70 000 | $M_r$ 10 000–600 000 |
| ≤ 250 µl | Superdex 75 HR 10/30 | Superdex 200 HR 10/30 |
| 250 µl–4.5 ml | HiLoad™ 16/60 Superdex 75 prep grade | HiLoad 16/60 Superdex 200 prep grade |
| 2.5 ml–12.5 ml* | HiLoad 26/60 Superdex 75 prep grade | HiLoad 26/60 Superdex 200 prep grade |

** If the sample has a volume greater than 12.5 ml, it should be concentrated or fractionated in several runs.*
*Note: The sample volume is a critical factor in the separation. Try to use a small sample volume for increased separation performance.*

Batch preparation procedures are frequently mentioned in the literature. However, the availability of prepacked columns and easily packed Glutathione Sepharose 4 Fast Flow provides faster, more convenient alternatives. Batch preparations are occasionally used if it appears that the GST tag is not fully accessible or when the concentration of protein in the bacterial lysate is very low (both could appear to give a low yield from the affinity purification step). A more convenient alternative to improve yield is to decrease the flow rate or pass the sample through the column several times (recirculation).

Purification steps should be monitored using one or more of the detection methods described in Chapter 5. The GST Detection Module, for example, is designed to identify GST fusion proteins using a biochemical or immunological assay, and is convenient to use for optimizing expression and monitoring steps in the purification of a GST fusion protein.

The yield of fusion protein in purified samples can be estimated by measuring the absorbance at 280 nm. The concentration of the GST affinity tag can be approximated by 1 $A_{280} \approx 0.5$ mg/ml. (This 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.)

The yield of protein in purified samples can 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 2000 volumes of 1× PBS to remove glutathione, which can interfere with protein measurement. The Bradford method can be performed in the presence of glutathione.

Re-use of purification columns and affinity media depends upon the nature of the sample and should only be performed with identical samples to prevent cross-contamination.

## Selecting equipment for purification

The choice of equipment will depend on the specific purification. Many purification steps can be carried out using simple methods and equipment as, for example, step-gradient elution using a syringe in combination with prepacked HiTrap™ columns. When more complex elution methods are necessary, such as linear gradients, or the same column is to be used for many runs in series, it is wise to use a dedicated system. Table 11 provides a guide to aid in selecting the correct purification format.

**Table 11.** Selection guide for purification equipment

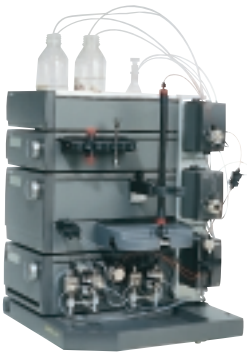
| Way of working | MicroSpin + Centrifugation | HiTrap + Syringe | Standard ÄKTA™design configurations | | | |
|---|---|---|---|---|---|---|
| | | | Explorer 100 | Purifier 10 | FPLC™ | Prime |
| Rapid, high-throughput screening | ✓ | | | | | |
| Simple, one-step purification | | ✓ | ✓ | ✓ | ✓ | ✓ |
| Reproducible performance for routine purification | | | ✓ | ✓ | ✓ | ✓ |
| Optimization of one-step purification to increase purity | | | ✓ | ✓ | ✓ | ✓ |
| System control and data handling for regulatory requirements, e.g. GLP | | | ✓ | ✓ | ✓ | |
| Automatic method development and optimization | | | ✓ | ✓ | ✓ | |
| Automatic buffer preparation | | | ✓ | ✓ | | |
| Automatic pH scouting | | | ✓ | ✓ | | |
| Automatic media or column scouting | | | ✓ | | | |
| Automatic multi-step purification | | | ✓ | | | |
| Scale-up, process development, and transfer to production | | | ✓ | | | |

For a single purification of a small quantity of product or for high-throughput screening, MicroSpin columns using centrifugation or MicroPlex 24 Vacuum are convenient and simple to use.

For purification of larger quantities of fusion proteins, GSTrap FF columns and GSTPrep FF 16/10 columns provide ideal formats. To increase capacity, use several GSTrap FF columns (1 ml or 5 ml) or two GSTPrep FF 16/10 columns (20 ml) in series or, for even larger capacity requirements, pack Glutathione Sepharose 4 Fast Flow into a suitable column.

For simple and reproducible purification, use of a chromatography system such as ÄKTAprime is advantageous since it can monitor and record the purification process, thus eliminating manual errors. For laboratory environments in which all experimental data must be recorded and traceable and where method development, optimization, and scale-up are needed, a computer-controlled ÄKTAdesign chromatography system is recommended. Experiments such as protein refolding or method optimization that require linear gradient elution steps can be performed only by a chromatography system.

ÄKTAexplorer



ÄKTApurifier

ÄKTAFPLC

ÄKTAprime

## Summary of procedures

Procedures are included for use of both prepacked columns and media available from Amersham Biosciences for GST fusion protein expression (Table 12). In several instances, alternative methods are provided.

**Table 12.** Procedures for purification of GST fusion proteins

| Procedure | | Description | Comments |
|---|---|---|---|
| 10 | 10.1 | Purification of multiple samples using GST MicroSpin columns with a microcentrifuge | Accommodates lysates from 2 ml to 12 ml of culture. Yield may be increased by repeating the elution step and pooling the eluates. |
| | 10.2 | High-throughput purification using GST MicroSpin columns with MicroPlex Vacuum | Rather than centrifugation, a vacuum source draws the liquid through the affinity medium. |
| 11 | 11.1 | Manual purification using GSTrap FF column with a syringe | One-step purification, 12 mg/1 ml column and 60 mg/5 ml column. Columns may be used in series to increase yield. |
| | 11.2 | Simple purification using a GSTrap FF column with ÄKTAprime | Automatic, preprogrammed application template for GST fusion proteins. |
| 12 | | Preparative purification using GSTPrep FF 16/10 column | One-step preparative purification of up to 240 mg of GST fusion protein. |
| 13 | 13.1 | Batch purification using Glutathione Sepharose 4B | Flexible method able to accommodate 50 µl to 10 ml of Glutathione Sepharose 4B. |
| | 13.2 | Batch/column purification using Glutathione Sepharose 4B | Can be scaled to purify 50 µg to 50 mg of GST fusion protein. Is a hybrid procedure that binds the protein in the batch method and elutes in a column. |
| 14 | | Column purification using Glutathione Sepharose 4 Fast Flow | Packing own columns and scaling-up. |

## Purification using the GST MicroSpin Purification Module

The GST MicroSpin Purification Module is useful for screening small or large numbers of bacterial lysates and for checking samples during the optimization of expression or purification conditions. Each module contains reagents sufficient for 50 purifications using MicroSpin columns prepacked with Glutathione Sepharose 4B. Sample application, washing, and elution can be performed using a standard microcentrifuge (Procedure 10.1) or in conjunction with MicroPlex 24 Vacuum (Procedure 10.2). GST fusion proteins can be purified from up to 48 samples in less than 1 h with the dual manifold system of MicroPlex 24 Vacuum.

Each MicroSpin column contains a 50 µl bed volume of Glutathione Sepharose 4B, sufficient for purifying up to 400 µg of recombinant GST.

## 10.1. Purification of multiple samples using GST MicroSpin columns with a microcentrifuge

Refer to page 27, General considerations for purification of GST fusion proteins, before beginning this procedure.

Do not apply more than 600 µl of sample at a time to a GST MicroSpin column. This procedure will accommodate lysates produced from 2 to 12 ml of culture.

**Components in GST MicroSpin Purification Module**

10× PBS: 1.4 M NaCl, 27 mM KCl, 100 mM $Na_2HPO_4$, 18 mM $KH_2PO_4$, pH 7.3. To prepare 1× PBS for use, dilute 10× PBS with sterile $H_2O$. Store at 4 °C.

Reduced glutathione: 0.154 g. To prepare elution buffer, pour the entire 50 ml volume of dilution buffer supplied with the module into the bottle containing the reduced glutathione. Shake until completely dissolved. Store as 1–20 ml aliquots at -20 °C.

Dilution buffer: 50 mM Tris-HCl, pH 8.0

IPTG: 500 mg. To prepare 100 mM IPTG, dissolve contents of the IPTG vial in 20 ml of sterile $H_2O$. Store as 1 ml aliquots at -20 °C.

MicroSpin columns: 50 units

Equipment required: Microcentrifuge

**Steps**

1. Resuspend the Glutathione Sepharose 4B in each column by vortexing gently.
2. Loosen the column caps one-fourth turn. Remove (and save) bottom closures.
3. Place each column into a clean 1.5 or 2 ml microcentrifuge tube. Spin for 1 min at 735 × g.
4. Discard the buffer from each centrifuge tube and replace the bottom closures.
5. Apply up to 600 µl of lysate to a column.
6. Recap each column securely and mix by gentle, repeated inversion. Incubate at room temperature for 5–10 min.
7. Remove (and save) the top caps and bottom closures. Place each column into a clean, pre-labelled 1.5 or 2 ml microcentrifuge tube.
8. Spin for 1 min at 735 × g to collect the flow-through.
9. Place each column into a clean, pre-labelled 1.5 or 2 ml microcentrifuge tube.
10. Apply 600 µl of 1× PBS wash buffer to each column and repeat the spin procedure. Additional 600 µl washes with 1× PBS can be performed if desired.
11. Add 100–200 µl of elution buffer to each column. Replace top caps and bottom closures. Incubate at room temperature for 5–10 min.
12. Remove and discard top caps and bottom closures and place each column into a clean 1.5 or 2 ml microcentrifuge tube.
13. Spin all columns again to collect the eluates. Save for analysis.

Yields of fusion protein can be increased by repeating the elution step two or three times and pooling the eluates.

## 10.2. High-throughput purification using GST MicroSpin columns with MicroPlex Vacuum

Refer to page 27, General considerations for purification of GST fusion proteins, before beginning this procedure.

Do not apply more than 600 µl of sample at a time to a GST MicroSpin column. This procedure will accommodate lysates produced from 2 to 12 ml of culture.

### Equipment required

Vacuum source capable of providing 220 mm Hg (e.g. a water vacuum)

Side-arm flask, 500 ml or 1 l

Single- or double-hole rubber stopper

Vacuum tubing

MicroPlex 24 Vacuum apparatus (one or two)

GST MicroSpin Purification Module (see Procedure 10.1 for listing of components)

### Steps

1. Assemble the MicroPlex 24 Vacuum according to the instructions supplied with the instrument.
2. Resuspend the Glutathione Sepharose 4B in each MicroSpin column by vortexing gently.
3. Remove the caps and snap off the bottom closures from the MicroSpin columns. Place the columns in the manifold, filling any unused holes with the plugs provided with MicroPlex 24 Vacuum.
4. Check the stopcock to make sure that it is in the closed position (i.e. perpendicular to the vacuum tubing) and that the manifold is placed squarely on the gasket.
5. Turn on the vacuum supply at the source. Open the stopcock (i.e. parallel to the vacuum tubing). After the column storage buffer has been drawn through all the columns into the collection tray, close the stopcock.
6. Allow 10–15 s for the vacuum pressure to dissipate. Remove the manifold and place it on a paper towel.
7. Apply up to 600 µl of lysate to the column and incubate at room temperature for 5–10 min.
8. Open the stopcock. After the lysates have been drawn through all the columns into the collection tray, close the stopcock.
9. Add 600 µl of 1× PBS wash buffer to each column. Open the stopcock. After the buffer has been drawn through all the columns into the collection tray, close the stopcock.
10. Allow 10–15 s for the vacuum pressure to dissipate. Remove the manifold and reassemble the apparatus with a clean collection tray.

Additional 600 µl 1× PBS washes can be performed if desired.

11. Add 200 µl of elution buffer to each column. Incubate at room temperature for 5–10 min.
12. Open the stopcock. After the elution buffer has been drawn through all the columns into the collection tray, close the stopcock.
13. Allow 10–15 s for the vacuum pressure to dissipate. Remove the manifold. Cover the eluates with sealing tape until required for analysis.

Yields of fusion protein can be increased by repeating the elution step two or three times and pooling the eluates.

# Purification using GSTrap FF 1 ml or 5 ml columns

GSTrap FF affinity columns provide a convenient format for fast and easy one-step purification of GST fusion proteins produced using the pGEX series of expression vectors, as well as other glutathione S-transferases, and glutathione binding proteins. The columns are specially designed 1 ml and 5 ml HiTrap columns prepacked with Glutathione Sepharose 4 Fast Flow, a high-capacity affinity medium with excellent flow properties. See Table 13.

**Table 13.** Column parameters of GSTrap FF columns

| | |
|---|---|
| Column type | GSTrap FF 1 ml and 5 ml prepacked columns |
| Prepacked medium | Glutathione Sepharose 4 Fast Flow |
| Column dimensions<br>(internal diameter × height) | 0.7 cm × 2.5 cm (GSTrap FF 1 ml)<br>1.6 cm × 2.5 cm (GSTrap FF 5 ml) |
| Column volume | 1 ml and 5 ml |
| Maximum back pressure | 0.3 MPa, 3 bar |
| Maximum flow rate | 4 ml/min (GSTrap FF 1 ml)<br>15 ml/min (GSTrap FF 5 ml) |
| Recommended flow rates | Sample loading:<br>0.2–1 ml/min (GSTrap FF 1 ml)<br>1–5 ml/min (GSTrap FF 5 ml)<br>Wash and elution:<br>1–2 ml/min (GSTrap FF 1 ml)<br>5–10 ml/min (GSTrap FF 5 ml) |

References 24–30 provide examples of use of GSTrap FF columns for purification of GST fusion proteins.

Sample application, washing, and elution can be performed using a syringe with a supplied adapter, a peristaltic pump, or a liquid chromatography system such as ÄKTAdesign (see Table 11 for equipment choices). For easy scale-up, two to three columns can be connected together in series simply by screwing the end of one column into the top of the next.

Figure 4 shows a schematic of the simple steps needed for successful purification using a 1 ml GSTrap FF column.

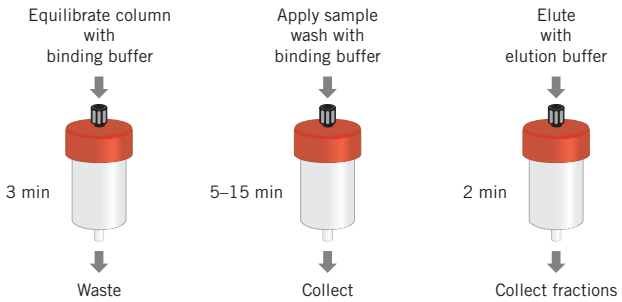



**Fig 4.** Simple purification of GST fusion proteins using a GSTrap FF column.

The column is made of polypropylene, which is biocompatible and non-interactive with biomolecules. The top and bottom frits are manufactured from porous polyethylene. Columns are delivered with a stopper on the inlet and a twist-off end on the outlet. Both ends have M6 connections (6 mm metric threads). Every package includes all necessary components for connection of the columns to different types of equipment.

GSTrap FF columns are directly compatible with existing purification protocols for GST fusion proteins, including on-column proteolytic cleavage methods. If removal of the GST moiety is required, the fusion protein can be digested with an appropriate site-specific protease while bound to the medium or, alternatively, after elution (see Chapter 6). On-column cleavage eliminates the extra step of separating the released protein from GST since the GST moiety remains bound (23).

GSTrap FF columns cannot be opened or refilled.

For quick scale-up of purifications, two or three GSTrap FF columns (1 ml or 5 ml) can be connected in series (back pressure will be higher).

Glutathione Sepharose 4 Fast Flow is also available in prepacked 20 ml GSTPrep FF 16/10 columns (Procedure 12) and as a loose medium for packing high-performance columns (Procedure 14).

Re-use of any purification column depends on the nature of the sample and should only be performed with identical fusion proteins to prevent cross-contamination.

## 11.1. Manual purification using GSTrap FF column with a syringe

One of the simplest methods for GST fusion protein purification is the use of GSTrap FF columns in combination with a syringe, as shown in Figure 5.


**Fig 5.** Using a GSTrap FF column with a syringe. A) Prepare buffers and sample. Remove the column's top cap and twist off the end. B) Load the sample and begin collecting fractions. C) Wash and elute and continue collecting fractions.

Refer to page 27, General considerations for purification of GST fusion proteins, before beginning this procedure.

**Reagents and equipment required**

Binding buffer: 1× PBS (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3)

Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

Syringe

**Steps**

1. Fill a syringe with binding buffer.
2. Connect the column to the syringe using the adapter supplied. Avoid introducing air into the column.
3. Remove the twist-off end.
4. Equilibrate the column with five column volumes of binding buffer.
5. Apply the sample using the syringe. For best results, maintain a flow rate of 0.2–1 ml/min (1 ml column) and 1–5 ml/min (5 ml column) as the sample is applied.*
6. Wash with 5–10 column volumes of binding buffer. Maintain flow rates of 1–2 ml/min (1 ml column) and 5–10 ml/min (5 ml column) during the wash.*

Optional: Collect the flow-through (in 1 ml fractions for the 1 ml column and 2 ml fractions for the 5 ml column) and reserve until the procedure has been successfully completed.

Retain a sample for analysis by SDS-PAGE or by CDNB assay to measure the efficiency of protein binding to the medium.

7. Elute with 5–10 column volumes of elution buffer and collect fractions. Maintain flow rates of 1–2 ml/min (1 ml column) and 5–10 ml/min (5 ml column) during elution.*

**One ml/min corresponds to approximately 30 drops/min when using a syringe with a HiTrap 1 ml column, and 5 ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column.*

*Note: The flow rate is critical for the yield. A lower flow rate may increase the binding capacity and yield.*

For large sample volumes, a simple peristaltic pump can be used to apply sample and buffers.

## 11.2. Simple purification using a GSTrap FF column with ÄKTAprime

ÄKTAprime, in combination with pre-installed templates for purifications and prepacked columns, is designed to perform the most common protein purification steps at the touch of a button. It provides significant advantages in speed, capacity, and fraction selection over manual methods. A set of cue cards includes detailed information on each procedure. Almost any sample volume can be loaded when using ÄKTAprime. High flow rates allow fast separations, and with on-line monitoring, UV, conductivity, or pH can be measured during a purification.

The pre-programmed application template in ÄKTAprime for purification of GST fusion proteins using a single GSTrap FF column is shown in Figure 6. This provides a standard purification protocol that can be followed exactly or optimized as required. Typical procedures and results are shown in Figures 7 and 8, respectively.

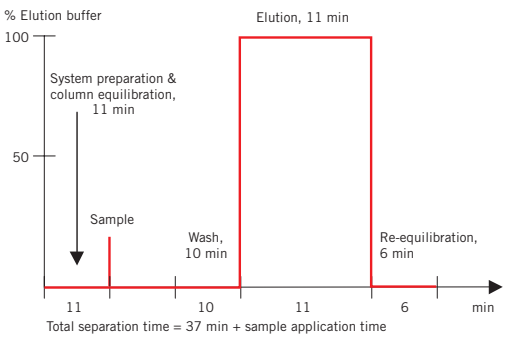



**Fig 6.** Purification of GST fusion proteins using a GSTrap FF column and ÄKTAprime.

Refer to page 27, General considerations for purification of GST fusion proteins, before beginning this procedure.

### Reagents required

Binding buffer: 20 mM sodium phosphate, 0.15 M NaCl, pH 7.3 (or the buffer used in Procedure 11.1)

Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

Prepare at least 500 ml of each buffer.

### Steps

1. Follow the instructions supplied on the ÄKTAprime cue card.
2. Select the Application Template.
3. Start the method.
4. Enter the sample volume and press OK to start.

Connecting the column.

Preparing the fraction collector.

**Fig 7.** Typical procedures when using ÄKTAprime.

A)

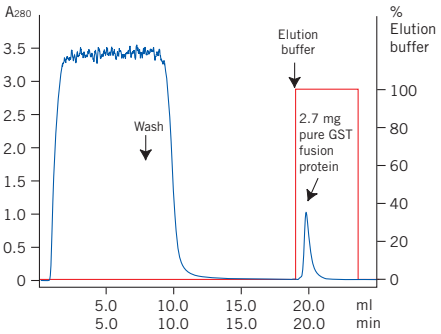
| | |
|---|---|
| *Column:* | GSTrap FF 1 ml |
| *Sample:* | 8 ml cytoplasmic extract from *E. coli* expressing a GST fusion protein |
| *Binding buffer:* | PBS, pH 7.3 |
| *Elution buffer:* | 50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione |
| *Flow:* | 1 ml/min |
| *Chromatographic procedure:* | 4 CV binding buffer, 8 ml sample, 10 CV binding buffer, 5 CV elution buffer, 5 CV binding buffer (CV = column volume) |

B)

Lane 1: Low Molecular Weight (LMW) Calibration kit, reduced, Amersham Biosciences (*10 µl prepared for silver stain*)

Lane 2: Cytoplasmic extract of *E. coli* expressing GST fusion protein, 1 g cell paste/10 ml (*5 µl sample from collect. fraction + 35 µl sample cocktail -> 10 µl applied*)

Lane 3: GST fusion protein eluted from GSTrap FF 1 ml (*5 µl sample from collect. fraction + 35 µl sample cocktail -> 10 µl applied*)



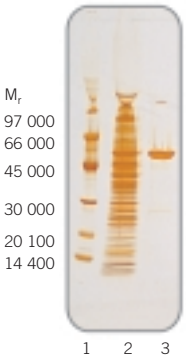




**Fig 8.** Purification of GST fusion protein on a GSTrap FF 1 ml column A) Chromatogram. B) SDS-PAGE on ExcelGel™ SDS Gradient 8–18% using Multiphor™ II (Amersham Biosciences) followed by silver staining.

## Preparative purification using GSTPrep FF 16/10 column

GSTPrep FF 16/10 columns are specially designed 20 ml HiPrep™ columns, ready to use for easy, one-step preparative purification of GST fusion proteins, other glutathione S-transferases, and glutathione binding proteins. Prepacked with Glutathione Sepharose 4 Fast Flow, the columns exhibit high binding capacity and excellent flow properties. For easy scale-up, columns can be connected in series.

The column is made of polypropylene, which is biocompatible and non-interactive with biomolecules. Both ends have M6 connections (6 mm metric threads), together with the included connectors. Separations can be easily achieved using a chromatography system such as ÄKTAdesign. Refer to Table 11 for a selection guide to purification equipment and to Table 14 for a list of GSTPrep FF 16/10 column parameters.

GSTPrep FF 16/10 columns cannot be opened or refilled.

For quick scale-up of purifications, two GSTPrep FF 16/10 columns can be connected in series (back pressure will be higher).

Glutathione Sepharose 4 Fast Flow is also available as prepacked 1 ml and 5 ml GSTrap FF columns (Procedures 11.1 and 11.2) and as a loose medium for packing high-performance columns (Procedure 14).

Re-use of any purification column depends on the nature of the sample and should only be performed with identical fusion proteins to prevent cross-contamination.

**Table 14.** Column parameters of GSTPrep FF 16/10 columns

| | |
|---|---|
| Column type | GSTPrep FF 16/10 prepacked columns |
| Prepacked medium | Glutathione Sepharose 4 Fast Flow |
| Column dimensions (internal diameter × height) | 16 × 100 mm |
| Column volume | 20 ml |
| Maximum pressure over the packed bed during operation | 0.15 MPa, 1.5 bar |
| HiPrep column hardware pressure limit | 0.5 MPa, 5 bar |
| Maximum flow rate | 10 ml/min (300 cm/h) |
| Recommended flow rates | 1–10 ml/min (30–300 cm/h) (protein dependent) |

## 12. Preparative purification using GSTPrep FF 16/10 column

Refer to page 27, General considerations for purification of GST fusion proteins, before beginning this procedure.

**Reagents required**

Binding buffer: 1× PBS (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3)

Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

**Initial purification/optimization**

1. Apply the centrifuged and/or filtered sample (in binding buffer) to the column at a flow rate of 1–5 ml/min (30–150 cm/h).
2. Wash the column with 5–10 column volumes of binding buffer at 2–10 ml/min (60–300 cm/h).
3. Elute the bound protein with 5–10 column volumes of elution buffer at a flow rate of 2–10 ml/min (60–300 cm/h).

Due to the relatively slow binding kinetics between GST and glutathione, it is important to keep the flow rate low during sample loading/elution. The binding capacity is protein dependent and therefore the yield will vary from protein to protein.

Optional: Collect the flow-through and reserve until the procedure has been successfully completed. Retain a sample for analysis by SDS-PAGE or by CDNB assay to measure the efficiency of protein binding to the medium

For cleaning, storage, and handling information, refer to Appendix 5.

## Purification using Glutathione Sepharose 4B medium

Glutathione Sepharose 4B is available in 10 ml, 300 ml, and function-tested 100 ml lab packs for affinity purification of GST fusion proteins in batch- or column-based methods. The batch method is very flexible, as purification can be performed with 10 µl to 10 ml of Glutathione Sepharose 4B. Both batch and batch/column purification schemes are presented below. For larger-scale column purifications, the high flow properties and binding capacity of Glutathione Sepharose 4 Fast Flow make it an excellent choice for scale-up.

### 13.1. Batch purification using Glutathione Sepharose 4B

The following batch protocol can be conveniently scaled to purify as little as 50 µg or as much as 50 mg of GST fusion protein using Glutathione Sepharose 4B.

Refer to page 27, General considerations for purification of GST fusion proteins, before beginning this procedure.

**Reagents required**

Glutathione Sepharose 4B

Binding buffer: 1× PBS (ice-cold) (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3)

Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0. It is possible to dispense in 1–10 ml aliquots and store at -20 °C until needed. Avoid more than five freeze/thaw cycles.

### Steps

#### Preparation of the medium

Glutathione Sepharose 4B, as supplied, is approximately a 75% slurry. The following procedure results in a 50% slurry.

1. Referring to Table 9, determine the bed volume of Glutathione Sepharose 4B required for your application.
2. Gently shake the bottle of Glutathione Sepharose 4B to resuspend the medium.
3. Use a pipet with a wide-bore tip to remove sufficient slurry, and transfer the slurry to an appropriate container/tube.
4. Sediment the medium by centrifuging at 500 × g for 5 min. Carefully decant the supernatant.
5. Wash the Glutathione Sepharose 4B by adding 10 ml of cold (4 °C) 1× PBS per 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed. Invert to mix.

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

6. Sediment the medium by centrifuging at 500 × g for 5 min. Decant the supernatant.
7. For each 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed, add 1 ml of 1× PBS. This results in a 50% slurry. Mix well prior to subsequent pipetting steps.

#### Batch purification

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

1. Add 2 ml of the 50% slurry of Glutathione Sepharose 4B equilibrated with 1× PBS to each 100 ml of bacterial sonicate (i.e. use a 1 ml bed volume per 100 ml of sonicate).
2. Incubate for 30 min at room temperature. Use gentle agitation such as end-over-end rotation.

At this stage, the medium with adsorbed fusion protein can be packed into a suitable column to facilitate washing and elution steps. If a column is used, refer to the column purification procedure in the following section for instructions on washing and elution.

3. Sediment the medium by centrifuging at 500 × g for 5 min. Carefully decant the supernatant (= flow-through).
4. Wash the medium with 10 bed volumes of 1× PBS. Invert to mix.
5. Sediment the medium by centrifuging at 500 × g for 5 min. Carefully decant the supernatant (= wash).
6. Repeat steps 4 and 5 twice for a total of three washes.

Optional: Reserve the flow-through and wash until the procedure has been successfully completed. Retain a sample for analysis by SDS-PAGE or by CDNB assay to measure the efficiency of protein binding to the medium.

Bound fusion protein can be eluted directly at this stage using elution buffer, or the protein can be cleaved on the medium to liberate the protein of interest from the GST moiety (see Chapter 6 for various options).

7. Elute the bound protein from the sedimented medium by adding 1.0 ml of elution buffer per 1 ml bed volume of the original slurry.
8. Mix gently to resuspend the medium. Incubate at room temperature for 10 min to elute the fusion protein from the medium. Use gentle agitation such as end-over-end rotation.

9. Sediment the medium by centrifuging at 500 × g for 5 min. Carefully decant the supernatant (= eluted protein) into a fresh centrifuge tube.
10. Repeat steps 7–9 twice for a total of three elutions. Check the three eluates separately for purified protein and pool those eluates containing protein.

## 13.2. Batch/column purification using Glutathione Sepharose 4B

Refer to page 27, General considerations for purification of GST fusion proteins, before beginning this procedure.

### Reagents and equipment required

Glutathione Sepharose 4B

Binding buffer: 1× PBS (ice-cold) (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3)

Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0. It is possible to dispense in 1–10 ml aliquots and store at -20 °C until needed. Avoid more than five freeze/thaw cycles.

Suitable disposable columns, for example Empty Disposable PD-10 Columns. These columns have a total volume capacity (including medium and sample) of ~ 13 ml, but they can be used with smaller volumes.

### Steps

#### Preparation of the medium

Steps 1–7 below are identical to those at the start of the batch procedure above.

Glutathione Sepharose 4B, as supplied, is approximately a 75% slurry. The following procedure results in a 50% slurry.

1. Referring to Table 9, determine the bed volume of Glutathione Sepharose 4B required for your application.
2. Gently shake the bottle of Glutathione Sepharose 4B to resuspend the medium.
3. Use a pipet to remove sufficient slurry for use and transfer the slurry to an appropriate container/tube.
4. Sediment the medium by centrifuging at 500 × g for 5 min. Carefully decant the supernatant.
5. Wash the Glutathione Sepharose 4B by adding 10 ml of cold (4 °C) 1× PBS per 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed. Invert to mix.

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

6. Sediment the medium by centrifuging at 500 × g for 5 min. Decant the supernatant.
7. For each 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed, add 1 ml of 1× PBS. This results in a 50% slurry. Mix well prior to subsequent pipetting steps.

#### Batch/column purification

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

1. Add 2 ml of the 50% slurry of Glutathione Sepharose 4B equilibrated with 1× PBS to each 100 ml of bacterial sonicate (i.e. use a 1 ml bed volume per 100 ml of sonicate).
2. Incubate for 30 min at room temperature. Use gentle agitation such as end-over-end rotation.
3. Use a pipet to transfer the medium to a disposable column (e.g. Empty Disposable PD-10 Column mounted in its rack folded from the package).
4. Tap the column to dislodge any trapped air bubbles in the medium bed. Allow the medium to settle.
5. Open the column outlet and allow the column to drain.

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

6. Wash the medium by adding 10 bed volumes of 1× PBS. Open the column outlet and allow the column to drain. Repeat twice more for a total of three washes.
7. After the column with bound protein has been washed and drained, close the column outlet.
8. Elute the fusion protein by adding 1 ml of elution buffer per 1 ml bed volume. Incubate the column at room temperature for 10 min to elute the fusion protein.
9. Open the column outlet and collect the eluate. This contains the fusion protein.
10. Repeat the elution and collection steps twice more. Pool the three eluates.

Optional: Collect the flow-through in fractions and reserve until the procedure has been successfully completed. Retain a sample for analysis by SDS-PAGE or by CDNB assay to measure the efficiency of protein binding to the medium.

Because of its superior flow rates, we recommend Glutathione Sepharose 4 Fast Flow (Procedure 14) for use when column purification is desired.

## Purification using Glutathione Sepharose 4 Fast Flow

Physical characteristics of Glutathione Sepharose 4 Fast Flow are listed in Table 8. For information on cleaning and storage of columns packed with Glutathione Sepharose 4 Fast Flow, refer to Appendix 5.

Suggested columns for this application include:

- HR 10/10 (10 mm i.d.) for bed volumes of 6.4–8.7 ml and bed heights of 8–11 cm.
- XK 16/20 (16 mm i.d.) for bed volumes of 2–34 ml and bed heights of 1–17 cm.
- XK 26/20 (26 mm i.d.) for bed volumes of 0–80 ml and bed heights of 0–15 cm.

Use the Packing Connector HR or XK to produce a well-packed column. A Column Packing Video is available to demonstrate appropriate packing techniques.

## 14. Column purification using Glutathione Sepharose 4 Fast Flow

Refer to page 27, General considerations for purification of GST fusion proteins, before beginning this procedure.

Glutathione Sepharose 4 Fast Flow is supplied in 20% ethanol as a bacteriostat. Decant the solution and replace it with binding buffer before use. In general, we recommend a bed height of 5–15 cm to allow the use of high flow rates.

Prepacked GSTrap FF 1 ml and GSTrap FF 5 ml columns (Procedures 11.1 and 11.2) and GSTPrep 16/10 columns (20 ml; Procedure 12) are also available.

**Reagents required**

Glutathione Sepharose 4 Fast Flow

Binding buffer: 1× PBS (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3)

Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

**Steps**

**Column packing**

1. Assemble the column (and packing reservoir if necessary).
2. Remove air from the column dead spaces by flushing the end-piece and adapter with binding buffer. Ensure that no air has been trapped under the column bed support. Close the column outlet, leaving the bed support covered with binding buffer.
3. Resuspend the medium stored in its container by shaking (avoid stirring the sedimented medium). Mix the binding buffer with the medium to form a 50–70% slurry (sedimented bed volume/slurry volume = 0.5–0.7).
4. Pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize air bubble formation.
5. If using a packing reservoir, immediately fill the remainder of the column and reservoir with binding buffer. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.
6. Open the bottom outlet of the column and set the pump to run at the desired flow rate. Ideally, Glutathione Sepharose 4 Fast Flow is packed at a constant pressure of approximately 0.1 MPa (1 bar).

If the packing equipment does not include a pressure gauge, use a packing flow velocity of approximately 450 cm/h (10 cm bed height, 25 °C, low-viscosity buffer). 450 cm/h corresponds to 6 ml/min in a HR 10/10 column or 15 ml/min in a XK 16/20 column. If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate allowed by the pump. This should also give a sufficiently well-packed bed.

Do not exceed 75% of the packing flow velocity in subsequent chromatographic procedures using the same pump.

7. When the bed has stabilized, close the bottom outlet, and stop the pump.
8. If using a packing reservoir, disconnect the reservoir, and fit the adapter to the column. If using a HR column, carefully place the top filter on top of the bed before fitting the adapter.
9. With the adapter inlet disconnected, push down the adapter approximately 2 mm into the bed, allowing the solution to flush the adapter inlet. The bottom outlet of the column should be closed.
10. Connect the pump, open the bottom outlet, and continue packing. The bed will be further compressed at this point and a space will be formed between the bed surface and the adapter.
11. Close the bottom outlet. Disconnect the column inlet, and lower the adapter approximately 2 mm into the bed. Connect the pump. The column is now ready to use.

**Column purification**

1. Equilibrate the column with approximately five column volumes of binding buffer (1× PBS).
2. Apply the centrifuged and/or filtered sample.
3. Wash the column with 5–10 column volumes of binding buffer or until no material appears in the flow-through.
4. Elute the bound protein with 5–10 column volumes of elution buffer.

Optional: Collect the flow-through and reserve until the procedure has been successfully completed. Retain a sample for analysis by SDS-PAGE or by CDNB assay.

The reuse of Glutathione Sepharose 4 Fast Flow depends on the nature of the sample and should only be performed with identical samples to prevent cross-contamination.

# Chapter 5
# Detection of GST fusion proteins

Several methods are available for detection of GST fusion proteins, with method selection largely depending on the experimental situation. For example, SDS-PAGE analysis, although frequently used for monitoring results during expression and purification (see Chapter 3), may not be the method of choice for routine monitoring of samples from high-throughput screening. Functional assays based on the properties of the protein of interest (and not the GST tag) are useful, but must be developed for each specific protein. These latter assays are not covered in this handbook. See Table 15 for a description of the procedures that follow.

## Summary of procedures

**Table 15.** Procedures for detection of GST fusion proteins

| Procedure | Description | Comments |
|---|---|---|
| 15 | GST 96-Well Detection Module for ELISA | Uses 100 µl of sample/well. Ideal for screening expression systems and chromatographic fractions. Useful when amount of expressed protein is unknown or when increased sensitivity is required. Gives estimate of relative level of expression. |
| 16 | GST Detection Module with CDNB enzymatic assay | Uses 5–50 µl of sample. Rapid assay; ideal for screening. Gives estimate of relative level of expression. |
| 17 | Western blot using anti-GST antibody | Uses 5–10 µl of sample. Highly specific, detects only GST fusion protein. Little or no background detectable when using detection systems with optimized concentrations of secondary HRP-conjugated antibody. ECL™ detection systems enhance detection in Western blots. ECL provides adequate sensitivity for most recombinant expression applications. For higher sensitivity, use ECL Plus. Provides information on size. |
| 18 | SDS-PAGE with Coomassie or silver staining | Uses 5–10 µl of sample. Provides information on size and percent purity. Detects fusion protein and contaminants. |

## 15. GST 96-Well Detection Module for ELISA

The GST 96-Well Detection Module provides a highly sensitive ELISA for testing clarified lysates and intermediate purification fractions for the presence of GST fusion proteins (see Figs 9 and 10). Samples are applied directly into the wells of the plates, and GST fusion proteins are captured by specific binding to anti-GST antibody that is immobilized on the walls of each well. Captured GST fusion proteins are then detected with HRP/Anti-GST conjugate provided in the module. Standard curves for quantitation of fusion proteins can be constructed using purified recombinant GST, which is included as a control.

Each detection module contains reagents sufficient for 96 detections. Each plate is an array of 12 strips with eight wells per strip, such that as few as eight samples (one strip) can be assayed at a time.

The GST 96-Well Detection Module can also be used with antibody directed against a GST fusion partner to screen and identify clones expressing the desired GST fusion protein.

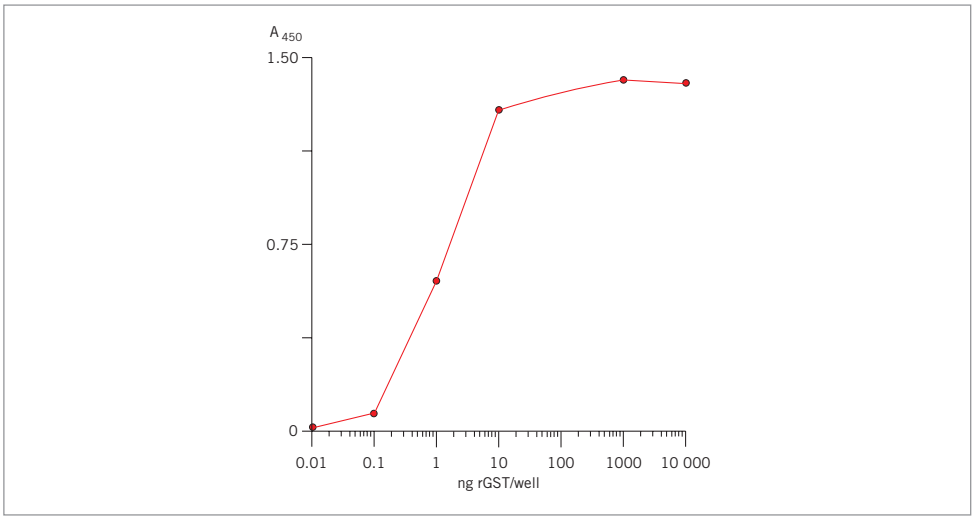



**Fig 9.** Sensitive detection of recombinant GST using the GST 96-Well Detection Module. Recombinant GST protein was prepared in 1× blocking buffer, and 100 µl volumes were applied directly to the wells of a GST 96-well capture plate. After 1 h binding at room temperature, the wells were washed in wash buffer and incubated with a 1:1000 dilution of HRP/Anti-GST conjugate for 1 h. Detection was performed using 3, 3',5,5'-tetramethyl benzidine (TMB) substrate, and the absorbance of each well was measured at 450 nm.

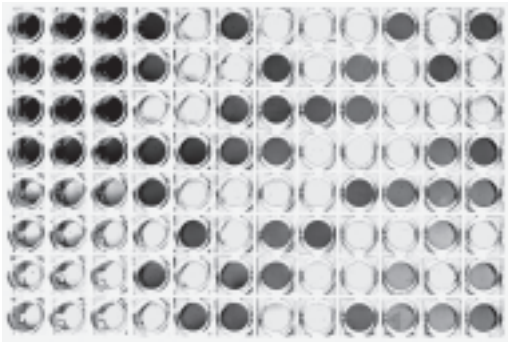


**Fig 10.** Screening of bacterial lysates for GST fusion protein expression using the GST 96-Well Detection Module.

Each fusion protein is captured uniquely; therefore, if quantitation is required, prepare standards of recombinant GST protein and the target fusion protein (if available) using a dilution series from 1 ng/µl to 10 pg/µl in 1× blocking buffer. Include recombinant GST protein as a standard control in every assay.

Prepare fresh buffers daily.

**Components of GST 96-Well Detection Module**

GST 96-Well Detection Plates (each well is coated with goat polyclonal anti-GST antibody, blocked, and dried)

Horseradish peroxidase conjugated to goat polyclonal anti-GST antibody (HRP/Anti-GST)

Purified recombinant glutathione S-transferase (GST) standard protein

**Additional reagents required for ELISA**

PBS (1×): 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3

Wash buffer: 0.05% Tween™ 20 in PBS (500 ml/96-well plate). Store at room temperature until needed.

Blocking buffer (1×): 3% non-fat dry milk in PBS with 0.05% Tween 20 (10 ml/96-well plate)

Blocking buffer (2×): 6% non-fat dry milk in PBS with 0.1% Tween 20 (5 ml/96-well plate)

Substrate

**Steps**

1. Bring each test sample to a final volume of 50 µl with 1× PBS.
2. Add 50 µl of 2× blocking buffer to each sample.
3. For screening, dilute the recombinant GST protein standard to 1 ng/100 µl in 1× blocking buffer.
4. For quantitation, prepare a dilution series from 1 ng/µl to 10 pg/µl in 1× blocking buffer for both the recombinant GST protein and the target fusion protein (when available).
5. Remove one 96-well plate from its foil pouch.

If using fewer than 96 wells, carefully remove the well strips from the holder by pushing up on the wells from below. Store unused well strips in the pouch with the desiccant provided.

6. Pipette 100 µl of sample into each well.
7. Incubate for 1 h at room temperature in a humidified container or incubator.
8. Invert the plate and flick sharply to empty the contents of the wells.

Biohazardous material should be pipetted or aspirated into a suitable container.

9. Blot the inverted plate or well strips onto a paper towel to remove excess liquid.
10. Wash each well five times with wash buffer by inverting and flicking out the contents each time.
11. Blot the inverted plate or well strips onto a paper towel to remove excess wash buffer.
12. Dilute the HRP/anti-GST conjugate 1:10 000 (1 µl:10 ml) in 1× blocking buffer.

One 96-well plate will require 10 ml of the diluted conjugate.

13. Add 100 µl of diluted HRP/anti-GST conjugate to each well and incubate for 1 h at room temperature in a humidified container or incubator.
14. Empty the well contents and wash twice with wash buffer as previously described.
15. Add soluble horseradish peroxidase substrate* to each well and incubate according to the supplier's instructions.

    **3,3',5,5'-tetramethyl benzidine ($A_{450}$) and 2',2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS™) ($A_{410}$) have been used successfully.*

16. Read plate absorbance in a microplate reader or spectrophotometer.

## 16. GST Detection Module with CDNB enzymatic assay

GST fusion proteins produced using pGEX vectors can be detected enzymatically using the GST substrate 1-chloro-2,4 dinitrobenzene (CDNB) (31, 32), included in the GST Detection Module. The GST-mediated reaction of CDNB with glutathione produces a conjugate that is measured by absorbance at 340 nm using either a plate reader or a UV/vis spectrophotometer, such as an Ultrospec™ 1100 *pro*. Assay results are available in less than 10 min for

crude bacterial sonicates, column eluates, or purified GST fusion protein. Figure 11 shows typical results from a CDNB assay. Each GST Detection Module contains reagents sufficient for 50 assays.

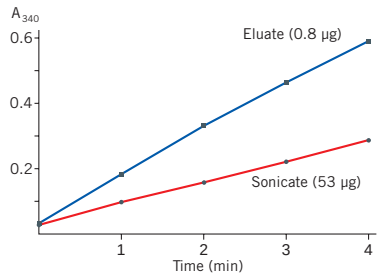



**Fig 11.** Typical results of a CDNB assay for GST fusion proteins. 53 µg of total protein from an *E. coli* TG1/pGEX-4T-Luc sonicate and 0.8 µg of total protein eluted from Glutathione Sepharose were assayed according to instructions included with the GST Detection Module.

**Components of GST Detection Module used with the CDNB enzymatic assay**

10× reaction buffer: 1 M $KH_2PO_4$ buffer, pH 6.5

CDNB: 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol

Reduced glutathione powder: Prepare a 100 mM solution by dissolving the reduced glutathione powder in sterile distilled $H_2O$. Aliquot into microcentrifuge tubes. Store at -20 °C. Avoid more than five freeze/thaw cycles.

CDNB is toxic. Avoid contact with eyes, skin and clothing. In case of accidental contact, flush affected area with water. In case of ingestion, seek immediate medical attention.

pGEX-bearing cells must be lysed prior to performing a CDNB assay.

**Steps**

1. In a microcentrifuge tube, combine the following:

| | |
|---|---|
| Distilled $H_2O$ | 880 µl |
| 10× reaction buffer | 100 µl |
| CDNB | 10 µl |
| Glutathione solution | 10 µl |
| Total volume | 1000 µl |

2. Cap the tube and mix the contents by inverting several times.

CDNB may cause the solution to become slightly cloudy. However, the solution should clear upon mixing.

3. Transfer 500 µl volumes of the above CDNB solution into two UV-transparent cuvettes labelled sample and blank. Add sample (5–50 µl) to the sample cuvette. To the blank cuvette, add 1× reaction buffer equal in volume to that of the sample in the sample cuvette.
4. Cover each cuvette with wax film and invert to mix.
5. Place the blank cuvette into the spectrophotometer and blank at 340 nm. Measure the absorbance of the sample cuvette at 340 nm and simultaneously start a stopwatch or other timer.
6. Record absorbance readings at 340 nm at 1 min intervals for 5 min by first blanking the spectrophotometer with the blank cuvette and then measuring the absorbance of the sample cuvette.

7. Calculate the $A_{340}$/min/ml sample as follows:

**Calculations**

$$\Delta A_{340}/\text{min/ml} = \frac{A_{340}(t_2) - A_{340}(t_1)}{(t_2 - t_1)(\text{ml sample added})}$$

Where: $A_{340}(t_2)$ = absorbance at 340 nm at time $t_2$ in min

$A_{340}(t_1)$ = absorbance at 340 nm at time $t_1$ in min

$\Delta A_{340}$/min/ml values can be used as a relative comparison of GST fusion protein content between samples of a given fusion protein.

Adapt the assay to give absolute fusion protein concentrations by constructing a standard curve of $\Delta A_{340}$/min versus fusion protein amount. Purified sample of the fusion protein is required to construct the curve.

Activity of the GST moiety can be affected by folding of the fusion partner. Absorbance readings obtained for a given fusion protein may not reflect the actual amount of fusion protein present.

## 17. Western blot using anti-GST antibody

Expression and purification of GST fusion proteins can also be monitored by Western blot analysis, using ECL or ECL Plus detection systems to enhance sensitivity.

**Reagents required**

Anti-GST antibody (goat polyclonal)

Blocking/incubation buffer: 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween 20 in 1× PBS (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3)

Wash buffer: 0.1% (v/v) Tween 20 in 1× PBS (as above)

Secondary antibody to detect the anti-GST antibody (such as anti-goat IgG HRP conjugate)

Appropriate membrane, such as Hybond ECL (for subsequent ECL detection) or Hybond P (for subsequent ECL or ECL Plus detection)

**Steps**

**Electrophoretic separation of proteins**

1. Separate the protein samples by SDS-PAGE.

Although anti-GST antibody from Amersham Biosciences has been cross-adsorbed with *E. coli* proteins, low levels of cross-reacting antibodies may remain. Samples of *E. coli* sonicates that do not contain a recombinant pGEX plasmid and samples that contain the parental pGEX plasmid should always be run as controls.

2. Transfer the separated proteins from the electrophoresis gel to membrane.

Electrophoresis and protein transfer can be accomplished using a variety of equipment and reagents. For further details, refer to the *Protein Electrophoresis Technical Manual* and *Hybond ECL Instruction Manual* from Amersham Biosciences.

### Blocking of membrane

1. Transfer the membrane onto which the proteins have been blotted into an appropriately sized container, such as a Petri dish.
2. Add 50–200 ml of blocking/incubation buffer to the container.
3. Incubate for 1–16 h at ambient temperature with gentle shaking.
4. Decant and discard the buffer.

Longer incubation times with blocking/incubation buffer may reduce background signal.

### Incubation of membrane blot with primary antibody

1. Prepare an appropriate dilution of anti-GST antibody with blocking/incubation buffer (e.g. 5–10 µl of antibody in 50 ml of buffer).

Refer to Amersham Biosciences Application Note 18-1139-13 for further information on optimization of antibody concentration for Western blotting.

2. Pour the antibody-buffer mixture into the container with the membrane.
3. Incubate for 1 h at ambient temperature with gentle shaking.
4. Decant and discard the antibody-buffer.
5. Rinse the membrane twice with 20–30 ml of blocking/incubation or wash buffer to remove most of the unbound antibody.
6. Decant and discard the rinse buffers.
7. Wash the membrane with 20–30 ml of blocking/incubation or wash buffer for 10–60 min at ambient temperature with gentle shaking.
8. Discard the buffer and repeat the wash from step 7.

### Incubation of membrane blot with secondary antibody

1. Dilute an appropriate anti-goat secondary antibody with blocking/incubation buffer according to the manufacturer's recommendation.

Refer to Amersham Biosciences Application Note 18-1139-13 for further information on optimization of antibody concentration for Western blotting.

2. Pour the antibody-buffer mixture into the container with the membrane.
3. Incubate for 1 h at ambient temperature with gentle shaking.
4. Decant and discard the antibody-buffer.
5. Rinse twice with 20–30 ml of blocking/incubation or wash buffer to remove most of the unbound antibody.
6. Decant and discard the rinse buffers.
7. Wash the membrane with 20–30 ml of blocking/incubation or wash buffer for 10–60 min at ambient temperature with gentle shaking.
8. Discard the buffer and repeat the wash step using wash buffer.

Use wash buffer not blocking/incubation buffer for step 9. The protein in blocking/incubation buffer would cause problems in the development step.

9. Develop the blot with a substrate that is appropriate for the conjugated secondary antibody.

ECL and ECL Plus detection systems require very little antibody to achieve a sufficient sensitivity; therefore, the amount of antibody (primary and secondary) used in the protocols can be minimized. Smaller quantities of antibody-buffer mixtures can be used by scaling down the protocol and performing the incubations in sealable plastic bags.

## 18. SDS-PAGE with Coomassie blue or silver staining

SDS-PAGE is useful for monitoring fusion protein levels during expression and purification. Transformants expressing the desired fusion protein are identified by the absence from total cellular proteins of the parental GST and by the presence of a novel, larger fusion protein. Parental pGEX vectors produce a $M_r$ 29 000 GST fusion protein containing amino acids coded for by the pGEX multiple cloning site.

### Reagents required

6× SDS loading buffer: 0.35 M Tris-HCl, 10.28% (w/v) SDS, 36% (v/v) glycerol, 0.6 M dithiothreitol (or 5% 2-mercaptoethanol), 0.012% (w/v) bromophenol blue, pH 6.8. Store in 0.5 ml aliquots at -80 °C.

### Steps

1. Add 1–2 µl of 6× SDS loading buffer to 5–10 µl of supernatant from crude extracts, cell lysates, or purified fractions, as appropriate.
2. Vortex briefly and heat for 5 min at 90–100 °C.
3. Centrifuge briefly, then load the samples onto an SDS-polyacrylamide gel.
4. Run the gel for the appropriate length of time and stain with Coomassie blue (Coomassie Blue R Tablets) or silver (PlusOne Silver Staining Kit, Protein).

The percentage of acrylamide in the SDS-gel should be selected based on the expected molecular weight of the protein of interest (see Table 16).

**Table 16.** Selecting the appropriate gel composition for protein separation

| Percent acrylamide in resolving gel | Separation size range ($M_r \times 10^3$) |
|---|---|
| **Single percentage** | |
| 5% | 36–200 |
| 7.5% | 24–200 |
| 10% | 14–200 |
| 12.5% | 14–100 |
| 15% | 14–60* |
| **Gradient** | |
| 5–15% | 14–200 |
| 5–20% | 10–200 |
| 10–20% | 10–150 |

**The larger proteins fail to move significantly into the gel.*

For information and advice on electrophoresis techniques, please refer to the section Additional reading on page 107.

# Chapter 6
# Removal of GST tag by enzymatic cleavage

In most cases, functional tests can be performed using the intact fusion with GST. However, if removal of the GST tag is necessary, fusion proteins containing a PreScission Protease, thrombin, or Factor Xa recognition site can be cleaved either while bound to Glutathione Sepharose or in solution after elution.

On-column cleavage is generally recommended as the method of choice since many potential contaminants can be washed out and the target protein eluted with a higher level of purity. Cleavage after elution is suggested if optimization of cleavage conditions is necessary.

It is highly recommended that fusion proteins be produced with a PreScission Protease cleavage site. The GST tag then can be removed and the protein purified in a single step on the column (see Fig 12 on next page). Because this protease is maximally active at 4 °C, cleavage can be performed at low temperatures, thus improving the stability of the target protein.

Thrombin or Factor Xa recognition sites can be cleaved either while the fusion protein is bound to the column or in solution after elution from the column (see Fig 13 on next page). For removal of thrombin and Factor Xa, GSTrap FF and HiTrap Benzamidine FF (high sub) columns can be connected in series so that cleaved product passes directly from the GSTrap FF into the HiTrap Benzamidine FF (high sub). Thus, samples are cleaved and proteases removed in a single step (see Fig 13 and application example 6 in Chapter 7) (see Procedure 22 for additional information).

Samples should be removed from the protease digest mixture at various time points and analyzed by SDS-PAGE to estimate the yield, purity, and extent of digestion.

**Table 17.** Approximate molecular weights for SDS-PAGE analysis

| Protease | Molecular weight |
|---|---|
| PreScission Protease* | 46 000 |
| Bovine thrombin | 37 000 |
| Bovine Factor Xa | 48 000 |

** PreScission Protease is a fusion protein of glutathione S-transferase and human rhinovirus type 14 3C protease (31).*

The amount of enzyme, temperature, and length of incubation required for complete digestion varies according to the specific GST fusion protein produced. Optimal conditions should always be determined in pilot experiments.

If protease inhibitors (see Table 18) have been used in the lysis solution, they must be removed prior to cleavage with PreScission Protease, thrombin, or Factor Xa. (The inhibitors will usually be eluted in the flow-through when sample is loaded onto a GSTrap FF column.)

## Cleavage of GST tag using PreScission Protease

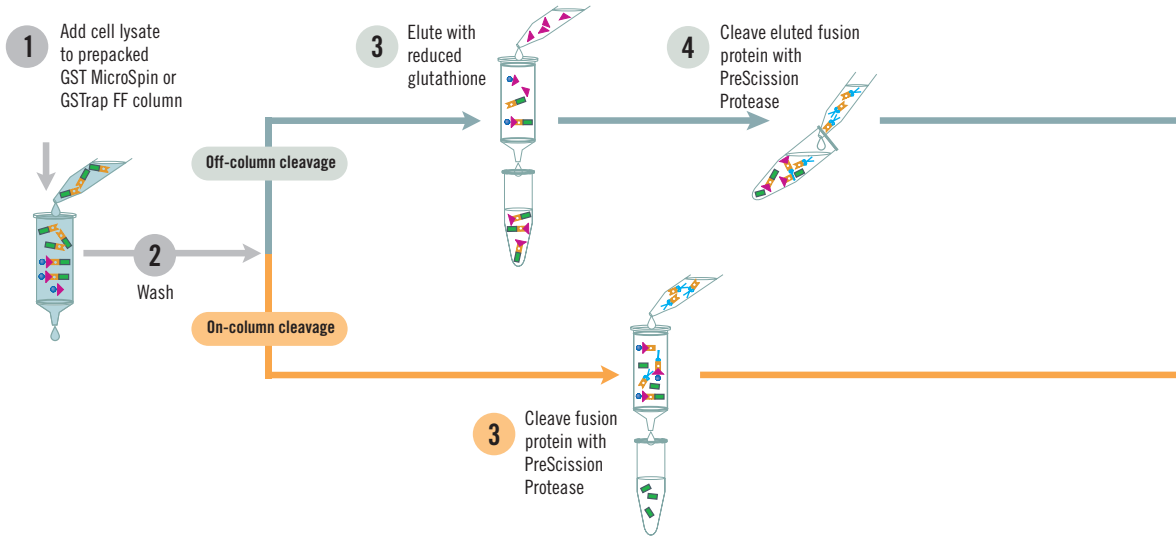



**Fig 12.** Flow chart of the affinity purification procedure and PreScission Protease cleavage of glutathione S-transferase fusion proteins.

## Cleavage of GST tag using thrombin or Factor Xa

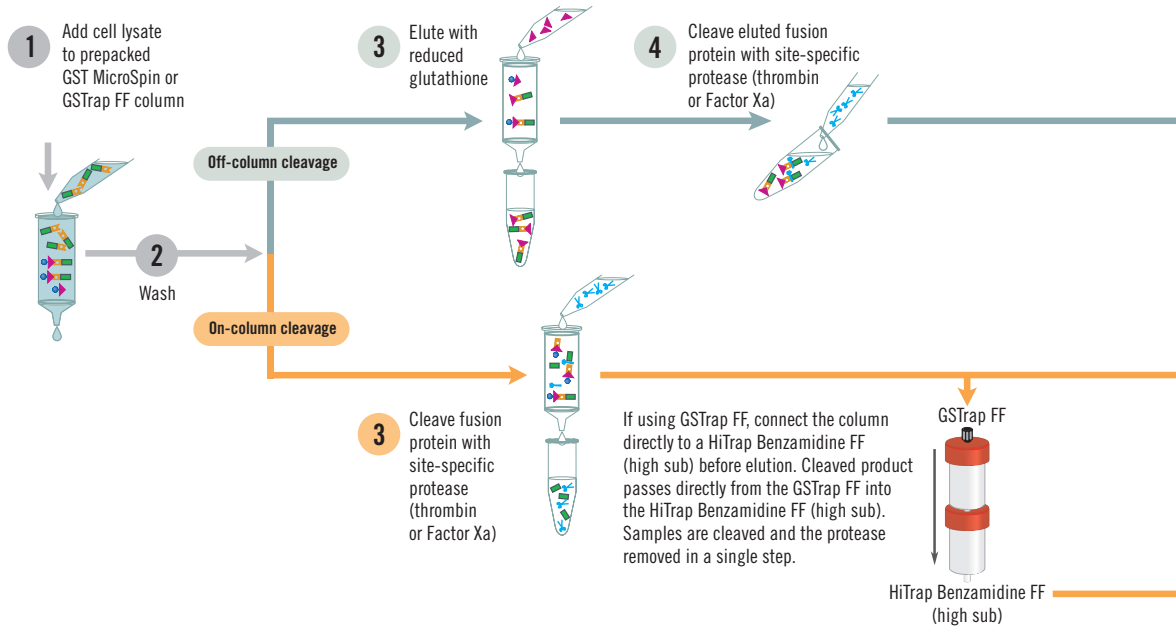
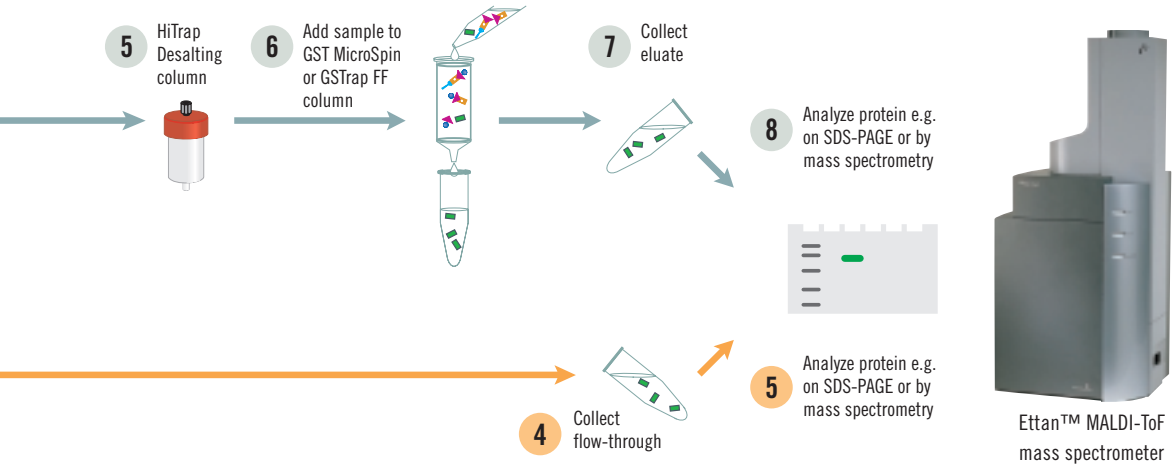



**Fig 13.** Flow chart of the affinity purification procedure and thrombin or Factor Xa cleavage of glutathione S-transferase fusion proteins.


5 HiTrap Desalting column
6 Add sample to GST MicroSpin or GSTrap FF column
7 Collect eluate
8 Analyze protein e.g. on SDS-PAGE or by mass spectrometry
5 Analyze protein e.g. on SDS-PAGE or by mass spectrometry
4 Collect flow-through
Ettan™ MALDI-ToF mass spectrometer

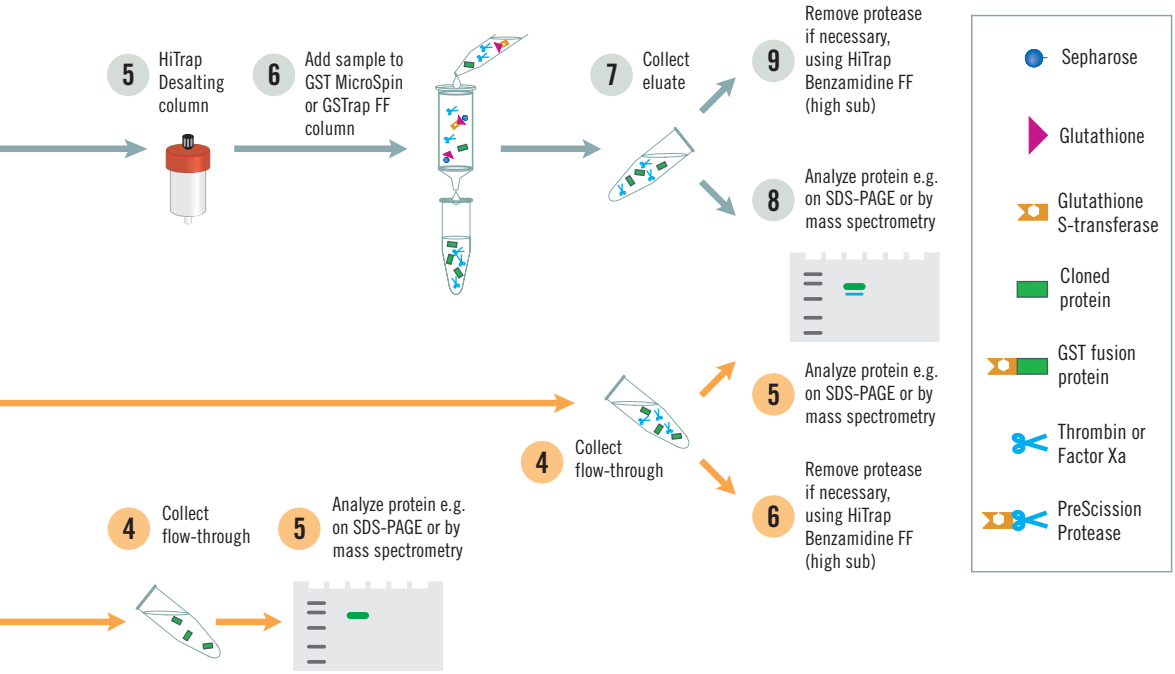

5 HiTrap Desalting column
6 Add sample to GST MicroSpin or GSTrap FF column
7 Collect eluate
9 Remove protease if necessary, using HiTrap Benzamidine FF (high sub)
8 Analyze protein e.g. on SDS-PAGE or by mass spectrometry
5 Analyze protein e.g. on SDS-PAGE or by mass spectrometry
4 Collect flow-through
6 Remove protease if necessary, using HiTrap Benzamidine FF (high sub)
4 Collect flow-through
5 Analyze protein e.g. on SDS-PAGE or by mass spectrometry
Sepharose
Glutathione
Glutathione S-transferase
Cloned protein
GST fusion protein
Thrombin or Factor Xa
PreScission Protease

**Table 18.** Inhibitors of the various proteases

| Enzyme | Inhibitor |
|---|---|
| PreScission Protease | 100 mM $ZnCl_2$ (> 50% inhibition)<br>100 µM chymostatin<br>4 mM Pefabloc™ |
| Factor Xa and thrombin | AEBSF, APMSF, antithrombin III, Antipain, α1-antitrypsin, aprotinin, chymostatin, hirudin, leupeptin, PMSF |
| Factor Xa only | Pefabloc FXa |
| Thrombin only | Pefabloc TH Benzamidine |

## Summary of procedures

Cleavage of fusion proteins is most commonly performed on milligram quantities of fusion protein suitable for purification on GSTrap FF columns. Protocols that follow describe manual cleavage and purification using a syringe and a 1 ml or 5 ml GSTrap FF column. The protocols can be adapted for use with GST MicroSpin columns to work at smaller scales.

For quick scale-up of purifications, two or three GSTrap FF columns can be connected in series (back pressure will be higher). Further scaling-up is possible using GSTPrep FF 16/10 columns or columns packed by the user with Glutathione Sepharose 4 Fast Flow. Protocols are included for column or batch format using this medium. Table 19 lists the cleavage procedures found in this chapter.

**Table 19.** Procedures for removal of GST by enzymatic cleavage

| Procedure | Description |
|---|---|
| **PreScission Protease cleavage and purification** | |
| 19.1 | PreScission Protease cleavage and purification of GST fusion protein bound to GSTrap FF |
| 19.2 | PreScission Protease cleavage and purification of GST fusion protein eluted from GSTrap FF |
| 19.3 | PreScission Protease cleavage and purification of GST fusion protein bound to Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B |
| 19.4 | PreScission Protease cleavage and purification of GST fusion protein eluted from Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B |
| **Thrombin cleavage and purification** | |
| 20.1 | Thrombin cleavage and purification of GST fusion protein bound to GSTrap FF |
| 20.2 | Thrombin cleavage and purification of GST fusion protein eluted from GSTrap FF |
| 20.3 | Thrombin cleavage and purification of GST fusion protein bound to Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B |
| 20.4 | Thrombin cleavage and purification of GST fusion protein eluted from Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B |
| **Factor Xa cleavage and purification** | |
| 21.1 | Factor Xa cleavage and purification of GST fusion protein bound to GSTrap FF |
| 21.2 | Factor Xa cleavage and purification of GST fusion protein eluted from GSTrap FF |
| 21.3 | Factor Xa cleavage and purification of GST fusion protein bound to Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B |
| 21.4 | Factor Xa cleavage and purification of GST fusion protein eluted from Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B |
| **Removal of proteases** | |
| 22 | Removal of proteases using HiTrap Benzamidine FF (high sub) |

One ml/min corresponds to approximately 30 drops/min when using a syringe with HiTrap 1 ml column, and 5 ml/min corresponds to approximately 120 drops/min when using HiTrap 5 ml column.

# PreScission Protease cleavage and purification

PreScission Protease is a fusion protein of GST and human rhinovirus 3C protease (33). The protease specifically recognizes the amino acid sequence Leu-Glu-Val-Leu-Phe-Gln↓Gly-Pro, cleaving between the Gln and Gly residues (34). Since the protease is fused to GST, it is easily removed from cleavage reactions using GSTrap FF or Glutathione Sepharose. Because this protease is maximally active at 4 °C, cleavage can be performed at low temperatures, thus improving the stability of the target protein.

## 19.1. PreScission Protease cleavage and purification of GST fusion protein bound to GSTrap FF

**Reagents required**

PreScission cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0

PreScission Protease

Binding buffer: 1× PBS (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3)

Cleavage should be complete following a 4 h treatment at 5 °C with at least 10 units of enzyme/mg of fusion protein. Incubation times can be reduced by adding more PreScission Protease.

**Steps**

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Fill the syringe with binding buffer.
2. Connect the column to the syringe using the adapter supplied. Avoid introducing air into the column.
3. Remove the twist-off end.
4. Equilibrate the column with five column volumes of binding buffer.
5. Apply the sample using the syringe. For best results, maintain a flow rate of 0.2–1 ml/min (for both 1 ml and 5 ml columns) as the sample is applied.
6. Wash the column with 5–10 column volumes of binding buffer. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column).
7. Wash the column with 10 column volumes of PreScission cleavage buffer.
8. Prepare the PreScission Protease mix:

   - For GSTrap FF 1 ml columns, mix 80 µl (160 units) of PreScission Protease with 920 µl of PreScission cleavage buffer at 5 °C.

   - For GSTrap FF 5 ml columns, mix 400 µl (800 units) of PreScission Protease with 4.6 ml of PreScission cleavage buffer at 5 °C.
9. Load the PreScission Protease mix onto the column using a syringe and the adapter supplied. Seal the column with the top cap and the domed nut supplied.
10. Incubate the column at 5 °C for 4 h.
11. Fill a syringe with 3 ml (1 ml column) or 15 ml (5 ml column) of PreScission cleavage buffer. Remove the top cap and domed nut from the column and attach the syringe. Avoid introducing air into the column.
12. Begin elution. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column), and collect the eluate (0.5–1 ml/tube for 1 ml column, 1–2 ml/tube for 5 ml column).

*Note: The eluate will contain the protein of interest, while the GST moiety of the fusion protein and the PreScission Protease will remain bound to GSTrap FF.*

## 19.2. PreScission Protease cleavage and purification of GST fusion protein eluted from GSTrap FF

### Reagents required

PreScission cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0

PreScission Protease

Binding buffer: 1× PBS (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3)

Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

### Steps

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Fill the syringe with binding buffer.
2. Connect the column to the syringe using the adapter supplied. Avoid introducing air into the column.
3. Remove the twist-off end.
4. Equilibrate the column with five column volumes of binding buffer.
5. Apply the sample using the syringe. For best results, maintain a flow rate of 0.2–1 ml/min (for both 1 ml and 5 ml columns) as the sample is applied.
6. Wash the column with 5–10 column volumes of binding buffer. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column).
7. Elute with 5–10 column volumes of elution buffer. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column). Collect the eluate (0.5–1 ml/tube for 1 ml column, 1–2 ml/tube for 5 ml column). Pool fractions containing the GST fusion protein (monitored by UV absorption at $A_{280}$).
8. Remove the free reduced glutathione from the eluate using a quick buffer exchange on HiTrap Desalting or HiPrep 26/10 Desalting, depending on the sample volume.

Desalting will remove the free glutathione. Glutathione bound to the GST protein can be removed by dialyzing the eluate against PreScission cleavage buffer.

9. Add 1 µl (2 units) of PreScission Protease for each 100 µg of fusion protein in the eluate.
10. Incubate at 5 °C for 4 h.
11. Once digestion is complete, apply the sample to an equilibrated GSTrap FF column as described above (steps 1–7) to remove the GST moiety of the fusion protein and the PreScission Protease.

*Note: The protein of interest will be found in the flow-through, while the GST moiety of the fusion protein and the PreScission Protease will remain bound to the column.*

If the amount of fusion protein in the eluate has not been determined, add 80 µl (160 units) of PreScission Protease for fusion protein eluted from a GSTrap FF 1 ml column or add 400 µl (800 units) of PreScission Protease for fusion protein eluted from a GSTrap FF 5 ml column.

## 19.3. PreScission Protease cleavage and purification of GST fusion protein bound to Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B

This procedure assumes that the GST fusion protein has been bound and washed as described in a purification procedure from Chapter 4.

**Reagents required**

PreScission Protease

PreScission cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.5

**Steps**

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Wash the fusion-protein-bound Glutathione Sepharose medium with 10 bed volumes of PreScission cleavage buffer. Bed volume is equal to 0.5× the volume of the 50% Glutathione Sepharose slurry used.
2. Prepare the PreScission Protease mix:
   For each ml of Glutathione Sepharose bed volume, prepare a mixture of 80 µl (160 units) of PreScission Protease and 920 µl of PreScission cleavage buffer at 5 °C.
3. Load the PreScission Protease mixture onto the column. Seal the column. If a batch format is used, centrifuge the suspension at 500 × g for 5 min, decant the supernatant, and add the PreScission Protease mixture to the Glutathione Sepharose pellet. Gently shake or rotate the suspension.
4. Incubate at 5 °C for 4 h.
5. Following incubation, elute the column with approximately three bed volumes of PreScission cleavage buffer. Collect the eluate in different tubes to avoid dilution of the fusion protein. Analyze the contents of each tube. If a batch format is used, centrifuge the suspension at 500 × g for 5 min to pellet the Glutathione Sepharose. Carefully transfer the eluate to a tube.

*Note: The eluate will contain the protein of interest, while the GST portion of the fusion protein and the PreScission Protease will remain bound to the Glutathione Sepharose medium.*

## 19.4. PreScission Protease cleavage and purification of GST fusion protein eluted from Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B

**Reagents required**

PreScission Protease

PreScission cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.5

**Steps**

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Following elution of the GST fusion protein from either a batch or column purification format as described in Chapter 4, remove the reduced glutathione from the eluate using a quick buffer exchange on HiTrap Desalting, a PD-10 disposable column, or HiPrep 26/10 Desalting, depending on sample volume.

Desalting will remove the free glutathione. Glutathione bound to the GST protein can be removed by dialyzing the eluate against PreScission cleavage buffer.

2. Add 1 µl (2 units) of PreScission Protease for each 100 µg of fusion protein in the eluate.

If the amount of fusion protein in the eluate has not been determined, add 80 µl (160 units) of PreScission Protease for each ml of Glutathione Sepharose bed volume from which the fusion protein was eluted.

3. Incubate at 5 °C for 4 h.
4. Once digestion is complete, apply the sample to washed and equilibrated Glutathione Sepharose to remove the GST moiety of the fusion protein and the PreScission Protease.
5. Incubate for 20–30 min at room temperature (22–25 °C).
6. Sediment the medium by centrifuging at 500 × g for 5 min and carefully transfer the supernatant to a new tube.

*Note: The protein of interest will be found in the supernatant, while the GST moiety and the PreScission Protease will remain bound to the Glutathione Sepharose medium.*

# Thrombin cleavage and purification

With a specific activity > 7500 units/mg protein, one unit of thrombin will digest > 90% of 100 µg of a test fusion protein in 16 h at 22 °C in elution buffer. One unit is approximately equal to 0.2 NIH units. Cleavage should be complete following overnight treatment with < 10 units of enzyme/mg of fusion protein.

Thrombin can be removed using Benzamidine Sepharose FF (high sub), a purification medium with a high specificity for serine proteases (see Procedure 22). A GSTrap FF column and a HiTrap Benzamidine FF (high sub) column can be connected in series so that cleaved product passes directly from the GSTrap FF into the HiTrap Benzamidine FF (high sub). Samples are cleaved and the thrombin removed in a single step.

## 20.1. Thrombin cleavage and purification of GST fusion protein bound to GSTrap FF

### Reagents required

1× PBS (binding buffer): 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3

Thrombin solution: Dissolve 500 units in 0.5 ml of 1× PBS pre-chilled to 4 °C. Swirl gently. Store solution in small aliquots at -80 °C to preserve activity.

### Steps

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Fill the syringe with binding buffer.
2. Connect the column to the syringe using the adapter supplied. Avoid introducing air into the column.
3. Remove the twist-off end.
4. Equilibrate the column with five column volumes of binding buffer.
5. Apply the sample using the syringe. For best results, maintain a flow rate of 0.2–1 ml/min (for both 1 ml and 5 ml columns) as the sample is applied.
6. Wash the column with 5–10 column volumes of binding buffer. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column).
7. Prepare the thrombin mix:

   - For GSTrap FF 1 ml columns, mix 80 µl (80 units) of thrombin solution with 920 µl of 1× PBS.

   - For GSTrap FF 5 ml columns, mix 400 µl (400 units) of thrombin solution with 4.6 ml of 1× PBS.
8. Load the thrombin mix onto the column using a syringe and the adapter supplied. Seal the column with the top cap and the domed nut supplied.
9. Incubate the column at room temperature (22–25 °C) for 2–16 h.

10. Fill a syringe with 3 ml (1 ml column) or 15 ml (5 ml column) of 1× PBS. Remove the top cap and domed nut from the column. Avoid introducing air into the column. Begin elution. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column).
11. Collect the eluate (0.5 ml–1 ml/tube for 1 ml column, 1–2 ml/tube for 5 ml column).

*Note: The eluate will contain the protein of interest and thrombin, while the GST moiety of the fusion protein will remain bound to GSTrap FF.*

After cleavage using thrombin, the enzyme can be removed from the eluted protein using HiTrap Benzamidine FF (high sub) (see Procedure 22).

## 20.2. Thrombin cleavage and purification of GST fusion protein eluted from GSTrap FF

### Reagents required

1× PBS (binding buffer): 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3

Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

Thrombin solution: Dissolve 500 units in 0.5 ml of 1× PBS pre-chilled to 4 °C. Swirl gently. Store solution in small aliquots at -80 °C to preserve activity.

### Steps

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Fill the syringe with binding buffer.
2. Connect the column to the syringe using the adapter supplied. Avoid introducing air into the column.
3. Remove the twist-off end.
4. Equilibrate the column with five column volumes of binding buffer.
5. Apply the sample using the syringe. For best results, maintain a flow rate of 0.2–1 ml/min (for both 1 ml and 5 ml columns) as the sample is applied.
6. Wash the column with 5–10 column volumes of binding buffer. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column).
7. Elute with 5–10 column volumes of elution buffer. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column). Collect the eluate (0.5–1 ml/tube for 1 ml column, 1–2 ml/tube for 5 ml column). Pool fractions containing the fusion protein (monitored by UV absorption at $A_{280}$).
8. Add 10 µl (10 units) of thrombin solution for each mg of fusion protein in the eluate.

If the amount of fusion protein in the eluate has not been determined, add 80 µl (80 units) of thrombin solution for fusion protein eluted from a GSTrap FF 1 ml column or add 400 µl (400 units) of thrombin solution for fusion protein eluted from a GSTrap FF 5 ml column.

9. Incubate at room temperature (22–25 °C) for 2–16 h.
10. Once digestion is complete, remove the reduced glutathione using a quick buffer exchange on HiTrap Desalting or HiPrep 26/10 Desalting, depending on the sample volume. Exchange with 1× PBS (binding buffer).

Desalting will remove the free glutathione. Glutathione bound to the GST protein can be removed by dialyzing the eluate against Thrombin cleavage buffer (1× PBS).

11. Apply the sample to an equilibrated GSTrap FF column to remove the GST moiety of the fusion protein.

*Note: The purified protein of interest and thrombin will be found in the flow-through.*

After cleavage using thrombin, the enzyme can be removed from the eluted protein using HiTrap Benzamidine FF (high sub) (see Procedure 22).

## 20.3. Thrombin cleavage and purification of GST fusion protein bound to Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B

This procedure assumes that the GST fusion protein has been bound and washed as described in a purification procedure from Chapter 4.

**Reagents required**

1× PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3

Thrombin solution: Dissolve 500 units in 0.5 ml of 1× PBS pre-chilled to 4 °C. Swirl gently. Store solution in small aliquots at -80 °C to preserve activity.

**Steps**

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Wash the fusion-protein-bound Glutathione Sepharose with 10 bed volumes of 1× PBS. Bed volume is equal to 0.5× the volume of the 50% Glutathione Sepharose slurry used.
2. Prepare the thrombin mix:
   For each ml of Glutathione Sepharose bed volume, prepare a mixture of 80 µl (80 units) of thrombin and 920 µl of 1× PBS.
3. Load the thrombin mixture onto the column. Seal the column. If a batch format is used, centrifuge the suspension at 500 × g for 5 min, decant the supernatant, and add the thrombin mixture to the Glutathione Sepharose pellet. Gently shake or rotate the suspension.
4. Incubate at room temperature (22–25 °C) for 2–16 h.
5. Following incubation, elute the column with approximately three bed volumes of 1× PBS. Collect the eluate in different tubes to avoid dilution of the fusion protein. Analyze the contents of each tube. If a batch format is used, centrifuge the suspension at 500 × g for 5 min to pellet the Glutathione Sepharose. Carefully transfer the eluate to a tube.

*Note: The eluate will contain the protein of interest and thrombin, while the GST portion of the fusion protein will remain bound to the Glutathione Sepharose medium.*

After cleavage using thrombin, the enzyme can be removed from the eluted protein using HiTrap Benzamidine FF (high sub) (see Procedure 22).

## 20.4. Thrombin cleavage and purification of GST fusion protein eluted from Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B

**Reagents required**

1× PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3

Thrombin solution: Dissolve 500 units in 0.5 ml of 1× PBS pre-chilled to 4 °C. Swirl gently. Store solution in small aliquots at -80 °C to preserve activity.

**Steps**

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Following elution of the GST fusion protein from either a batch or column purification format as described in Chapter 4, remove the reduced glutathione from the eluate using a quick buffer exchange on HiTrap Desalting, a PD-10 column, or HiPrep 26/10 Desalting, depending on the sample volume.
2. Add 10 µl (10 units) of thrombin solution for each mg of fusion protein in the eluate.

If the amount of fusion protein in the eluate has not been determined, add 80 µl (80 units) of thrombin solution for each ml of Glutathione Sepharose bed volume from which the fusion protein was eluted.

3. Incubate at room temperature (22–25 °C) for 2–16 h.
4. Once digestion is complete, remove the reduced glutathione using a quick buffer exchange on HiTrap Desalting, a PD-10 column, or HiPrep 26/10 Desalting, depending on the sample volume.

Desalting will remove the free glutathione. Glutathione bound to the GST protein can be removed by dialyzing the eluate against thrombin cleavage buffer (1× PBS).

5. Apply the sample to washed and equilibrated Glutathione Sepharose to remove the GST moiety of the fusion protein.
6. Incubate for 20–30 min at room temperature (22–25 °C).
7. Sediment the medium by centrifuging at 500 × g for 5 min and carefully transfer the supernatant to a new tube.

*Note: The supernatant will contain the protein of interest and thrombin, while the GST portion of the fusion protein will remain bound to the Glutathione Sepharose medium.*

After cleavage using thrombin, the enzyme can be removed from the eluted protein using HiTrap Benzamidine FF (high sub) (see Procedure 22).

## Factor Xa cleavage and purification

With a specific activity of > 800 units/mg protein, one unit of Factor Xa will digest > 90% of 100 µg of a test fusion protein in 16 h at 22 °C in Factor Xa cleavage buffer. Cleavage should be complete following overnight treatment at 22 °C with a Factor Xa to substrate ratio of at least 1% (w/w).

Factor Xa can be removed using Benzamidine Sepharose FF (high sub), a purification medium with a high specificity for serine proteases (see Procedure 22). A GSTrap FF column and a HiTrap Benzamidine FF (high sub) column can be connected in series so that cleaved product passes directly from the GSTrap FF into the HiTrap Benzamidine FF (high sub). Samples are cleaved and the Factor Xa removed in a single step. Heparin Sepharose has also been used for this application; however, since benzamidine has a higher specificity for Factor Xa, the protease will be removed more efficiently.

Factor Xa consists of two subunits linked by disulfide bridges. Since glutathione can disrupt disulfide bridges, it should be removed from the sample prior to the cleavage reaction.

Free glutathione can be easily and rapidly removed from the sample using a desalting column with Factor Xa cleavage buffer as eluent, followed by dialysis to remove any glutathione bound to the GST protein.

## 21.1. Factor Xa cleavage and purification of GST fusion protein bound to GSTrap FF

### Reagents required

1× PBS (binding buffer): 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3

Factor Xa cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5

Factor Xa solution: Dissolve 400 units of Factor Xa in 4 °C $H_2O$ to give a final solution of 1 unit/µl. Swirl gently. Store solution in small aliquots at -80 °C to preserve activity.

### Steps

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Fill the syringe with binding buffer.
2. Connect the column to the syringe using the adapter supplied. Avoid introducing air into the column.
3. Remove the twist-off end.
4. Equilibrate the column with five column volumes of binding buffer.
5. Apply the sample using the syringe. For best results, maintain a flow rate of 0.2–1 ml/min (for both 1 ml and 5 ml columns) as the sample is applied.
6. Wash the column with 5–10 column volumes of binding buffer. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column).
7. Wash the column with 10 column volumes of Factor Xa cleavage buffer.
8. Prepare the Factor Xa mix:

   - For GSTrap FF 1 ml columns, mix 80 µl (80 units) of Factor Xa solution with 920 µl of Factor Xa cleavage buffer.

   - For GSTrap FF 5 ml columns, mix 400 µl (400 units) of Factor Xa solution with 4.6 ml of Factor Xa cleavage buffer.
9. Load the Factor Xa mix onto the column using a syringe and the adapter supplied. Seal the column with the top cap and the domed nut.
10. Incubate the column at room temperature (22–25 °C) for 2–16 h.
11. Fill a syringe with 3 ml (1 ml column) or 15 ml (5 ml column) of Factor Xa cleavage buffer. Remove the top cap and domed nut from the column. Avoid introducing air into the column. Begin the elution. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column).
12. Collect the eluate (0.5–1 ml/tube for 1 ml column, 1–2 ml/tube for 5 ml column).

*Note: The eluate will contain the protein of interest and Factor Xa, while the GST moiety of the fusion protein will remain bound to GSTrap FF.*

After cleavage using Factor Xa, the enzyme can be removed from the eluted protein using HiTrap Benzamidine FF (high sub) (see Procedure 22).

## 21.2. Factor Xa cleavage and purification of GST fusion protein eluted from GSTrap FF

**Reagents required**

1× PBS (binding buffer): 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3

Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

Factor Xa cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5

Factor Xa solution: Dissolve 400 units of Factor Xa in 4 °C $H_2O$ to give a final solution of 1 unit/µl. Swirl gently. Store solution in small aliquots at -80 °C to preserve activity.

**Steps**

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Fill the syringe with binding buffer.
2. Connect the column to the syringe using the adapter supplied. Avoid introducing air into the column.
3. Remove the twist-off end.
4. Equilibrate the column with five column volumes of binding buffer.
5. Apply the sample using the syringe. For best results, maintain a flow rate of 0.2–1 ml/min (for both 1 ml and 5 ml columns) as the sample is applied.
6. Wash the column with 5–10 column volumes of binding buffer. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column).
7. Elute with 5–10 column volumes of elution buffer. Maintain flow rates of 1–2 ml/min (1 ml column) or 1–5 ml/min (5 ml column). Collect the eluate (0.5 ml/tube for 1 ml column, 1–2 ml/tube for 5 ml column). Pool fractions containing the GST fusion protein (monitored by UV absorption at $A_{280}$).
8. Remove the reduced glutathione from the eluate using a quick buffer exchange on HiTrap Desalting or HiPrep 26/10 Desalting, depending on sample volume. Exchange with 1× PBS (binding buffer).

Desalting will remove the free glutathione. Glutathione bound to the GST protein can be removed by dialyzing the eluate against cleavage buffer.

9. Add 10 units of Factor Xa solution for each mg of fusion protein in the eluate.

If the amount of fusion protein in the eluate has not been determined, add 80 µl (80 units) of Factor Xa solution for fusion protein eluted from a GSTrap FF 1 ml column or add 400 µl (400 units) of Factor Xa solution for fusion protein eluted from a GSTrap FF 5 ml column.

10. Incubate at room temperature (22–25 °C) for 2–16 h.
11. Once digestion is complete, apply the sample to an equilibrated GSTrap FF column as described above (steps 1–7) to remove the GST moiety of the fusion.

*Note: The protein of interest will be found in the flow-through together with Factor Xa.*

After cleavage using Factor Xa, the enzyme can be removed from the eluted protein using HiTrap Benzamidine FF (high sub) (see Procedure 22).

## 21.3. Factor Xa cleavage and purification of GST fusion protein bound to Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B

This procedure assumes that the GST fusion protein has been bound and washed as described in a purification procedure from Chapter 4.

**Reagents required**

Factor Xa cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5

Factor Xa solution: Dissolve 400 units of Factor Xa in 4 °C $H_2O$ to give a final solution of 1 unit/µl. Swirl gently. Store solution in small aliquots at -80 °C to preserve activity.

**Steps**

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Wash the fusion-protein-bound Glutathione Sepharose medium with 10 bed volumes of Factor Xa cleavage buffer. Bed volume is equal to 0.5× the volume of the 50% Glutathione Sepharose slurry used.
2. Prepare the Factor Xa mix:
   For each ml of Glutathione Sepharose bed volume, prepare a mixture of 80 µl (80 units) of Factor Xa and 920 µl of Factor Xa cleavage buffer.
3. Load the Factor Xa mix onto the column. Seal the column. If a batch format is used, centrifuge the suspension at 500 × g for 5 min, decant the supernatant, and add the Factor Xa mix to the Glutathione Sepharose pellet. Gently shake or rotate the suspension.
4. Incubate at room temperature (22–25 °C) for 2–16 h.
5. Following incubation, elute the column with approximately three bed volumes of Factor Xa cleavage buffer. Collect the eluate in different tubes to avoid dilution of the fusion protein and analyze it. If a batch format is used, centrifuge the suspension at 500 × g for 5 min to pellet the Glutathione Sepharose medium. Carefully transfer the eluate to a tube.

*Note: The eluate will contain the protein of interest and Factor Xa, while the GST portion of the fusion protein will remain bound to the Glutathione Sepharose medium.*

After cleavage using Factor Xa, the enzyme can be removed from the eluted protein using HiTrap Benzamidine FF (high sub) (see Procedure 22).

## 21.4. Factor Xa cleavage and purification of GST fusion protein eluted from Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B

**Reagents required**

Factor Xa cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5

Factor Xa solution: Dissolve 400 units of Factor Xa in 4 °C $H_2O$ to give a final solution of 1 unit/µl. Swirl gently. Store solution in small aliquots at -80 °C to preserve activity.

**Steps**

*Assume: 8 mg GST fusion protein bound/ml medium.*

1. Following elution of the GST fusion protein from either a batch or column purification format as described in Chapter 4, remove reduced glutathione from the eluate using a quick buffer exchange on HiTrap Desalting, a PD-10 column, or HiPrep 26/10 Desalting, depending on sample volume.

Desalting will remove the free glutathione. Glutathione bound to the GST protein can be removed by dialyzing the eluate against Factor Xa cleavage buffer.

2. Add 10 µl (10 units) of Factor Xa solution for each mg of fusion protein in the eluate.

If the amount of fusion protein in the eluate has not been determined, add 80 µl (80 units) of Factor Xa solution for each ml of Glutathione Sepharose bed volume from which the fusion protein was eluted.

3. Incubate at room temperature (22–25 °C) for 2–16 h.
4. Once digestion is complete, apply the sample to washed and equilibrated Glutathione Sepharose to remove the GST moiety of the fusion protein.
5. Incubate for 20–30 min at room temperature.
6. Sediment the medium by centrifuging at 500 × g for 5 min and carefully transfer the supernatant to a new tube.

*Note: The protein of interest will be found in the supernatant together with Factor Xa.*

After cleavage using Factor Xa, the enzyme can be removed from the eluted protein using HiTrap Benzamidine FF (high sub) (see Procedure 22).

## Removal of proteases using Benzamidine Sepharose 4 Fast Flow (high sub)

To protect the fusion protein from proteolytic degradation prior to enzymatic cleavage with PreScission Protease, thrombin, or Factor Xa, it may be necessary to remove proteases from the sample. Additionally, following enzymatic cleavage, it may be necessary to remove thrombin or Factor Xa from the sample. Benzamidine Sepharose 4 Fast Flow (high sub) provides a convenient and highly specific medium for the removal of trypsin and trypsin-like serine proteases, not only from enzymatic digests but also from cell culture supernatants, bacterial lysates, or serum.

In the procedure that follows, the GST fusion protein is present in the flow-through and wash, whereas the protease remains bound to the medium until eluted.

Elution buffers are listed in the procedure so that after the GST fusion protein has been collected, the bound protease can be eluted. This step is necessary if the medium is to be re-used. See Appendix 5 for more information on eluting the protease from the column.

Benzamidine Sepharose 4 Fast Flow is available in either prepacked 1 ml or 5 ml HiTrap columns or in packages for scaling up purifications. HiTrap columns can be operated with a syringe together with the supplied adapters, a pump, or a liquid chromatography system, e.g. ÄKTAdesign. See Table 20 for a selection guide of purification options.

Characteristics of HiTrap Benzamidine FF (high sub) are summarized in Table 21.

**Table 20.** Selection guide for purification options to remove thrombin and Factor Xa

| Column (prepacked) or medium | Binding capacity for trypsin | Comments |
|---|---|---|
| HiTrap Benzamidine FF (high sub), 1 ml | > 35 mg trypsin | Prepacked 1 ml column |
| HiTrap Benzamidine FF (high sub), 5 ml | > 175 mg trypsin | Prepacked 5 ml column |
| Benzamidine Sepharose 4 Fast Flow (high sub) | > 35 mg trypsin/ml medium | For column packing and scale-up |

**Table 21.** Characteristics of HiTrap Benzamidine FF (high sub)

| | |
|---|---|
| Column dimensions (i.d. × h) | 0.7 × 2.5 cm (1 ml) and 1.6 × 2.5 cm (5 ml) |
| Column volumes | 1 ml and 5 ml |
| Ligand | p-Aminobenzamidine (pABA) |
| Spacer | 14-atom |
| Ligand concentration | ≥ 12 µmol p-Aminobenzamidine/ml medium |
| Binding capacity | ≥ 35 mg trypsin/ml medium |
| Mean particle size | 90 µm |
| Bead structure | Highly cross-linked agarose, 4% |
| Maximum back pressure | 0.3 MPa, 3 bar |
| Recommended flow rates | 1 ml/min (1 ml column) and 5 ml/min (5 ml column) |
| Maximum flow rates | 4 ml/min (1 ml column) and 20 ml/min (5 ml column) |
| Chemical stability | All commonly used aqueous buffers |
| pH stability short term* | pH 1–9 |
| pH stability long term* | pH 2–8 |
| Storage temperature | 4–8 °C |
| Storage buffer | 20% ethanol in 0.05 M acetate buffer, pH 4 |

**The ranges given are estimates based on our knowledge and experience. Please note the following:*
***pH stability, short term*** *refers to the pH interval for regeneration, cleaning-in-place, and sanitization procedures.*
***pH stability, long term*** *refers to the pH interval where the medium is stable over a long period of time without adverse effects on its chromatographic performance.*

The column is made of polypropylene, which is biocompatible and non-interactive with biomolecules. The top and bottom frits are manufactured from porous polyethylene. Columns are delivered with a stopper on the inlet and a twist-off end on the outlet. Both ends have M6 connections (6 mm metric threads).

The column cannot be opened or refilled.

### Buffer and sample preparation

Water and chemicals used for buffer preparation should be of high purity. Buffers should be filtered through a 0.45 µm filter and de-gassed before use.

Samples should be centrifuged and/or filtered through a 0.45 µm filter immediately before applying to the column. If the sample is too viscous, dilute it with binding buffer to prevent column clogging.

### Recommended binding and wash buffers

HiTrap Benzamidine FF (high sub) has some ionic binding characteristics; therefore, binding and wash buffers should contain at least 0.5 M salt and have a pH of 7.4–8. An appropriate buffer would be 0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4.

If a lower salt concentration is used, include a high-salt wash step prior to elution of the bound protease.

### Recommended elution buffers

See Appendix 5 for a discussion of elution options for removing bound protease from the medium.

# 22. Removal of thrombin and Factor Xa using HiTrap Benzamidine FF (high sub)

**Reagents required**

Binding buffer: 0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4

Elution buffer alternatives (for removing the protease after the GST fusion protein has come through in the flow-through and wash; see Appendix 5):

- 0.05 M glycine-HCl, pH 3.0
- 10 mM HCl, 0.5 M NaCl, pH 2.0
- 20 mM p-Aminobenzamidine in binding buffer (competitive elution)
- 8 M urea or 6 M guanidine hydrochloride (denaturing solutions)

**Steps**

Recommended flow rates are 1 ml/min (1 ml column) or 5 ml/min (5 ml column).

1. Fill the pump tubing or syringe with distilled $H_2O$. Connect the column to the syringe, using the adapter supplied, or to the pump tubing. Avoid introducing air into the column.
2. Remove the twist-off end.
3. Wash the column with five column volumes of distilled $H_2O$ to remove the storage buffer (0.05 M acetate buffer, pH 4, containing 20% ethanol).
4. Equilibrate the column with five column volumes of binding buffer.
5. Apply the sample using a syringe fitted to the luer adapter or by pumping it onto the column. Recommended flow rates for sample application are 1 ml/min for 1 ml column and 5 ml/min for 5 ml column. Collect any flow-through and reserve.
6. Wash the column with 5–10 column volumes of binding buffer, collecting fractions (0.5–1 ml fractions for 1 ml column and 1–3 ml fractions for 5 ml column) until no material appears in the effluent (monitored by UV absorption at $A_{280}$).
7. Pool fractions from flow-through and wash that contain the GST fusion protein (monitored by UV absorption at $A_{280}$).
8. For re-use of column, elute the bound protease with 5–10 column volumes of the elution buffer of choice (see above and Appendix 5). If the eluted thrombin or Factor Xa is to be retained, buffer-exchange the fractions containing the protease using HiTrap Desalting or PD-10 Desalting column. If a low pH elution buffer has been used, collect fractions in neutralization buffer.
9. After all protease has been eluted, wash the column with binding buffer so it is ready for re-use. See Appendix 5 for additional cleaning and storage information.

   *Since elution conditions are quite harsh, collect fractions into neutralization buffer (60–200 µl of 1 M Tris-HCl, pH 9.0 per ml fraction collected), so that the final pH of the fractions will be approximately neutral.

An example showing isolation of a GST fusion protein on a GSTrap FF column and on-column cleavage with thrombin, followed by purification on HiTrap Benzamidine FF (high sub), can be found in Chapter 7.

# Chapter 7
# Applications

Examples of the use of the GST gene fusion system to purify, cleave, and detect GST fusion proteins include the following:

**Purification**

- Rapid purification of GST fusion proteins using GSTrap FF 1 ml and 5 ml columns
- Rapid purification using Glutathione Sepharose 4 Fast Flow in XK 16/20 column
- High-throughput purification of GST fusion proteins using the MicroSpin GST Purification Module

**Purification and cleavage**

- On-column cleavage and sample clean-up

**Detection of GST fusion proteins**

- Detection of GST fusion proteins in bacterial lysates using a 96-well Detection Plate

For more details, refer to the source material listed at the end of each example.

## Purification

GSTrap FF columns prepacked with Glutathione Sepharose 4 Fast Flow provide a convenient format that is easy to use for one-step purification of GST fusion proteins. In the applications described below, examples are presented showing typical purifications using these columns. Applications are also included for large sample volume purification using Glutathione Sepharose 4 Fast Flow packed in a XK 16/20 column and for rapid, high-throughput sample processing using MicroSpin GST Purification Module in conjunction with MicroPlex Vacuum.

### Rapid purification of GST fusion proteins using GSTrap FF 1 ml and 5 ml columns

#### Example 1. Purification of a phosphatase SH2 domain GST fusion protein

GSTrap FF 1 ml and 5 ml prepacked columns were used in conjunction with ÄKTAexplorer 10 to purify a fusion protein containing the SH2 domain of a phosphatase fused with GST (SH2-GST) ($M_r$ 37 000). Approximately 2 mg of fusion protein was recovered from 2 ml of clarified *E. coli* homogenate using GSTrap FF 1 ml for estimation of expression level (Fig 14A). The eluted material contained mostly SH2-GST, with only a small amount of GST detected (Fig 14B). No detectable contaminants were observed using SDS-PAGE and silver staining.

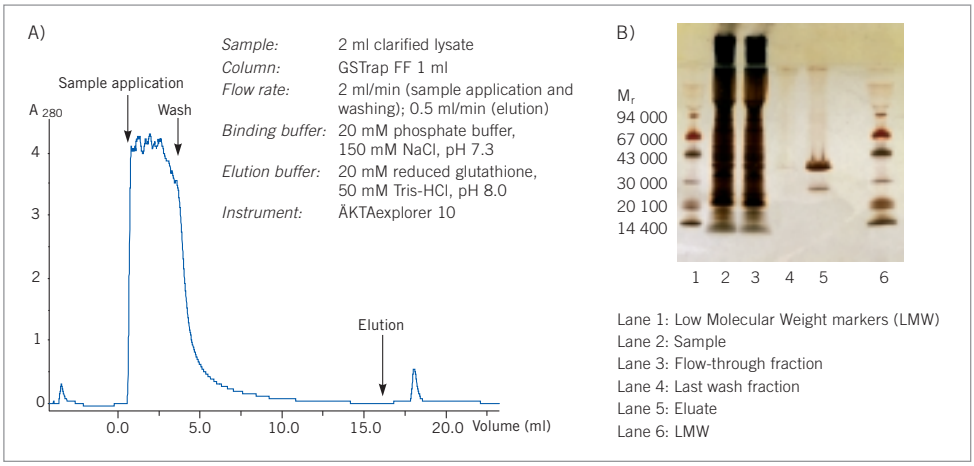



**Fig 14.** Purification of SH2 phosphatase domain-GST fusion protein using GSTrap FF 1 ml and ÄKTAexplorer 10. A) Two ml of clarified *E. coli* homogenate containing a $M_r$ 37 000 SH2-GST fusion protein was applied to the column and the resulting chromatogram recorded. B) Fractions were analyzed by SDS-PAGE on an 8–25% PhastGel™ with silver staining for detection.

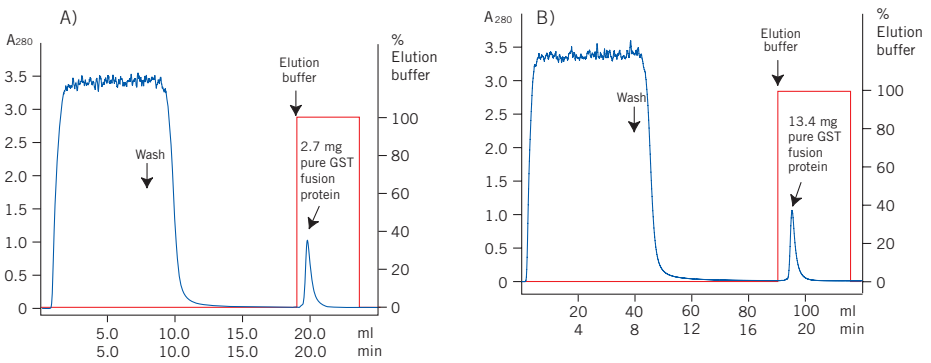
Source: Haneskog, L. *et al.*, Scientific poster: Rapid purification of GST-fusion proteins from large sample volumes, Amersham Biosciences, code number 18-1139-51.

### Example 2. Scaling-up purification of a GST fusion protein

A GST fusion protein was purified from 8 ml and 40 ml of a clarified cell lysate using GSTrap FF 1 ml and 5 ml columns, respectively. Samples were applied to columns pre-equilibrated with 1× PBS, pH 7.3. After washing the columns with 10 column volumes (CV) of 1× PBS, GST fusion protein was eluted using reduced glutathione (Fig 15). Each run was completed in 25 min using ÄKTAexplorer 10. Analysis by SDS-PAGE indicated the isolation of highly pure GST fusion protein (Fig 27, lanes 3–4). Fusion protein yields were 2.7 mg from GSTrap FF 1 ml and 13.4 mg from GSTrap FF 5 ml.

### Example 3. GSTrap FF column purification of open reading frames cloned as GST fusions

As part of a structural genomics project where the aim was to determine three-dimensional structures of proteins at high speed, four open reading frames (ORFs) were cloned as GST fusions and expressed in *E. coli*. The proteins were purified on a small scale using GSTrap FF 1 ml columns and ÄKTAprime. These conditions allowed quick purification of the proteins from cell extracts, making it possible to easily evaluate the expression, purity, and stability of each protein. Although protein expression levels were good, under these non-optimized conditions a degradation pattern was observed for all the purified proteins (Fig 16).




| | | | |
|---|---|---|---|
| *Sample:* | 8 ml clarified lysate | *Sample:* | 40 ml clarified lysate |
| *Column:* | GSTrap FF 1ml | *Column:* | GSTrap FF 5 ml |
| *Binding buffer:* | 1× PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.3) | *Binding buffer:* | 1× PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.3) |
| *Elution buffer:* | 10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0 | *Elution buffer:* | 10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0 |
| *Flow rate:* | 1 ml/min | *Flow rate:* | 5 ml/min |
| *Chromatographic procedure:* | 4 column volumes (CV) binding buffer, 8 ml sample 10 CV binding buffer, 5 CV elution buffer 5 CV binding buffer | *Chromatographic procedure:* | 4 column volumes (CV) binding buffer, 40 ml sample 10 CV binding buffer, 5 CV elution buffer 5 CV binding buffer |
| *Instrument:* | ÄKTAexplorer 10 | *Instrument:* | ÄKTAexplorer 10 |

**Fig 15.** Purification of a GST fusion protein on GSTrap FF 1 ml and 5 ml columns in combination with ÄKTAexplorer 10. Eight and 40 ml of cytoplasmic extract from *E. coli* expressing a GST fusion protein were applied to GSTrap FF 1 ml (A) and GSTrap FF 5 ml (B), respectively.

Sources: Data File: GSTrap FF 1 ml and 5 ml, Glutathione Sepharose 4 Fast Flow, Amersham Biosciences, code number 18-1136-89.

Haneskog, L. *et al.*, Fast and simple purification of GST fusion proteins using prepacked GSTrap affinity columns, *Life Science News* **4**, 16 (2000). See also online *Life Science News* archive.

Refer to the SDS-PAGE analysis of the above GST fusion protein in Figure 27, lanes 1–4 (page 81).

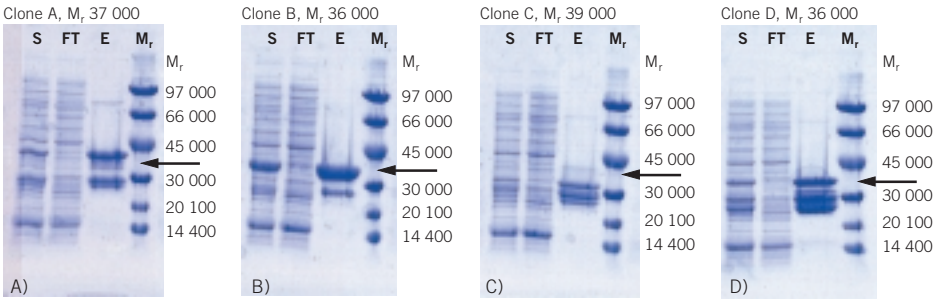



**Fig 16.** SDS-PAGE analysis of four different human open reading frames (A-D) expressed as GST fusions. Proteins were loaded onto ExcelGel SDS Gradient, 8–18% for electrophoresis and stained with Coomassie blue. S = sample loaded onto the GSTrap FF column; FT = flow-through during sample load and wash; E = eluted fusion protein; $M_r$ = low molecular weight marker proteins. The arrow indicates the location of the GST fusion protein.

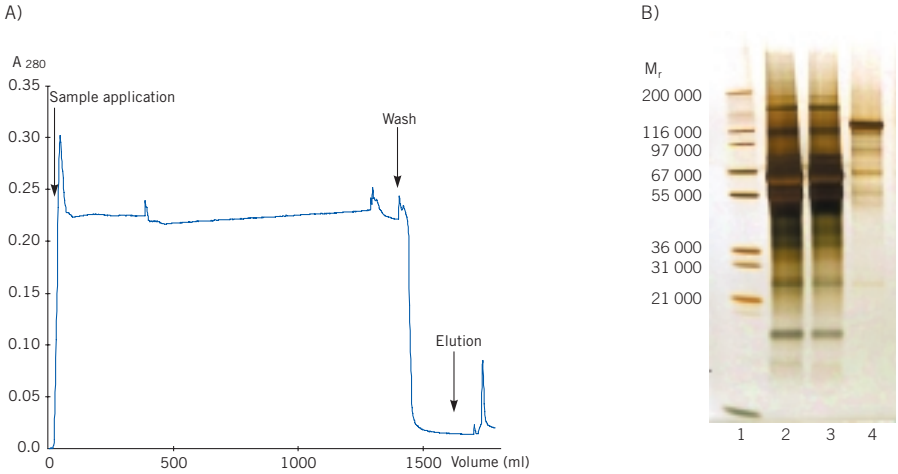
Source: Sigrell, J. A., Scientific poster: Purification of GST fusion proteins, on-column cleavage and sample clean-up. Initial purification screen within a structural genomics program, Amersham Biosciences, code number 18-1150-20.

## Rapid purification using Glutathione Sepharose 4 Fast Flow packed in XK 16/20 column

### Example 4. Purification of eukaryotic GST fusion protein present at low levels in large sample volumes

For this application, 34 ml of Glutathione Sepharose 4 Fast Flow medium was packed in a XK 16/20 column and used for purification of a eukaryotic GST fusion protein expressed at low levels. This method provides a realistic alternative to optimizing a fermentation process or preparing a new gene construct to obtain higher expression levels.

A large volume (1.5 l) of clarified cell culture medium from human embryo kidney cells (HEK293 cells) expressing small amounts of a $M_r$ 120 000 glycosylated and secreted protein was applied to the Glutathione Sepharose 4 Fast Flow column. Preliminary data had shown expression levels of 0.5–1.5 µg of the GST fusion protein per ml of culture medium. The column was washed with binding buffer, and 1 mg of pure protein was eluted using a step-gradient of glutathione (Fig 17), all within a period of 5 h. The protein was concentrated by ultrafiltration (cut-off $M_r$ 10 000) and used for successful crystallization trials (Fig 18).




*Sample:* 1500 ml clarified cell culture medium from HEK293 expressing a $M_r$ 120 000 glycosylated and secreted protein
*Column:* Glutathione Sepharose 4 Fast Flow (34 ml, 1.6 × 17 cm) packed in XK 16/20 column
*Flow rate:* 10 ml/min (sample application and washing); 1 ml/min (elution)
*Binding buffer:* 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3
*Elution buffer:* 20 mM reduced glutathione, 50 mM NaCl, 50 mM Tris-HCl, pH 8.0
*Instrument:* ÄKTAexplorer 100

Lane 1: Molecular weight markers
Lane 2: Sample
Lane 3: Flow-through
Lane 4: Eluate

**Fig 17.** Purification (A) and SDS-PAGE analysis using silver staining (B) of a eukaryotic protein present at low levels in large sample volumes.

**Fig 18.** Crystals of the eukaryotic GST fusion protein obtained in the initial crystallization trial.

Source: Haneskog, L. *et al.*, Scientific poster: Rapid purification of GST-fusion proteins from large sample volumes, Amersham Biosciences, code number 18-1139-51.

## High-throughput purification of GST fusion proteins using the MicroSpin GST Purification Module

### Example 5. Purification of human myoglobin GST fusion protein

The MicroSpin GST Purification Module provides an ideal format for the simple and rapid purification of GST fusion proteins from large numbers of small-scale bacterial lysates, especially when it is used in conjunction with MicroPlex 24 Vacuum. By connecting two MicroPlex 24 Vacuum units in parallel, up to 48 Glutathione Sepharose 4B MicroSpin Columns can be processed simultaneously.

In this application, GST fusion proteins from 24 randomly selected transformants were purified according to the instructions provided with the module and analyzed by SDS-PAGE (Fig 19). Seven of the 24 colonies tested expressed a GST fusion protein corresponding in molecular weight to that of GST-myoglobin. Each remaining colony expressed a GST-sized protein consistent with that encoded by the parent vector without an insert.

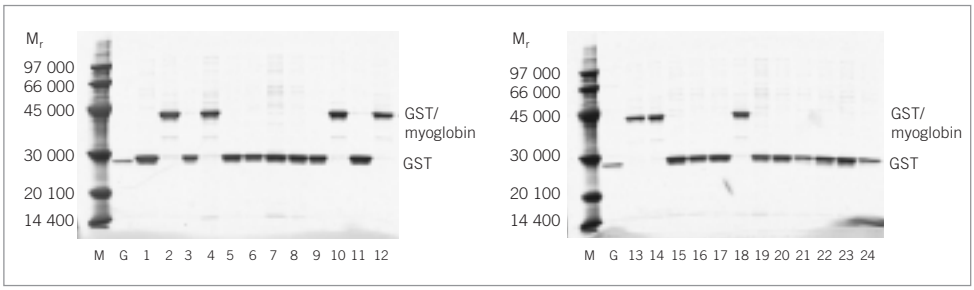



**Fig 19.** Rapid screening of randomly selected *E. coli* transformants for expression of human myoglobin GST fusion protein. Aliquots (15 µl) of each eluted product were loaded into the lanes of a 12% polyacrylamide Tris-glycine-SDS gel and run for 2 h at 90 V. M = LMW Marker Kit, G = purified recombinant GST. Lanes 1–24 contain products eluted from the MicroSpin Columns using reduced glutathione.

Source: See Figure 20.

When purified GST-myoglobin fusion protein obtained from the rapid screening procedure was treated with Factor Xa, two protein products were observed, a $M_r$ 29 000 product and a $M_r$ 14 000 product corresponding in size to that of the expected GST domain and human myoglobin, respectively (Fig 20). These results demonstrate not only expression of the desired GST-myoglobin fusion protein by the selected clone, but also that fusion proteins purified during rapid screening with the Glutathione Sepharose 4B MicroSpin Columns can be cleaved with site-specific proteases.

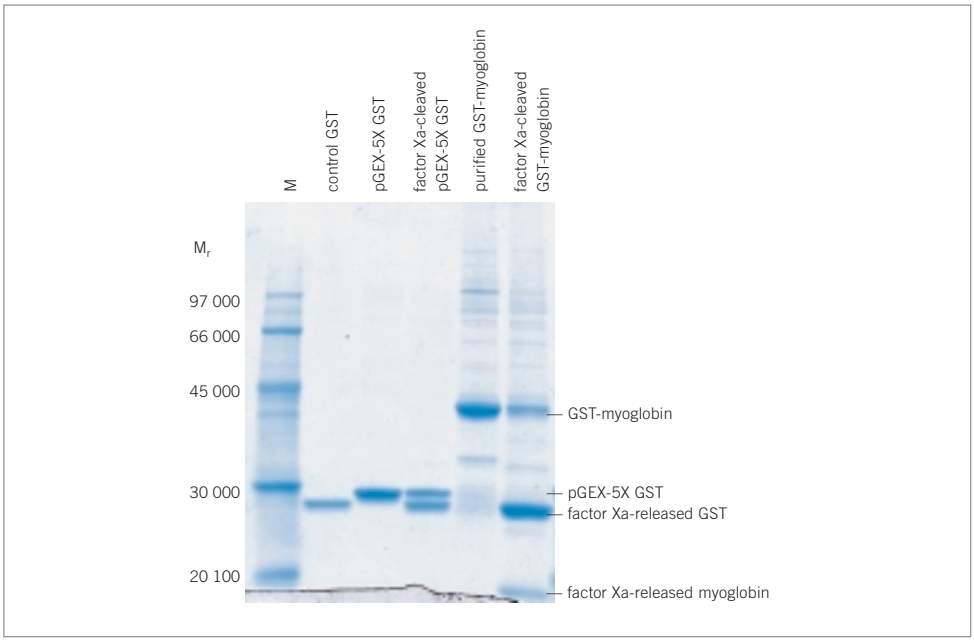



**Fig 20.** Cleavage of putative GST-myoglobin fusion protein with factor Xa. After purification using the MicroSpin GST Purification Module, protein from a representative parent clone and GST-myoglobin fusion protein clone were dialyzed with two changes of 1× PBS overnight, then treated with factor Xa for 20 h at room temperature. M = LMW Marker Kit.

Sources: Bell, P. A. *et al*., Rapid screening of multiple clones for GST fusion protein expression, *Life Science News* **1**, 14 (1998). See also online *Life Science News* archive.

Data File: MicroSpin GST Purification Module, Amersham Biosciences, code number 18-1128-13.

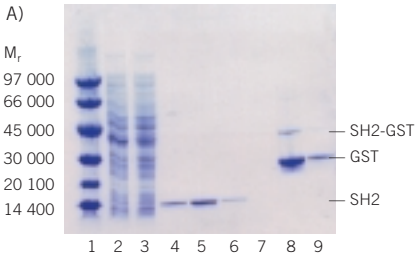
# Purification and cleavage

## On-column cleavage and sample clean-up

### Example 6. Purification and on-column cleavage of GST fusion SH2 domain using thrombin and GSTrap FF. Sample clean-up using HiTrap Benzamidine FF (high sub) column in series with GSTrap FF

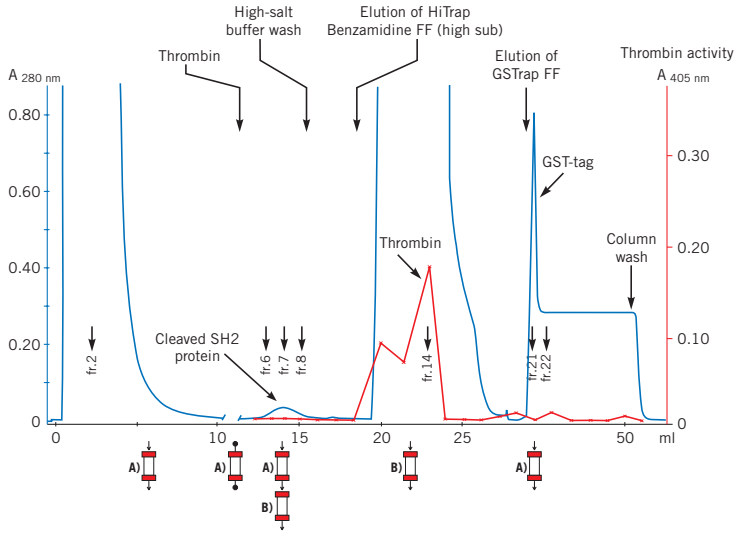
The following application describes the purification of a $M_r$ 37 000 SH2-GST fusion protein on a GSTrap FF 1 ml column, followed by on-column cleavage with thrombin (Fig 21). After the thrombin incubation step, a HiTrap Benzamidine FF (high sub) 1 ml column was placed in series below the GSTrap FF column. As the columns were washed with binding buffer and later with high salt buffer, the cleaved SH2 fusion protein and thrombin were washed from the GSTrap FF column onto the HiTrap Benzamidine FF (high sub) column. Thrombin was captured by this second column, thus enabling the collection of pure thrombin-free protein in the eluent (Fig 21A). Complete removal of thrombin was verified using the chromogenic substrate S-2238 (Chromogenix, Haemochrom Diagnostica AB; supplier in US is DiaPharma) for detection of thrombin activity (Fig 21B). This whole procedure could be completed in less than one day.

A)




Gel: ExcelGel SDS Gradient 8–18%, Coomassie blue staining
Lane 1: Low Molecular Weight Marker Kit (LMW)
Lane 2: Clarified *E. coli* homogenate containing SH2-GST fusion protein with a thrombin cleavage site
Lane 3: Flow-through from GSTrap FF (fraction 2)
Lane 4: SH2 domain (GST-tag cleaved off), washed out with binding buffer through both columns (fraction 6)
Lane 5: Same as lane 4 (fraction 7)
Lane 6: Same as lane 4 (fraction 8)
Lane 7: Elution of thrombin from HiTrap Benzamidine FF (high sub)
Lane 8: Elution of GST-tag and some non-cleaved SH2-GST from GSTrap FF (fraction 21)
Lane 9: Same as lane 8 (fraction 22)

B)




**A)** GSTrap FF, 1 ml
**B)** HiTrap Benzamidine FF (high sub), 1 ml

| | |
|---|---|
| *Sample:* | 2 ml clarified *E. coli* homogenate containing a $M_r$ 37 000 SH2-GST fusion protein with a thrombin cleavage site |
| *Columns:* | GSTrap FF 1 ml and HiTrap Benzamidine FF (high sub) 1 ml |
| *Binding buffer:* | 20 mM sodium phosphate, 0.15 M NaCl, pH 7.5 |
| *High salt wash buffer:* | 20 mM sodium phosphate, 1.0 M NaCl, pH 7.5 |
| *Benzamidine elution buffer:* | 20 mM p-Aminobenzamidine in binding buffer |
| *GST elution buffer:* | 20 mM reduced glutathione, 50 mM Tris, pH 8.0 |
| *Flow rate:* | 0.5 ml/min |
| *System:* | ÄKTAprime |
| *Protease treatment:* | 20 U/ml thrombin protease (Amersham Biosciences) for 2 h at room temperature |
| *Thrombin activity:* | Measured at 405 nm using S-2238 (Chromogenix, Haemochrom Diagnostica AB; supplier in US is DiaPharma) as substrate |

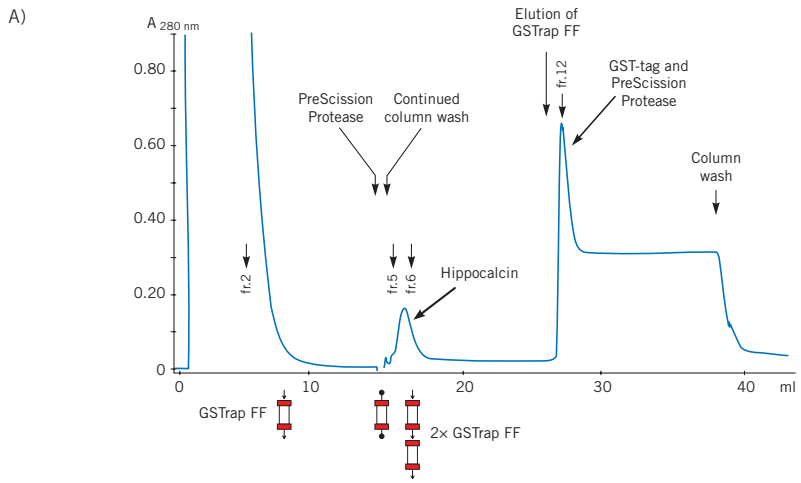
**Fig 21.** Purification of SH2 domain-GST fusion protein with on-column cleavage and post-cleavage removal of thrombin using GSTrap FF and HiTrap Benzamidine FF (high sub) columns. A) SDS-PAGE analysis of various sample processing steps. B) Chromatogram and thrombin activity curve demonstrating all steps in the purification of the SH2 domain.

Sources: Sigrell, J. A., Scientific poster: Purification of GST fusion proteins, on-column cleavage and sample clean-up, Amersham Biosciences, code number 18-1150-20.

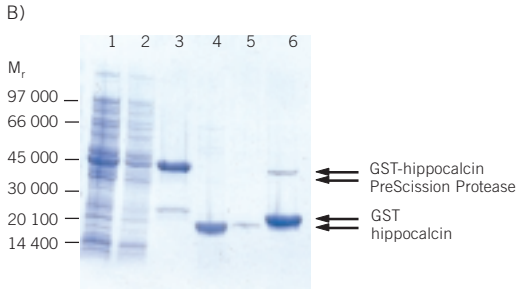
Data File: HiTrap Benzamidine FF (high sub) and Benzamidine Sepharose 4 Fast Flow (high sub), Amersham Biosciences, code number 18-1139-38.

### Example 7. Purification of human hippocalcin using GSTrap FF columns in series with on-column cleavage by PreScission Protease

The gene for human hippocalcin, a member of the neurone-specific calcium-binding protein family, was cloned into a pGEX vector containing a PreScission Protease site adjacent to the GST-tag. The expressed fusion protein was captured on a GSTrap FF 1 ml column. The column was then incubated overnight at 4 °C and for an additional 2 h at room temperature with PreScission Protease (which is GST-tagged itself). Following on-column cleavage, a second GSTrap FF 1 ml column was placed in series after the first to remove any PreScission Protease, uncleaved GST-fusion, or free GST-tag that could co-elute with the sample during the additional wash with binding buffer (Fig 22). For every gram of wet *E. coli* cells, 10 mg of pure, non-tagged hippocalcin was obtained.




| | |
|---|---|
| *Sample:* | 2 ml clarified *E. coli* homogenate containing expressed GST-hippocalcin, $M_r$ 43 000 |
| *Columns:* | GSTrap FF 1 ml |
| *Binding and wash buffer:* | 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM DTT, 10% glycerol |
| *GST elution buffer:* | 20 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0 |
| *Flow rate:* | 0.5 ml/min |
| *Instrument:* | ÄKTAprime |
| *Protease treatment:* | 80 U/ml PreScission Protease overnight at 4 °C and then 2 h at room temperature |




| | |
|---|---|
| Gel: | ExcelGel SDS Gradient, 8–18%, Coomassie blue staining |
| Lane 1: | Clarified *E. coli* homogenate containing expressed GST-hippocalcin |
| Lane 2: | Flow-through (fraction 2) |
| Lane 3: | GST-hippocalcin |
| Lane 4: | Pure hippocalcin after on-column cleavage (fraction 5) |
| Lane 5: | Same as lane 4, but fraction 6 |
| Lane 6: | Eluted fraction from GSTrap FF containing PreScission Protease and GST-tag released by cleavage (fraction 12) |

**Fig 22.** Purification of human hippocalcin-GST fusion protein with on-column cleavage and post-cleavage removal of PreScission Protease using GSTrap FF columns. A) Chromatogram showing purification of hippocalcin. B) SDS-PAGE analysis of various sample processing steps.

Source: Sigrell, J. A., Scientific poster: Purification of GST fusion proteins, on-column cleavage and sample clean-up, Amersham Biosciences, code number 18-1150-20.

### Example 8. Purification and on-column cleavage of TLP40-GST fusion protein using GSTrap FF columns and PreScission Protease

The gene coding for TLP40 protein was subcloned into pGEX-6P-1 and transformed into *E. coli* BL21. GST fusion proteins were purified from clarified lysates using two GSTrap FF 5 ml columns connected in series and ÄKTAexplorer 10. 1× PBS was used as binding buffer. The flow rate for loading was 1 ml/min, a rate found optimal for fusion protein binding. Loaded columns were washed with 1× PBS until the absorbance baseline stabilized, after which the buffer was changed to PreScission buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0). Columns were equilibrated with PreScission buffer until both the UV absorbance and conductivity baselines stabilized, after which buffer flow was stopped.

For PreScission Protease digestion, 2 units of enzyme/100 µg of bound GST-fusion protein was used. PreScission Protease was diluted in PreScission buffer (9 ml total volume for two columns in series) and manually injected into the columns at a flow rate of 5–7 ml/min. This increased flow allows for uniform distribution of enzyme through the GSTrap FF 5 ml columns. Following injection, the columns were closed, sealed and incubated for 12–16 h at 4 °C.

Prior to elution, a 1 ml GSTrap FF column (pre-equilibrated with PreScission buffer) was connected downstream to the GSTrap FF proteolytic cleavage columns. This configuration minimized loss of cleaved product and allowed for rapid baseline recalibration before peak elution. The 1 ml column also acted as a filter to capture any released cleaved GST protein, uncleaved GST-fusion protein and unbound PreScission Protease.

Elution of cleaved protein occurred immediately upon flow start-up (Fig 23A). The eluted material contained TLP40 cleavage product with no contaminating proteins detected (Fig 23B). After target protein elution, GST, unbound GST-fusion protein, and PreScission Protease were eluted with reduced glutathione [as applied in a full one-step gradient (100%)]. SDS-PAGE analysis of various fractions showed isolation of highly pure TLP40 after on-column cleavage (Fig 23B).

This application is reproduced with kind permission of Dr. Darcy Birse, University of Stockholm, Sweden.

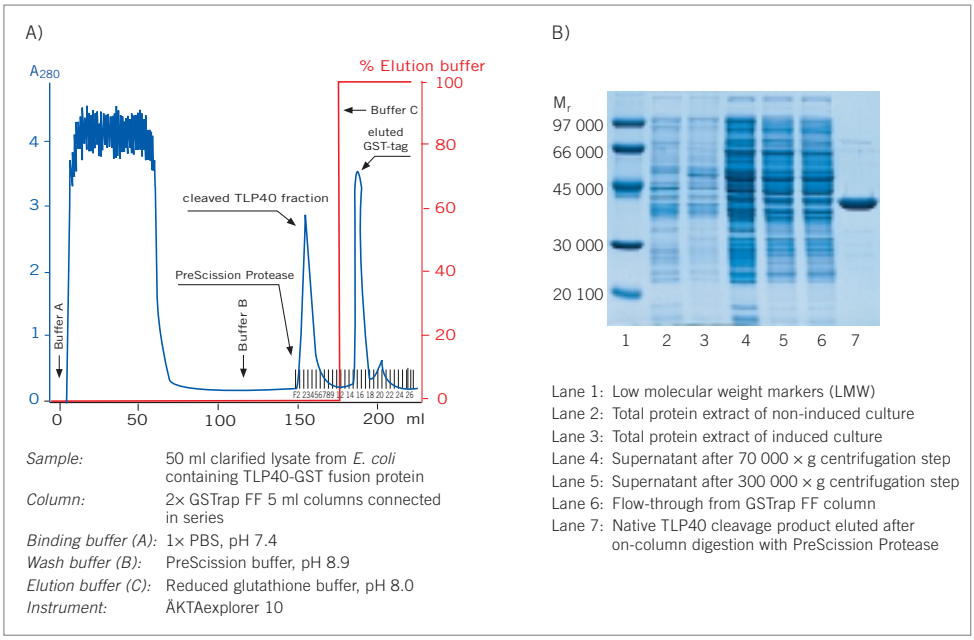



**Fig 23.** Purification and SDS-PAGE analysis of TLP40-GST fusion protein. A) Purification and on-column cleavage of fusion protein using GSTrap FF 5 ml and PreScission Protease in combination with ÄKTAexplorer 10. The flow rate for sample loading and injecting the protease were 1 ml/min and 5–7 ml/min, respectively. B) Fractions from the purification steps were analyzed by SDS-PAGE using a 3.5–12% polyacrylamide gel. The gel was stained with Coomassie blue.

Sources: Application Note: Efficient, rapid protein purification and on-column cleavage using GSTrap FF columns, Amersham Biosciences, code number 18-1146-70.

Knaust, R. *et al.*, An efficient and rapid protein purification and on-column cleavage strategy using GSTrap FF columns, *Life Science News* **6,** 12–13 (2000). See also online *Life Science News* archive.

Dian, C. *et al.*, in Abstracts of the 4th Annual CHI Protein Expression Meeting, McLean, VA (April 4–6, 2001).

### Example 9. Scaling-up purification of a phosphatase SH2 domain GST fusion protein and on-column cleavage using thrombin

To obtain the pure SH2 domain without the GST tag, chromatography was scaled-up using 100 ml of clarified *E. coli* homogenate and GSTrap FF 5 ml, and on-column cleavage was carried out overnight with thrombin prior to elution of released SH2 domain with binding buffer (Fig 24). The eluted SH2 domain fraction contained 2 mg of protein, while the GST fraction that was subsequently eluted with reduced glutathione contained 4 mg. SDS-PAGE analysis and silver staining indicated that the SH2 domain was pure and that protease cleavage was complete (Fig 24). Mass spectrometry revealed essentially two peaks corresponding to the single-charged (m/z 12 472) and double-charged (m/z 6 241) protein (Fig 25); this agrees with the expected $M_r$ of the SH2 domain (Fig 24). The spectra contained no other peak in the m/z window used (inset in Fig 25).

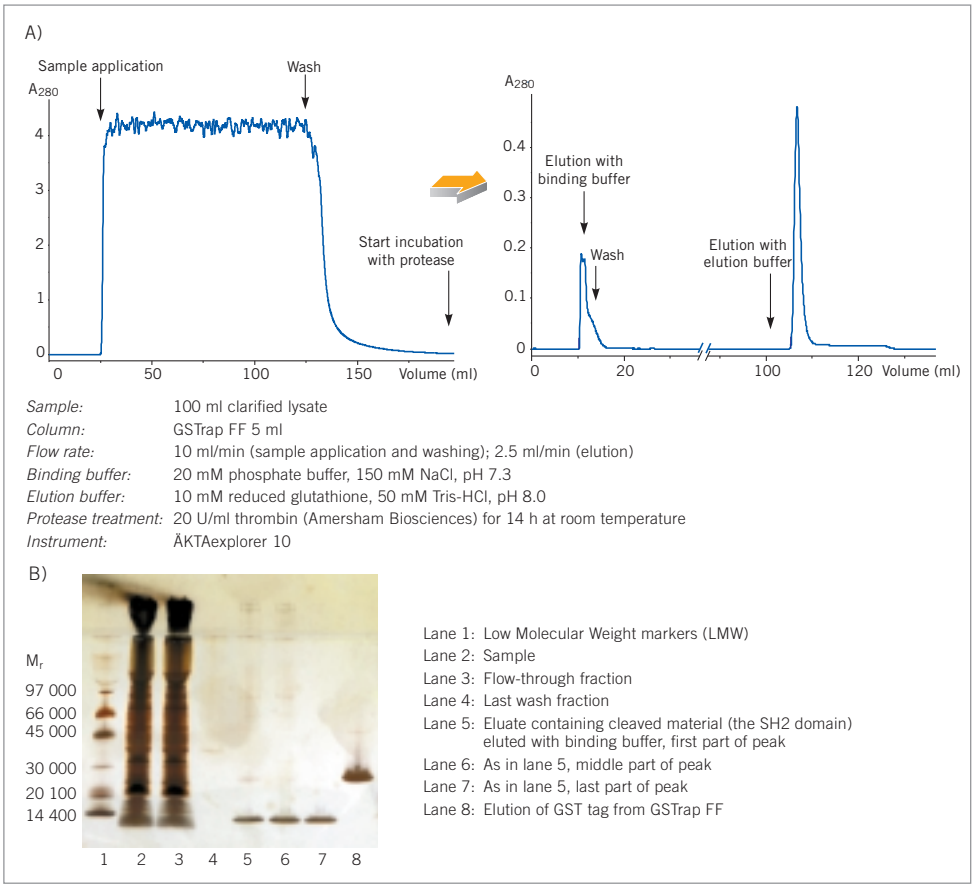



**Fig 24.** Purification of SH2 domain using GSTrap FF 5 ml and ÄKTAexplorer 10 with on-column cleavage of the GST tag using thrombin. A) One hundred ml of clarified *E. coli* homogenate containing a $M_r$ 37 000 SH2-GST fusion protein was applied to the column. The GST tag was removed with thrombin prior to elution of released SH2 domain with binding buffer and the resulting chromatogram recorded. B) Fractions were analyzed by SDS-PAGE on 8–25% PhastGel with silver staining for detection.

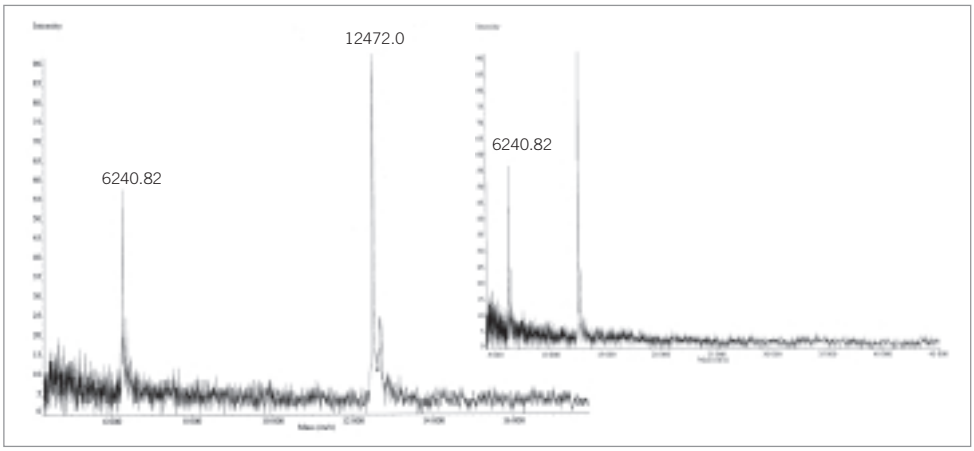



**Fig 25.** MALDI-ToF MS analysis of the SH2 domain.

Source: Haneskog, L. *et al.*, Scientific poster: Rapid purification of GST-fusion proteins from large sample volumes, Amersham Biosciences, code number 18-1139-51.

### Example 10. On-column cleavage of a GST fusion protein using thrombin

To demonstrate the efficiency of on-column cleavage in conjunction with purification, a GST fusion protein containing the recognition sequence for thrombin was applied to GSTrap FF 1 ml. After washing, the column was filled by syringe with 1 ml of thrombin solution (20 U/ml in 1× PBS) and sealed using the supplied connectors. After incubation for 16 h at room temperature, the target protein minus the GST moiety was eluted using 1× PBS, and the bound GST was subsequently eluted using elution buffer (Fig 26). The cleavage reaction yield was 100%. Intact GST fusion protein was not detected in the eluate by SDS-PAGE (Fig 27, lane 5).

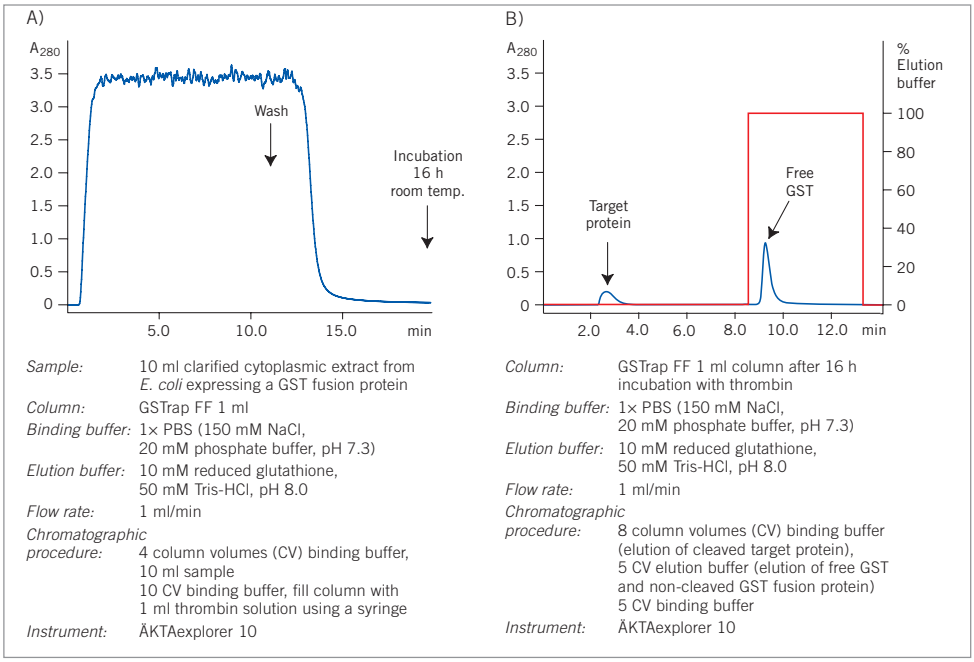


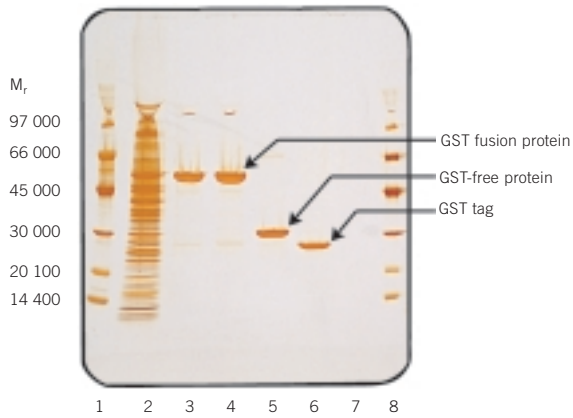

**Fig 26.** On-column thrombin cleavage of a GST fusion protein. A) Equilibration, sample application, and washing of a GST fusion protein on GSTrap FF 1 ml were performed using ÄKTAexplorer 10. After washing, the column was filled by syringe with 1 ml of thrombin (20 U/ml 1× PBS) and incubated for 16 h at room temperature. B) GST-free target protein was eluted using 1× PBS. GST was eluted using 10 mM reduced glutathione. The GST-free target protein fraction also contained a small amount of thrombin (not detectable by SDS-PAGE; see Fig 27, lane 6). The thrombin can be removed using a HiTrap Benzamidine FF (high sub) column.

Source: See Figure 27.

## Detection of GST fusion proteins

### Example 11. Detection of GST fusion proteins in bacterial lysates using the GST 96-Well Detection Module

The GST 96-Well Detection Module provides a convenient format for rapidly screening as many as 96 samples per plate for the presence of GST fusion proteins. The module uses immobilized goat polyclonal anti-GST antibody to capture GST fusion proteins from complex mixtures and exhibits very low, non-specific background binding. Using a chromogenic substrate, the system can detect as little as 1 ng of recombinant GST (Fig 28), providing a level of sensitivity that is 10 to 100 times greater than capture plates using immobilized glutathione.




Lane 1: Low Molecular Weight (LMW) Calibration kit, reduced
Lane 2: Cytoplasmic extract of *E. coli* containing GST fusion protein, 1 g cell paste/10 ml
Lane 3: GST fusion protein eluted from GSTrap FF 1 ml (from Fig 15A)
Lane 4: GST fusion protein eluted from GSTrap FF 5 ml (from Fig 15B)
Lane 5: GST-free target protein eluted from GSTrap FF 1 ml after 16 h thrombin cleavage (from Fig 26)
Lane 6: Free GST eluted from GSTrap FF 1 ml after thrombin cleavage (from Fig 26)
Lane 7: Thrombin solution (20 U/ml)
Lane 8: LMW, reduced

**Fig 27.** Analysis of fractions from Figs 15 and 26 by SDS-PAGE on ExcelGel SDS Gradient 8–18% using Multiphor II followed by silver staining. Fusion protein was eluted using 10 mM reduced glutathione (lanes 3 and 4). On-column cleavage with thrombin (20 U/ml) was performed with GSTrap FF 1 ml loaded with fusion protein. The GST-free target protein was eluted with 1× PBS (lane 5), and the GST moiety was eluted with 10 mM glutathione (lane 6).

Sources: Data File: GSTrap FF 1 ml and 5 ml Glutathione Sepharose 4 Fast Flow, Amersham Biosciences, code number 18-1136-89.

Haneskog, L. *et al.*, Fast and simple purification of GST fusion proteins using prepacked GSTrap affinity columns, *Life Science News* **4,** 16 (2000). See also online *Life Science News* archive.

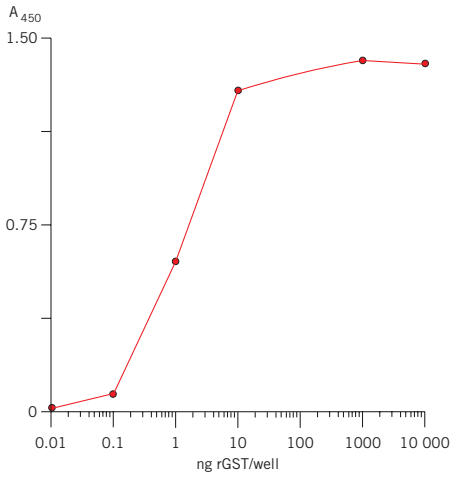



**Fig 28.** Detection of recombinant GST using the GST 96-Well Detection Module. The indicated amounts of recombinant GST Protein were prepared in 1× blocking buffer, and 100 µl volumes of each were transferred to the wells of a GST 96-Well Detection Plate. After 1 h binding at room temperature, the wells were washed and then incubated with a 1:1000 dilution of HRP/Anti-GST Conjugate for 1 h. Using the substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) for detection, the absorbance of each well was measured at 450 nm.

Source: See Figure 29.

The following application demonstrates that fusion proteins immobilized through their GST domains can be detected with antibodies directed against the fusion partner. A luciferase gene fragment was inserted into the multiple cloning site of the GST gene fusion vector pGEX-6P-1. Lysates from 64 randomly selected transformant colonies of *E. coli* BL21 were screened for GST fusion expression. Aliquots (50 µl) of each lysate were mixed 1:1 (v/v) with blocking buffer and applied to the wells of a GST 96-Well Detection Plate. Serial dilutions of recombinant GST were used as controls. After a 1 h incubation at room temperature, wells containing culture lysates were washed and incubated with rabbit anti-luciferase, followed by HRP/anti-rabbit IgG conjugate for detection. Control rGST wells were incubated with HRP/Anti-GST Conjugate. All wells were developed using TMB, and the $A_{450}$ of each well was measured in a 96-well plate reader. Wells containing luciferase GST fusion protein are evident by their strong positive signal (Fig 29).

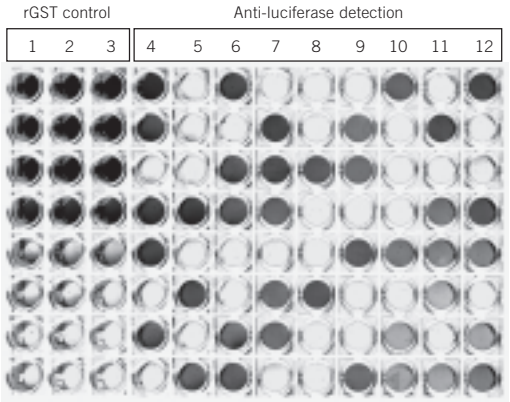



**Fig 29.** Screening of bacterial lysates for luciferase GST fusion protein expression using the GST 96-Well Detection Module. Cultures of randomly selected *E. coli* colonies transformed with a pGEX-6P-1/luciferase construct were grown, induced and lysed in a 96-well plate. Aliquots (50 µl) of each cleared lysate were transferred to the wells in columns 4–12 of a GST 96-Well Detection Plate to capture expressed GST-luciferase fusion proteins. Captured fusion proteins were detected using rabbit anti-luciferase, anti-rabbit IgG/peroxidase conjugate and TMB substrate. Columns 1–3 contain serial dilutions of recombinant GST. Recombinant GST was detected as described in Fig 28.

Sources: Bell, P. A. *et al*., Rapid screening of multiple clones for GST fusion protein expression, *Life Science News* **1**, 14 (1998). See also online *Life Science News* archive.

Data File: GST 96-Well Detection Module, Amersham Biosciences, code number 18-1128-14.

# Troubleshooting guide

## Protein expression

### A high basal level of expression is observed

Basal level expression (i.e. expression in the absence of an inducer, such as IPTG) is associated with most inducible promoters. It can affect the outcome of cloning experiments for toxic inserts by selecting against inserts cloned in the proper orientation. Basal level expression can be minimized by catabolite repression (e.g. growth in the presence of glucose). The *tac* promoter responsible for the expression of the fusion protein in pGEX vectors is not subject to catabolite repression. However, with the pGEX vector system there is a *lac* promoter located upstream between the 3'-end of the *lac*$I^q$ gene and the *tac* promoter. This *lac* promoter might contribute to the basal level of expression of inserts cloned into the pGEX multiple cloning site, and it is subject to catabolite repression.

- Add 2% glucose to the growth medium. This will decrease the basal level expression 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.

### No protein is detected in the bacterial sonicate

- Check DNA sequences. It is essential that protein-coding DNA sequences are cloned in the proper translation frame in the vectors. Cloning junctions should be sequenced to verify that inserts are in-frame. For convenience, use the pGEX 5' and 3' Sequencing Primers (see Appendix 4 for more information on the primers). The reading frame of the multiple cloning site for each pGEX vector is shown in Figure 2, Chapter 2.
- Optimize culture conditions to improve yield. Cell strain, medium composition, incubation temperature, and induction conditions can all affect yield. Exact conditions will vary for each fusion protein expressed.
- Analyze a small aliquot of an overnight culture by SDS-PAGE. Generally, a highly expressed protein will be visible by Coomassie blue staining when 5–10 µl of an induced culture with an $A_{600}$ of ~ 1.0 is loaded onto the gel. Non-transformed host *E. coli* cells and cells transformed with the parental vector should be run in parallel as negative and positive controls, respectively. If fusion protein is present in this total cell preparation and absent from a clarified sonicate, this may indicate the presence of inclusion bodies (see next page).
- Check for expression by immunoblotting, which is generally more sensitive than stained gels. Some fusion proteins may be masked on an SDS-polyacrylamide gel by a bacterial protein of approximately the same molecular weight. Immunoblotting can be used to identify fusion proteins in most of these cases. Run an SDS-polyacrylamide gel of induced cells and transfer the proteins to a nitrocellulose or PVDF membrane (such as Hybond-C or Hybond-P). Detect fusion protein using anti-GST antibody. Alternatively, purify the extract using GSTrap FF, GSTPrep FF 16/10, or Glutathione Sepharose 4 Fast Flow prior to SDS-PAGE analysis.
- Select a new, independently transformed isolate and check for expression.

**Most of the fusion protein is in the post-sonicate pellet**

- Check the cell disruption procedure. Cell disruption is indicated by partial clearing of the suspension or 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 might improve results. Avoid frothing as this might denature the fusion protein.

- Reduce sonication since over-sonication can lead to co-purification of host proteins with the fusion protein.

- Fusion protein may be produced as insoluble inclusion bodies. Try altering the growth conditions to slow the rate of translation, as suggested below. It may be necessary to combine these approaches. Exact conditions must be determined empirically for each fusion protein.

  – Lower the growth temperature (within the range of 20–30 °C) to improve solubility (35, 36).
  – Decrease the IPTG concentration to < 0.1 mM to alter induction level.
  – Alter the time 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.

- Alter extraction conditions to improve solubilization of inclusion bodies. Protein can sometimes be solubilized from inclusion bodies using common denaturants such as 4–6 M guanidine hydrochloride, 4–8 M urea, alkaline pH > 9, organic solvents (20, 37), 0.5–2% Triton X-100, 0.5–2% N-lauroylsarcosine (Sarcosyl) (38, 39), or other detergents. Other variables that affect solubilization include time, temperature, ionic strength, the ratio of denaturants to protein, and the presence of reducing reagents (20, 37). For reviews, see references 20, 35, 37, and 40.

- Following solubilization with denaturants, proteins must be correctly refolded to regain function. Denaturants can be removed by desalting or by dilution or dialysis to allow refolding of the protein and formation of the correct intramolecular associations. Detergent-solubilized proteins may have retained their native structure. Critical parameters during refolding include pH, the presence of reducing reagents, and the speed of denaturant removal (20, 37, 41). Once refolded, protein can be purified by ion exchange, gel filtration, or affinity chromatography.

- Fusion proteins can be purified to some extent while denatured. In some instances when GST fusion proteins form inclusion bodies, solubilization and binding to GSTrap FF columns can be achieved in the presence of 2–3 M guanidine hydrochloride or by using up to 2% Tween 20. Binding to Glutathione Sepharose can also be achieved in the presence of 1% CTAB, 10 mM DTT or 0.03% SDS (19). Successful purification in the presence of these agents may depend on the nature of the fusion protein.

# Purification and detection

### The column has clogged

- Cell debris in the sample may clog the column. Clean the column using the protocol outlined in Appendix 5 and make sure that samples have been filtered using a 0.45 µm filter and centrifuged at > 30 000 × g. Dilute samples if they are too viscous.

### The fusion protein does not bind to the purification medium

- Decrease the flow rate to improve binding.
- Over-sonication may have denatured the fusion protein. Check the lysate microscopically to monitor cell breakage. Use mild sonication conditions during cell lysis.
- 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 as this may denature the fusion protein.
- Add 1–10 mM DTT prior to cell lysis. This can significantly increase binding of some GST fusion proteins to Glutathione Sepharose. The optimal concentration must be determined empirically for each fusion protein.
- 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.
- If re-using a column, check that the column has been cleaned and regenerated correctly (see Appendix 5). Replace with fresh Glutathione Sepharose or a new prepacked column if binding capacity does not return after regeneration.
- Check the binding of a cell sonicate prepared from the parental pGEX plasmid. 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. Try reducing the binding temperature to 4 °C and limit the number of washes.
- 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.
- Fusion protein may be in inclusion bodies. See discussion on page 84.

### The fusion protein is poorly eluted

- Decrease the flow rate to improve elution.
- Increase the concentration of glutathione in the elution buffer. Above 15 mM glutathione, the buffer concentration should be increased to maintain pH (42). As an example, try 50 mM Tris-HCl, 20–40 mM reduced glutathione, pH 8 as elution buffer.
- Increase the pH of the elution buffer. Values up to pH 9 may improve elution without requiring an increase in the concentration of glutathione.
- Increase the ionic strength of the elution buffer by adding 0.1–0.2 M NaCl. Note 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).

- Increase the volume of elution buffer. In some cases, especially after on-column cleavage of fusion protein, a larger volume of buffer may be necessary to elute the fusion protein.
- Add a non-ionic detergent (e.g. 0.1% Triton X-100 or 2% N-octyl glucoside) to the elution buffer to reduce non-specific hydrophobic interactions that may prevent solubilization and elution of fusion proteins (39).
- Try overnight elution at room temperature or 4 °C. *Note:* The longer the duration of purification, the greater the risk of protein degradation.

### Multiple bands are seen on SDS-PAGE or Western blot analysis

Multiple bands result from partial degradation of fusion proteins by proteases, or denaturation and co-purification of host proteins with the GST fusion protein due to over-sonication.

- Check that a protease-deficient host such as *E. coli* B21 has been used.
- Add protease inhibitors such as 1 mM PMSF to the lysis solution. A non-toxic, water soluble alternative to PMSF is 4-(2-amino-ethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) (43), commercially available as Pefabloc SC from Roche Molecular Biochemicals. *Note:* Serine protease inhibitors must be removed prior to cleavage by thrombin or Factor Xa. Use HiTrap Benzamidine FF (high sub) (see Procedure 22).
- Use prepacked GSTrap FF columns or Glutathione Sepharose 4 Fast Flow. These can be used at higher flow rates (compared with gravity columns) to process samples more quickly and thus avoid degradation.
- Decrease sonication. 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 as this may denature the fusion protein.
- Include an additional purification step. A variety of proteins known as chaperonins are involved in the correct folding of nascent proteins in *E. coli* (44) and may co-purify with GST fusion proteins. These include, but are not limited to: DnaK ($M_r$ ~ 70 000, reference 45), DnaJ ($M_r$ ~ 37 000, reference 46), GrpE ($M_r$ ~ 40 000, reference 47), GroEL ($M_r$ ~ 57 000, reference 48), and GroES ($M_r$ ~ 10 000, reference 49). Several methods for purifying GST fusion proteins from these co-purifying proteins have been described (see below).
- Anti-GST antibody from Amersham Biosciences has been cross-absorbed against *E. coli* proteins and tested for its lack of non-specific background binding in a Western blot. However, other sources of the anti-GST antibody may contain antibodies that react with various *E. coli* proteins present in the fusion protein sample. Cross-adsorb the antibody with an *E. coli* sonicate to remove anti-*E. coli* antibodies. The *E. coli* used for cross-absorption must not contain the pGEX plasmid. See Appendix 6 for procedural details.

### $M_r$ 70 000 protein co-purifies with the GST fusion protein

Pre-incubate the protein solution with 2 mM ATP, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.4 for 10 min at 37 °C prior to purification to dissociate the complex. This $M_r$ 70 000 protein is probably a product of the *E. coli* gene *dnaK* and involved in the degradation of "abnormal" proteins in *E. coli* (50). Reports suggest that this protein can be removed by ion exchange chromatography (50) or by passage of the sample over ATP agarose (51).

Various reports (50, 52) suggest washing the column with ATP or GroES rather than using a subsequent ion exchange step.

# Detection

### Results with the GST Detection Module are problematic

- The reaction rate of the CDNB assay is linear provided that an $A_{340}$ of ~ 0.8 is not exceeded during the 5-min time course. Plot initial results to verify that the reaction rate is linear over the time course. Adjust the amount of sample containing the GST fusion protein to maintain a linear reaction rate.
- Depending on how the fusion protein is folded, some GST fusions will show very low activity with the CDNB assay. Whether for this or for any other reason, if a low absorbance is obtained using the CDNB assay, a Western blot using anti-GST antibody may reveal high levels of fusion protein expression.
- Under standard assay conditions at 22 °C and in the absence of GST, glutathione and CDNB react spontaneously to form a chemical moiety that produces a baseline drift at $\Delta A_{340}$ /min of ~ 0.003 (or 0.015 in 5 min). Correct for baseline drift by blanking the spectrophotometer with the blank cuvette before each reading of the sample cuvette.

### Results with the GST 96-Well Detection Module are problematic

- If low absorbance is detected in the samples, check that host cells were sufficiently induced and the samples were sufficiently lysed (see Troubleshooting protein expression).
- If clarified lysate is being tested, mix the initial GST sample with 2× blocking buffer to give a final concentration of 1× blocking buffer.
- If poor day-to-day reproducibility between identical samples is experienced, verify that all incubation times are consistent. GST capture incubation time can be decreased with slightly reduced signal, but do not incubate for less than 30 min. Every 15-min decrease in HRP/anti-GST conjugate incubation time can significantly reduce signal.

# Cleavage

## PreScission Protease

### PreScission Protease cleavage is incomplete

- Check that the ratio of PreScission Protease to fusion protein is correct.
- Increase the incubation time to 20 h or longer at 5 °C, and increase the amount of PreScission Protease used in the reaction.
- Verify that a PreScission Protease cleavage site is present in the fusion protein. Compare the DNA sequence of the construct with the known PreScission Protease cleavage sequence. Verify that the optimal PreScission Protease recognition site, Leu-Glu-Val-Leu-Phe-Gln↓Gly-Pro, has not been altered.
- Remove possible PreScission Protease inhibitors by extensive washing of the purification column before cleaving with PreScission Protease. The presence of $Zn^{2+}$, as well as Pefabloc SC or chymostatin, may interfere with PreScission Protease activity.

### Following cleavage and SDS gel analysis, multiple bands are seen on the gel

- Determine when the bands appear. Additional bands seen prior to PreScission Protease cleavage may be the result of proteolysis in the host bacteria or in the purification steps leading up to the cleavage steps. *E. coli* BL21 is a protease-deficient strain that is recommended for use.
- Check the sequence of the fusion partner for the presence of additional PreScission Protease recognition sites. PreScission Protease optimally recognizes the sequence Leu-Glu-Val-Leu-Phe-Gln↓Gly-Pro and cleaves between the Gln and Gly residues; however, similar secondary sites may exhibit some propensity for cleavage. Adjusting reaction conditions (e.g. time, temperature, salt concentration) may provide selective cleavage at the desired site. If adjustment of the conditions does not correct the problem, reclone the insert into a pGEX-T (thrombin) or pGEX-X (Factor Xa) expression vector.

### The fusion partner is contaminated with PreScission Protease after purification

- Pass the sample over a new GSTrap FF column or fresh Glutathione Sepharose to remove residual PreScission Protease. It is possible that the Glutathione Sepharose may have been saturated with GST fusion protein in the first purification, although saturation of the purification column is rarely a problem.

## Thrombin

### Cleavage with thrombin is incomplete

- Check that the ratio of thrombin to fusion protein is correct.
- Increase the reaction time to 20 h at 22–25 °C, and increase the amount of thrombin used in the reaction.
- Check the DNA sequence of the construct to verify the presence of the thrombin site. Verify that the thrombin site has not been altered.
- Check that protease inhibitors have been removed.

### Following cleavage and SDS gel analysis, multiple bands are seen on the gel

- Determine when the bands appear. Additional bands seen prior to thrombin cleavage may be the result of proteolysis in the host bacteria or in the purification steps leading up to the cleavage step. *E. coli* BL21 is a protease-deficient strain that is recommended for use.
- Check the sequence of the fusion partner for the presence of additional thrombin recognition sites. Optimum cleavage sites for thrombin are given in 1) and 2) below.

  1) P4-P3-Pro-Arg/Lys↓P1'-P2' where P3 and P4 are hydrophobic amino acids and P1' and P2' are non-acidic amino acids. The Arg/Lys↓P1' bond is cleaved.

Examples:

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

In A, the Arg↓Gly bond is cleaved very quickly by thrombin. In B, the Lys↓Leu bond is

cleaved. C is the recognition sequence found on the thrombin series of pGEX plasmids; the Arg↓Gly bond is cleaved.

2) P2-Arg/Lys↓P1', where either P2 or P1' is Gly. The Arg/Lys↓P1' bond is cleaved.

Examples:

| | P2 | R/K↓P1' |
|---|---|---|
| A) | Ala | Arg↓Gly |
| B) | Gly | Lys↓Ala |

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

Adjusting time and temperature of digestion can result in selective cleavage at the desired thrombin site. If adjustment of conditions does not correct the problem, reclone the insert into a pGEX-6P (PreScission) or pGEX-X (Factor Xa) expression vector.

## Factor Xa

### Cleavage with Factor Xa is incomplete

- Factor Xa requires activation of Factor X with Russell's viper venom. Factor Xa from Amersham Biosciences has been preactivated, but other sources may not be activated. To activate, incubate Factor Xa with Russell's viper venom at a ratio of 1% (w/w) in 70 mM NaCl, 8 mM $CaCl_2$, 8 mM Tris-HCl, pH 8.0 at 37 °C for 5 min.
- Check the DNA sequence of the construct to verify the presence of the Factor Xa site. Verify that the Factor Xa site has not been altered. The recognition sequence for Factor Xa is Ile-Glu-Gly-Arg↓X, where X can be any amino acid except Arg or Pro.
- Check that the Factor Xa to fusion protein ratio is correct.
- Check that glutathione has been removed as recommended.
- In some cases, increasing the substrate concentration up to 1 mg/ml may improve results.
- Add < 0.5% w/v SDS to the reaction buffer. This can significantly improve Factor Xa cleavage with some fusion proteins. Various concentrations of SDS should be tested to find the optimum concentration.
- Increase incubation time to 20 h or longer at 22 °C and increase the amount of Factor Xa (for some fusion proteins, Factor Xa can be increased up to 5%).
- Check that protease inhibitors have been removed.

### Following cleavage and SDS gel analysis, multiple bands are seen on the gel

- Determine when the bands appear. Additional bands seen prior to cleavage may be the result of proteolysis in the host bacteria or in the purification steps leading up to the cleavage step. *E. coli* BL21 is a protease-deficient strain that is recommended for use.
- Check the sequence of the fusion partner for the presence of additional Factor Xa recognition sites. Factor Xa is highly specific for the recognition sequence Ile-Glu-Gly-Arg↓. The bond following the Arg residue is cleaved. Adjusting the time and temperature of digestion can result in selective cleavage at the desired Factor Xa site. If adjustment of conditions does not correct the problem, reclone the insert into a pGEX-6P (PreScission) or pGEX-T (thrombin) expression vector.

# Appendix 1

## Characteristics of GST and of host bacterial strain

| **Properties of Glutathione S-transferase** | |
|---|---|
| Glutathione S-transferase is a naturally occurring $M_r$ 26 000 protein that can be expressed in *E. coli* with full enzymatic activity. The properties below were determined in pGEX-1N (6). | |
| Dimer molecular weight | $M_r$ 58 500 |
| $K_m$ (glutathione) | 0.43 ± 0.07 mM |
| $K_m$ (CDNB) | 2.68 ± 0.77 mM |
| pI (chromatofocusing) | 5.0 |
| GST class | hybrid of Alpha and Mu characteristics |

| **Properties and handling of *E. coli* BL21** | |
|---|---|
| Genotype | F-, *ompT*, *hsdS* ($r_B$-, $m_B$-), *gal* (53, 54). |
| Growth conditions | Resuspend lyophilized cultures in 1 ml of LB medium*. Grow overnight at 37 °C before plating onto LB agar plates. |
| Long-term storage | Mix equal volumes of stationary phase culture (grown in LB medium) and glycerol. Store at -70 °C. Revive frozen glycerol stocks of BL21 by streaking onto LB agar plates. |
| Recommended usage | The protease-minus nature of BL21 makes it useful as an expression host. Since BL21 does not transform well, use an alternate strain for cloning and maintenance of the vector. |

** LB medium (prepared fresh): 10 g tryptone, 5 g yeast extract, 10 g NaCl. Combine tryptone, yeast extract, and NaCl in 900 ml $H_2O$. Stir to dissolve, and adjust volume to 1 l. Sterilize by autoclaving. To prepare as a solid medium, add 1.2–1.5% agar.*

# Appendix 2

## Control regions for pGEX vectors

| SELECTION GUIDE – pGEX Vector Control Regions | pGEX-2TK 27-4587-01 | pGEX-4T-1 27-4580-01 | pGEX-4T-2 27-4581-01 | pGEX-4T-3 27-4583-01 | pGEX-5X-1 27-4584-01 | pGEX-5X-2 27-4585-01 | pGEX-5X-3 27-4586-01 | pGEX-6P-1 27-4597-01 | pGEX-6P-2 27-4598-01 | pGEX-6P-3 27-4599-01 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Glutathione S- Transferase Region** | | | | | | | | | | |
| *tac* promoter | | | | | | | | | | |
| –10 | 205–211 | 205–211 | 205–211 | 205–211 | 205–211 | 205–211 | 205–211 | 205–211 | 205–211 | 205–211 |
| –35 | 183–188 | 183–188 | 183–188 | 183–188 | 183–188 | 183–188 | 183–188 | 183–188 | 183–188 | 183–188 |
| *lac* operator | 217–237 | 217–237 | 217–237 | 217–237 | 217–237 | 217–237 | 217–237 | 217–237 | 217–237 | 217–237 |
| Ribosome binding site for GST | 244 | 244 | 244 | 244 | 244 | 244 | 244 | 244 | 244 | 244 |
| Start codon (ATG) for GST | 258 | 258 | 258 | 258 | 258 | 258 | 258 | 258 | 258 | 258 |
| Coding region for thrombin cleavage | 918–935 | 918–935 | 918–935 | 918–935 | NA | NA | NA | NA | NA | NA |
| Coding region for factor Xa cleavage | NA | NA | NA | NA | 921–932 | 921–932 | 921–932 | NA | NA | NA |
| Coding region for PreScission Protease cleavage | NA | NA | NA | NA | NA | NA | NA | 918–938 | 918–938 | 918–938 |
| Coding for kinase recognition site | 936–950 | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| Multiple Cloning Site | 951–966 | 930–966 | 930–967 | 930–965 | 934–969 | 934–970 | 934–971 | 945–981 | 945–982 | 945–980 |
| **β-lactamase (Amp$^r$) Gene Region** | | | | | | | | | | |
| Promoter | | | | | | | | | | |
| –10 | 1330–1335 | 1330–1335 | 1331–1336 | 1329–1334 | 1333–1338 | 1334–1339 | 1335–1340 | 1345–1350 | 1346–1351 | 1344–1349 |
| –35 | 1307–1312 | 1307–1312 | 1308–1313 | 1306–1311 | 1310–1315 | 1311–1316 | 1312–1317 | 1322–1327 | 1323–1328 | 1321–1326 |
| Start codon (ATG) | 1377 | 1377 | 1378 | 1376 | 1380 | 1381 | 1382 | 1392 | 1393 | 1391 |
| Stop codon (TAA) | 2235 | 2235 | 2236 | 2234 | 2238 | 2239 | 2240 | 2250 | 2251 | 2249 |
| ***LacI$^q$* Gene Region** | | | | | | | | | | |
| Start codon (GTG) | 3318 | 3318 | 3319 | 3317 | 3321 | 3322 | 3323 | 3333 | 3334 | 3332 |
| Stop codon (TGA) | 4398 | 4398 | 4399 | 4397 | 4401 | 4402 | 4403 | 4413 | 4414 | 4412 |
| **Plasmid Replication Region** | | | | | | | | | | |
| Site of replication initiation | 2995 | 2995 | 2996 | 2994 | 2998 | 2999 | 3000 | 3010 | 3011 | 3009 |
| Region necessary for replication | 2302–2998 | 2302–2998 | 2303–2999 | 2301–2997 | 2305–3001 | 2306–3002 | 2307–3003 | 2317–3013 | 2318–3014 | 3216–3012 |
| **Sequencing Primers** | | | | | | | | | | |
| 5' pGEX Sequencing Primer binding | 869–891 | 869–891 | 869–891 | 869–891 | 869–891 | 869–891 | 869–891 | 869–891 | 869–891 | 869–891 |
| 3' pGEX Sequencing Primer binding | 1041–1019 | 1041–1019 | 1042–1020 | 1040–1018 | 1044–1022 | 1045–1023 | 1046–1024 | 1056–1034 | 1057–1035 | 1055–1033 |
| **GenBank Accession Number** | U13851 | U13853 | U13854 | U13855 | U13856 | U13857 | U13858 | U78872 | U78873 | U78874 |

**Complete DNA sequences and restriction site data are available at the Amersham Biosciences web site (http:// www.amershambiosciences.com).**

# Appendix 3

# Electroporation

## Preparation of cells

### Reagents required

2× YT medium: Dissolve 16 g tryptone, 10 g yeast extract, and 5 g NaCl in 900 ml of distilled $H_2O$. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 l with distilled $H_2O$. Sterilize by autoclaving for 20 min. To prepare as a solid medium, add 1.2–1.5% agar.

1 mM HEPES: 0.26 g HEPES, sodium salt. Dissolve in 900 ml distilled, deionized $H_2O$. Adjust the pH to 7.0. Adjust the volume to 1 l with distilled $H_2O$. Sterilize by autoclaving.

10% glycerol in 1 mM HEPES, pH 7.0: Aseptically add 10 ml sterile glycerol to 90 ml sterile 1 mM HEPES, pH 7.0.

10% glycerol in distilled, deionized $H_2O$: Add 10 ml glycerol to 90 ml distilled, deionized $H_2O$. Sterilize by autoclaving.

Isopropanol

TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA

Phenol: Redistilled phenol saturated with TE buffer containing 8-hydroxy quinoline (17)

Chloroform/isoamyl alcohol: Reagent-grade chloroform and isoamyl alcohol, mixed 24:1

Phenol/chloroform: Equal parts of redistilled phenol and chloroform/isoamyl alcohol (24:1), each prepared as described above

3 M sodium acetate, pH 5.4: Aqueous solution

Ethanol, 70%, 95%

### Steps

1. Inoculate 10 ml of 2× YT medium with an *E. coli* host strain from an LB or 2× YT medium plate. Incubate at 37 °C overnight with shaking.
2. Inoculate 1 l of 2× YT medium with the 10 ml overnight culture of host cells. Incubate for 2–2.5 h at 37 °C with shaking at 250 rpm until an $A_{600}$ of 0.5–0.7 is achieved.
3. Place the flask on ice for 15–30 min.
4. Spin at 4000 × g for 20 min at 4 °C.
5. Decant the supernatant and resuspend the cells in 1 l of ice-cold sterile 1 mM HEPES, pH 7.0.
6. Spin as described above. Decant the supernatant and resuspend the cells in 500 ml of ice-cold sterile 1 mM HEPES, pH 7.0.
7. Spin as described above. Decant the supernatant. Wash the cells in 20 ml of sterile 1 mM HEPES, pH 7.0, containing 10% glycerol.
8. Spin as described above. Decant the supernatant. Resuspend the cells in a total volume of 2–3 ml of sterile 10% glycerol in distilled, deionized $H_2O$.
9. Dispense in 50–100 µl aliquots and proceed to the Electroporation protocol or freeze on dry ice and store at -70 °C.
10. Extract the ligated pGEX vector (as well as the uncut vector) once with an equal volume of phenol/chloroform and once with an equal volume of chloroform/isoamyl alcohol.
11. Remove the aqueous phase and add 1/10 volume of 3 M sodium acetate, pH 5.4 and 2.5 volumes of 95% ethanol.
12. Place on dry ice for 15 min and then spin in a microcentrifuge for 5 min to pellet the DNA.

13. Remove the supernatant and wash the pellet with 1 ml of 70% ethanol. Spin for 5 min, discard the supernatant, and dry the pellet.
14. Resuspend each DNA pellet in 20 µl of sterile distilled $H_2O$. Alternatively, the DNA can be gel band-purified.

The DNA must be completely free of salt prior to electroporation.

## Electroporation

We recommend that 1 ng of uncut (supercoiled) vector DNA be transformed in parallel with insert/pGEX ligations to determine the efficiency of each competent cell preparation.

### Reagents required

The following protocol was developed using a Bio-Rad Gene Pulser™.

Salt-free DNA

SOC medium: To 20 g of tryptone, 5 g of yeast extract and 0.5 g of NaCl, add distilled $H_2O$ to 1 l. Dispense into bottles at 100 ml per bottle and autoclave for 20 min. When cool, add 1 ml of sterile 1 M $MgCl_2$, 1 ml of sterile 250 mM KCl and 2.78 ml of sterile 2 M (36%) glucose (see below) for each 100 ml of medium.

LBAG plates: Prepare LBG medium by dissolving 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 900 ml of distilled $H_2O$. Sterilize by autoclaving. After the medium has cooled to 50 °C, add 10 ml of sterile 2 M glucose (see below), then aseptically add 1 ml of a 100 mg/ml ampicillin stock solution (final concentration 100 µg/ml), see below. Adjust to 1 l with sterile distilled $H_2O$. To prepare as a solid medium, add 1.2–1.5% agar.

2 M glucose: Dissolve 36 g in 70 ml of $H_2O$. Add $H_2O$ to 100 ml and filter-sterilize using a 0.2 µm filter.

Ampicillin stock solution: Dissolve 400 mg of the sodium salt of ampicillin in 4 ml of $H_2O$. Sterilize by filtration and store in small aliquots at -20 °C.

### Steps

1. If electroporation-competent cells have been frozen, thaw vials on ice. Otherwise, proceed directly to the following step using freshly prepared cells.
2. Transfer 50 µl of cells to a pre-chilled 0.2 cm cuvette.
3. Add 2 µl of salt-free DNA from the insert/pGEX ligations (from step 14 above) or 1 ng of supercoiled control DNA (e.g. pUC 18) and disperse. Place on ice for 1 min.
4. Program the electroporator to give 25 µF, 2.5 kV at 200 ohms. Dry the cuvette with a tissue and place it into the electroporation chamber. Pulse once (should yield a pulse with a time constant of 4.5–5 msec).
5. Immediately add 1 ml of fresh SOC medium to the cuvette and invert to resuspend the cells. Transfer the contents of the cuvette to a 15 ml disposable culture tube.

   *Note: For a negative control, repeat steps 2–5 without adding DNA.*
6. Incubate all tubes for 1 h at 37 °C with shaking (250 rpm).
7. Plate 100 µl of the transformed cells from the ligated samples and 100 µl of the negative control onto separate LBAG plates. Dilute the sample of the transformed cells from the uncut vector 10-fold and plate 10 µl onto a LBAG plate. Incubate the plates at 37 °C overnight.

# Appendix 4

## Sequencing of pGEX fusions

### Sequencing

pGEX vectors can be sequenced using the pGEX 5' and 3' Sequencing Primers. The sequences and the binding regions of these primers are given below:

### pGEX 5' Sequencing Primer

5'-d[GGGCTGGCAAGCCACGTTTGGTG]-3'
The pGEX 5' Sequencing Primer binds at nucleotides 869–891 on all ten pGEX vectors.

### pGEX 3' Sequencing Primer

5'-d[CCGGGAGCTGCATGTGTCAGAGG]-3'
The pGEX 3' Sequencing Primer binds at the following locations on the pGEX vectors:

| Vector | Binding site |
|---|---|
| pGEX-2TK | 1041–1019 |
| pGEX-4T-1 | 1041–1019 |
| pGEX-4T-2 | 1042–1020 |
| pGEX-4T-3 | 1040–1018 |
| pGEX-5X-1 | 1044–1022 |
| pGEX-5X-2 | 1045–1023 |
| pGEX-5X-3 | 1046–1024 |
| pGEX-6P-1 | 1056–1034 |
| pGEX-6P-2 | 1057–1035 |
| pGEX-6P-3 | 1055–1033 |

*For information concerning control regions in the pGEX vectors, see Appendix 2.*

# Appendix 5

## Cleaning, storage, and handling of media/columns

### Glutathione Sepharose 4B and Glutathione Sepharose 4 FF media

- Glutathione Sepharose 4B is recommended for packing small gravity-flow columns and for batch purifications.
- Glutathione Sepharose Fast Flow is excellent for packing high-performance columns for use with purification systems and for scaling-up.

See Chapter 4, Table 8 for the physical characteristics of these media.

#### Chemical stability

Glutathione Sepharose shows no significant loss of binding capacity when exposed to 70% ethanol or 6 M guanidine hydrochloride for 2 h at room temperature or to 1% SDS for 14 d.

#### Cleaning

Re-use of purification columns and media depends upon the nature of the sample and should only be performed with identical samples to prevent cross-contamination.

If Glutathione Sepharose appears to be losing binding capacity, it may be due to an accumulation of precipitated, denatured or non-specifically bound proteins.

**To remove precipitated or denatured substances:**

1. Wash with two column volumes of 6 M guanidine hydrochloride.
2. Immediately wash with five column volumes of 1× PBS, pH 7.4.

**To remove hydrophobically bound substances:**

1. Wash with 3–4 column volumes of 70% ethanol or two column volumes of 1% Triton X-100.
2. Immediately wash with five column volumes of 1× PBS, pH 7.4.

**For storage:**

Wash medium and column twice with 20% ethanol at neutral pH (use approximately five column volumes for packed medium) and store at 4–8 °C.

### GSTPrep FF 16/10 columns

The column is supplied in 20% ethanol. If the column is to be stored for more than 2 d after use, clean and store as described below.

See Chapter 4, Table 14 for the physical characteristics of GSTPrep FF 16/10 columns.

Re-use of GSTPrep FF 16/10 columns depends upon the nature of the sample and should only be performed with identical samples to prevent cross-contamination.

Do not open the column.

### Buffer and solvent resistance

- De-gas and filter all solutions through a 0.45 µm filter to increase column lifetime.
- Daily use: All commonly used aqueous buffers, pH 6–9.
- Cleaning: Guanidine hydrochloride, up to 6 M for 1 h at room temperature. Ethanol up to 70%. Non-ionic detergents, e.g. Triton X-100.
- Avoid: Solutions < pH 6.

### Cleaning in place

If the column appears to be losing binding capacity, it may be due to an accumulation of precipitated, denatured or non-specifically bound proteins.

#### To remove precipitated or denatured substances:

1. Wash with 40 ml of 6 M guanidine hydrochloride.
2. Immediately wash with 100 ml of 1× PBS, pH 7.4, at a flow rate of 5 ml/min.

#### To remove hydrophobically bound substances:

1. Wash with 60–80 ml of 70% ethanol or 40 ml of 1% Triton X-100.
2. Immediately wash with 100 ml of 1× PBS, pH 7.4, at a flow rate of 5 ml/min.

#### For storage:

Wash column with at least 100 ml of 20% ethanol at neutral pH at a flow rate of 5 ml/min and store at 4–8 °C.

### Handling

Many chromatography systems are equipped with pressure gauges to measure the pressure at a particular point in the system, usually just after the pumps. The pressure measured here is the sum of the pre-column pressure, the pressure drop over the gel bed, and the post-column pressure. It is always higher than the pressure drop over the bed alone. We recommend keeping the pressure drop over the bed below 1.5 bar. Setting the upper limit of your pressure gauge to 1.5 bar will ensure the pump shuts down before the gel is over-pressured. If necessary, post-column pressure of up to 3.5 bar can be added to the limit without exceeding the column hardware limit. To determine post-column pressure, proceed as follows:

To avoid breaking the column, the post-column pressure must never exceed 3.5 bar.

1. Connect a piece of tubing in place of the column.
2. Run the pump at the maximum flow you intend to use for chromatography. Use a buffer with the same viscosity as you intend to use for chromatography. Note this back pressure as total pressure.
3. Disconnect the tubing and run the same flow rate used in step 2. Note this back pressure as pre-column pressure.

4. Calculate the post-column pressure as total pressure minus pre-column pressure.

If the post-column pressure is higher than 3.5 bar, take steps to reduce it (shorten tubing, clear clogged tubing, or change flow restrictors) and perform steps 1–4 again until the post-column pressure is below 3.5 bar. When the post-column pressure is satisfactory, add the post-column pressure to 1.5 bar and set this as the upper pressure limit on the chromatography system.

## Benzamidine Sepharose 4 Fast Flow

With Benzamidine Sepharose 4 Fast Flow (high sub) columns, the GST fusion protein is present in the flow-through and wash. The protease (thrombin or Factor Xa) remains bound to the column until eluted using one of the buffers described below.

### Recommended elution buffers

Elution of the bound protease can be performed using either a step or a continuous gradient.

1. Substances bound through ionic interactions can be eluted by increasing the salt concentration to 1.0 M.

2. Affinity-bound substances can be eluted in different ways:

- Low pH elution buffers: 0.05 M glycine, pH 3.0 or 10 mM HCl, 0.5 M NaCl, pH 2.0. For each ml of fraction to be collected, add 60–200 µl of 1 M Tris-HCl, pH 9 to the collection tube. This will prevent denaturation of the eluted protein at low pH.
- Competitive elution buffer: 20 mM p-Aminobenzamidine in binding buffer. *Note:* This elution buffer has a very high $A_{280}$. Therefore, the eluted protein must be detected by methods other than absorbance, such as activity measurement (if possible), total protein, or SDS-PAGE analysis. The advantage of competitive elution is that pH can be kept constant during the run.

3. Other possible elution buffers: Denaturing agents such as 8 M urea or 6 M guanidine hydrochloride.

After elution of the protease, wash the column with five column volumes of binding buffer (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4). For longer-term storage, store in a buffer containing 20% ethanol in 0.05 M acetate buffer, pH 4 at 4–8 °C.

# Appendix 6

## Cross-adsorption of anti-GST antiserum with *E. coli* proteins

Some sources of anti-GST antibody may contain anti-*E. coli* antibodies that will react with *E. coli* proteins contaminating a fusion protein sample. Use the following protocols to prepare an immobilized *E. coli* sonicate that can be used to cross-adsorb anti-*E. coli* antibodies. The antibody available from Amersham Biosciences has been cross-adsorbed with *E. coli* BL21 proteins and therefore requires no additional cross-adsorption. The following protocols will treat 240 ml of anti-GST antiserum.

### Preparation of sonicate

#### Reagents required

2× YT medium: Dissolve 16 g tryptone, 10 g yeast extract, and 5 g NaCl in 900 ml of distilled $H_2O$. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 l with distilled $H_2O$. Sterilize by autoclaving for 20 min. To prepare as a solid medium, add 1.2–1.5% agar.

Coupling buffer: Dissolve 29 g NaCl and 8.4 g $NaHCO_3$ in 800 ml distilled, deionized $H_2O$. Adjust the pH to 8.3 with HCl. Bring final volume to 1 l with distilled, deionized $H_2O$. Store at room temperature for no longer than 1 week.

#### Steps

1. Use a single colony of non-transformed *E. coli* cells to inoculate 30 ml of 2× YT medium.
2. Incubate for 12–15 h at 37 °C with vigorous shaking.
3. Transfer 25 ml of the culture into 2 l of pre-warmed 2× YT medium contained in a 4-l flask. Incubate at 37 °C with vigorous shaking until the $A_{600}$ reaches 2.5.
4. Transfer the culture to 42 °C and continue incubating for an additional hour.
5. Transfer the culture to appropriate centrifuge bottles and centrifuge at 7700 × g for 10 min (or 5000 × g for 30 min) at 4 °C to sediment the cells.
6. Discard the supernatant and resuspend the cells in coupling buffer to an $A_{600}$ of 80. The final volume should be approximately 50–75 ml.
7. Transfer the cell suspension to a container appropriate for sonication.
8. Place the container on ice and disrupt the cells using an appropriately equipped sonicator.

Sonication should achieve > 90% cell lysis as determined by microscopic examination.

9. Store the sonicate at -70 °C until needed.

### Preparation of immobilized sonicate

#### Reagents required

CNBr-activated Sepharose 4B

Coupling buffer: Dissolve 29 g NaCl and 8.4 g $NaHCO_3$ in 800 ml distilled, deionized $H_2O$. Adjust the pH to 8.3 with HCl. Bring the final volume to 1 l with distilled, deionized $H_2O$. Store at room temperature for no longer than 1 week.

Acetate buffer: Dissolve 8.2 g sodium acetate, 29 g NaCl in 800 ml distilled, deionized $H_2O$. Adjust the pH to 4.0 with acetic acid. Bring the final volume to 1 l with distilled, deionized $H_2O$. Store at room temperature.

Tris buffer: Dissolve 12.1 g Tris-base, 29 g NaCl in 800 ml distilled, deionized $H_2O$. Adjust the pH to 8.0 with HCl. Bring the final volume to 1 l with distilled, deionized $H_2O$. Store at room temperature.

1× PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3

**Steps**

1. Prepare 15 g of CNBr-activated Sepharose 4B according to the manufacturer's instructions.
2. To the 15 g of prepared CNBr-activated Sepharose 4B, add 45 ml of *E. coli* sonicate (thawed if necessary). Close the container securely and incubate at 4 °C for 12–16 h with gentle shaking (do not use magnetic stirring).
3. Transfer the suspension to an appropriately sized sintered filter funnel attached to a vacuum source. Apply a light vacuum to remove the solution.
4. Wash the medium with 500 ml of coupling buffer by slowly pouring the buffer into the funnel while stirring the medium with a glass stir rod. Apply a light vacuum to remove the solution.
5. Turn off the vacuum. Add 40 ml of acetate buffer. Stir for 5 min with a glass stir rod. Apply a light vacuum to remove the solution.
6. Turn off the vacuum. Add 40 ml of Tris buffer. Stir for 5 min with a glass stir rod. Apply a light vacuum to remove the solution.
7. Repeat steps 5 and 6 for a total of three times.
8. Add 100 ml of 1× PBS to the medium. Stir to suspend.
9. Split the suspension equally into four 100 ml centrifuge bottles. Add 50 ml of 1× PBS to each of the four bottles. Centrifuge at 500 × g for 10 min at 4 °C. Aspirate the supernatant, taking care not to disturb the medium.

## Cross-adsorption of anti-GST antiserum with immobilized *E. coli* sonicate

1. Add 60 ml of crude anti-GST antiserum to each of the four bottles containing the immobilized *E. coli* sonicate.
2. Incubate at room temperature for 1 h with gentle shaking (do not use magnetic stirring).
3. Remove the medium by filtering the serum-medium suspension over Whatman™ 40 ashless filter paper.
4. The supernatant contains the cross-adsorbed anti-GST antiserum, which should be stored at 4 °C.

# Appendix 7

## Converting from linear flow (cm/h) to volumetric flow rates (ml/min) and *vice versa*

When comparing results for columns of different sizes, it is often convenient to express flow as linear flow (cm/h). However, flow is usually measured in volumetric flow rate (ml/min). To convert between linear flow and volumetric flow rate, use one of the following formulas.

### From linear flow (cm/h) to volumetric flow rate (ml/min)

$$\text{Volumetric flow rate (ml/min)} = \frac{\text{Linear flow (cm/h)}}{60} \times \text{column cross-sectional area (cm}^2\text{)}$$

$$= \frac{Y}{60} \times \frac{\pi \times d^2}{4}$$

where

Y = linear flow in cm/h

d = column inner diameter in cm

*Example:*

What is the volumetric flow rate in an XK 16/70 column (i.d. 1.6 cm) when the linear flow is 150 cm/h?

Y = linear flow = 150 cm/h

d = inner diameter of the column = 1.6 cm

$$\text{Volumetric flow rate} = \frac{150 \times \pi \times 1.6 \times 1.6}{60 \times 4} \text{ ml/min}$$

$$= 5.03 \text{ ml/min}$$

### From volumetric flow rate (ml/min) to linear flow (cm/h)

$$\text{Linear flow (cm/h)} = \frac{\text{Volumetric flow rate (ml/min)} \times 60}{\text{column cross-sectional area (cm}^2\text{)}}$$

$$= Z \times 60 \times \frac{4}{\pi \times d^2}$$

where

Z = volumetric flow rate in ml/min

d = column inner diameter in cm

*Example:*

What is the linear flow in an HR 5/5 column (i.d. 0.5 cm) when the volumetric flow rate is 1 ml/min?

Z = Volumetric flow rate = 1 ml/min

d = column inner diameter = 0.5 cm

$$\text{Linear flow} = 1 \times 60 \times \frac{4}{\pi \times 0.5 \times 0.5} \text{ cm/h}$$

$$= 305.6 \text{ cm/h}$$

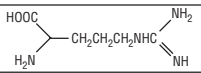
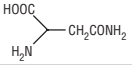
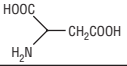
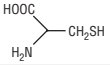
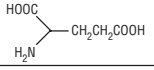
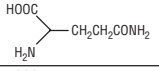
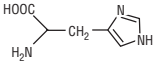
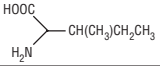
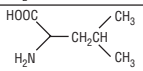
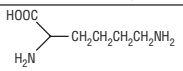
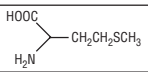
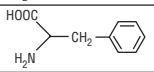
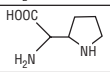
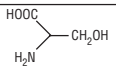
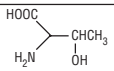
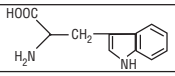
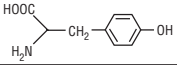
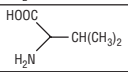
### From ml/min to using a syringe

1 ml/min = approximately 30 drops/min on a HiTrap 1 ml column

5 ml/min = approximately 120 drops/min on a HiTrap 5 ml column

# Appendix 8

## Amino acids table

| Amino acid | Three-letter code | Single-letter code | Structure |
|---|---|---|---|
| Alanine | Ala | A |  |
| Arginine | Arg | R |  |
| Asparagine | Asn | N |  |
| Aspartic Acid | Asp | D |  |
| Cysteine | Cys | C |  |
| Glutamic Acid | Glu | E |  |
| Glutamine | Gln | Q |  |
| Glycine | Gly | G |  |
| Histidine | His | H |  |
| Isoleucine | Ile | I |  |
| Leucine | Leu | L |  |
| Lysine | Lys | K |  |
| Methionine | Met | M |  |
| Phenylalanine | Phe | F |  |
| Proline | Pro | P |  |
| Serine | Ser | S |  |
| Threonine | Thr | T |  |
| Tryptophan | Trp | W |  |
| Tyrosine | Tyr | Y |  |
| Valine | Val | V |  |

| Formula | $M_r$ | Middle unit residue ($-H_2O$) Formula | $M_r$ | Charge at pH 6.0–7.0 | Hydrophobic (non-polar) | Uncharged (polar) | Hydrophilic (polar) |
|---|---|---|---|---|---|---|---|
| $C_3H_7NO_2$ | 89.1 | $C_3H_5NO$ | 71.1 | Neutral | ■ | | |
| $C_6H_{14}N_4O_2$ | 174.2 | $C_6H_{12}N_4O$ | 156.2 | Basic (+ve) | | | ■ |
| $C_4H_8N_2O_3$ | 132.1 | $C_4H_6N_2O_2$ | 114.1 | Neutral | | ■ | |
| $C_4H_7NO_4$ | 133.1 | $C_4H_5NO_3$ | 115.1 | Acidic(-ve) | | | ■ |
| $C_3H_7NO_2S$ | 121.2 | $C_3H_5NOS$ | 103.2 | Neutral | | ■ | |
| $C_5H_9NO_4$ | 147.1 | $C_5H_7NO_3$ | 129.1 | Acidic (-ve) | | | ■ |
| $C_5H_{10}N_2O_3$ | 146.1 | $C_5H_8N_2O_2$ | 128.1 | Neutral | | ■ | |
| $C_2H_5NO_2$ | 75.1 | $C_2H_3NO$ | 57.1 | Neutral | | ■ | |
| $C_6H_9N_3O_2$ | 155.2 | $C_6H_7N_3O$ | 137.2 | Basic (+ve) | | | ■ |
| $C_6H_{13}NO_2$ | 131.2 | $C_6H_{11}NO$ | 113.2 | Neutral | ■ | | |
| $C_6H_{13}NO_2$ | 131.2 | $C_6H_{11}NO$ | 113.2 | Neutral | ■ | | |
| $C_6H_{14}N_2O_2$ | 146.2 | $C_6H_{12}N_2O$ | 128.2 | Basic(+ve) | | | ■ |
| $C_5H_{11}NO_2S$ | 149.2 | $C_5H_9NOS$ | 131.2 | Neutral | ■ | | |
| $C_9H_{11}NO_2$ | 165.2 | $C_9H_9NO$ | 147.2 | Neutral | ■ | | |
| $C_5H_9NO_2$ | 115.1 | $C_5H_7NO$ | 97.1 | Neutral | ■ | | |
| $C_3H_7NO_3$ | 105.1 | $C_3H_5NO_2$ | 87.1 | Neutral | | ■ | |
| $C_4H_9NO_3$ | 119.1 | $C_4H_7NO_2$ | 101.1 | Neutral | | ■ | |
| $C_{11}H_{12}N_2O_2$ | 204.2 | $C_{11}H_{10}N_2O$ | 186.2 | Neutral | ■ | | |
| $C_9H_{11}NO_3$ | 181.2 | $C_9H_9NO_2$ | 163.2 | Neutral | | ■ | |
| $C_5H_{11}NO_2$ | 117.1 | $C_5H_9NO$ | 99.1 | Neutral | ■ | | |

# Appendix 9

## Protein conversion data

| Mass (g/mol) | 1 µg | 1 nmol |
|---|---|---|
| 10 000 | 100 pmol; $6 \times 10^{13}$ molecules | 10 µg |
| 50 000 | 20 pmol; $1.2 \times 10^{13}$ molecules | 50 µg |
| 100 000 | 10 pmol; $6.0 \times 10^{12}$ molecules | 100 µg |
| 150 000 | 6.7 pmol; $4.0 \times 10^{12}$ molecules | 150 µg |

| | | |
|---|---|---|
| 1 kb of DNA | = | 333 amino acids of coding capacity |
| | = | 37 000 g/mol |
| 270 bp DNA | = | 10 000 g/mol |
| 1.35 kb DNA | = | 50 000 g/mol |
| 2.70 kb DNA | = | 100 000 g/mol |

*Average molecular weight of an amino acid = 120 g/mol.*

| Protein | $A_{280}$ for 1 mg/ml |
|---|---|
| IgG | 1.35 |
| IgM | 1.20 |
| IgA | 1.30 |
| Protein A | 0.17 |
| Avidin | 1.50 |
| Streptavidin | 3.40 |
| Bovine Serum Albumin | 0.70 |

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# Additional reading

| | Code No. |
|---|---|
| **GST fusion system** | |
| Data File, "GSTrap FF and Glutathione Sepharose 4 Fast Flow" | 18-1136-89 |
| Data File, "MicroSpin GST Purification Module" | 18-1128-13 |
| Scientific poster, "Rapid purification of GST-fusion proteins from large sample volumes" | 18-1139-51 |
| Application Note, "Efficient, rapid protein purification and on-column cleavage using GSTrap FF columns" | 18-1146-70 |
| Scientific poster, "Purification of GST fusion proteins, on-column cleavage, and sample clean-up" | 18-1150-20 |
| Application Note, "ECL Western and ECL Plus Western blotting" | 18-1139-13 |
| Data File, "GST 96-Well Detection Module" | 18-1128-14 |

*Note: See also the* Life Science News *articles listed in Chapter 7.*

| | |
|---|---|
| **Handbooks** | |
| *Recombinant Protein Handbook: Protein Amplification and Simple Purification** | 18-1142-75 |
| *Antibody Purification Handbook* | 18-1037-46 |
| *Affinity Chromatography Handbook, Principles and Methods* | 18-1022-29 |
| *Expanded Bed Adsorption Handbook, Principles and Methods* | 18-1124-26 |
| *Gel Filtration Handbook, Principles and Methods* | 18-1022-18 |
| *Hydrophobic Interaction Chromatography Handbook, Principles and Methods* | 18-1020-90 |
| *Ion Exchange Chromatography Handbook, Principles and Methods* | 18-1114-21 |
| *Protein Purification Handbook* | 18-1132-29 |
| *Reversed Phase Chromatography Handbook, Principles and Methods* | 18-1134-16 |
| Protein Electrophoresis Technical Manual | 80-6013-88 |

** The* Recombinant Protein Handbook *includes information on recombinant protein expression and purification, including GST fusion proteins. It has less information specifically on the GST Gene Fusion System than this handbook, but includes more general advice and information for working with His-tagged protein purification.*

| | |
|---|---|
| **Column packing** | |
| Column Packing Video PAL | 17-0893-01 |
| Column Packing Video NTSC | 17-0894-01 |
| **Additional reading** | |
| Data File, "HiTrap Benzamidine FF (high sub) and Benzamidine Sepharose 4 Fast Flow" | 18-1139-38 |
| Application Note, "Purification of a labile, oxygen sensitive enzyme for crystallization and 3-D structural determination" | 18-1128-91 |
| Application Note, "Purification of a recombinant phosphatase using preprogrammed generic templates for different chromatographic techniques" | 18-1142-32 |
| ÄKTAdesign Brochure | 18-1129-05 |
| Convenient Purification, HiTrap Column Guide | 18-1129-81 |
| Affinity Chromatography Columns and Media, Product Profile | 18-1121-86 |
| Protein and Peptide Purification, Technique Selection Guide | 18-1128-63 |
| Fast Desalting and Buffer Exchange of Proteins and Peptides | 18-1128-62 |
| Gel Media Guide (electrophoresis) | 18-1129-79 |
| Protein and Peptide Purification Technique Selection | 18-1128-63 |
| *Protein Purification, Principles, High Resolution Methods and Applications,* J.C. Janson and L. Rydén, 1998, 2nd ed. Wiley VCH | 18-1128-68 |

# Ordering information

| Product | Quantity | Code No. |
|---|---|---|
| **Cloning and expression** | | |
| pGEX- 6P-1 | 25 µg | 27-4597-01 |
| pGEX- 6P-2 | 25 µg | 27-4598-01 |
| pGEX- 6P-3 | 25 µg | 27-4599-01 |
| pGEX- 5X-1 | 25 µg | 27-4584-01 |
| pGEX- 5X-2 | 25 µg | 27-4585-01 |
| pGEX- 5X-3 | 25 µg | 27-4586-01 |
| pGEX- 4T-1 | 25 µg | 27-4580-01 |
| pGEX- 4T-2 | 25 µg | 27-4581-01 |
| pGEX- 4T-3 | 25 µg | 27-4583-01 |
| pGEX 2TK | 25 µg | 27-4587-01 |
| All vectors include *E. coli* B21 (*E. coli* BL21 also available separately; see below under Companion products.) | | |
| **Purification** | | |
| **Prepacked columns and media** | | |
| GST MicroSpin Purification Module | 50 purifications | 27-4570-03 |
| GSTrap FF | 2 × 1 ml | 17-5130-02 |
| | 5 × 1 ml | 17-5130-01 |
| | 1 × 5 ml | 17-5131-01 |
| GSTPrep FF 16/10 | 1 × 20 ml | 17-5234-01 |
| Glutathione Sepharose 4 Fast Flow | 25 ml | 17-5132-01 |
| | 100 ml | 17-5132-02 |
| | 500 ml | 17-5132-03 |
| Glutathione Sepharose 4B | 10 ml | 17-0756-01 |
| | 100 ml | 27-4574-01 |
| | 300 ml | 17-0756-04 |
| **Detection** | | |
| GST 96-Well Detection Module | 96 reactions | 27-4592-01 |
| GST Detection Module | 50 reactions | 27-4590-01 |
| Anti-GST Antibody | 0.5 ml | 27-4577-01 |
| **Cleavage enzymes and media for removal of thrombin and Factor Xa** | | |
| PreScission Protease | 500 units | 27-0843-01 |
| Factor Xa | 400 units | 27-0849-01 |
| Thrombin | 500 units | 27-0846-01 |
| HiTrap Benzamidine FF (high sub) | 2 × 1 ml | 17-5143-02 |
| | 5 × 1 ml | 17-5143-01 |
| | 1 × 5 ml | 17-5144-01 |
| Benzamidine Sepharose 4 Fast Flow (high sub) | 25 ml | 17-5123-10 |
| **Companion products** | | |
| **Reagents** | | |
| *E. coli* BL21 (All pGEX vectors include *E. coli* B21) | 1 vial | 27-1542-01 |
| Isopropyl β-D-thiogalactoside (IPTG) | 1 g | 27-3054-03 |
| | 5 g | 27-3054-04 |
| | 10 g | 27-3054-05 |
| Sephaglas BandPrep Kit | 100 average purifications | 27-9285-01 |
| T4 DNA Ligase, FPLC*pure*, Cloned | 200 units | 27-0870-03 |
| | 1000 units | 27-0870-04 |

| Product | Quantity | Code No. |
|---|---|---|
| **Companion products (continued)** | | |
| **Reagents** | | |
| Ready-To-Go T4 DNA Ligase | 50 reactions | 27-0361-01 |
| Adenosine 5'-Triphosphate, 100 mM Solution (ATP) | 25 µmol | 27-2056-01 |
| Ready-To-Go PCR Beads (0.2 ml tubes/plate) | 96 reactions<br>5 × 96 reactions | 27-9553-01<br>27-9553-02 |
| Ready-To-Go PCR Beads (0.5 ml tubes) | 100 reactions | 27-9555-01 |
| *Taq* DNA Polymerase (cloned) | 50 units†<br>250 units†<br>5000 units†<br>10 × 50 units†<br>5 × 250 units†<br>250 units‡<br>4 × 250 units‡<br>10 × 250 units‡<br>25 000 units‡ | T0303Y<br>T0303V<br>T0303X<br>RPN0303Y<br>RPN0303Z<br>27-0798-04<br>27-0798-05<br>27-0798-06<br>27-0798-64 |

*† Supplied with: 10× PCR buffer containing 100 mM Tris-HCl (pH 8.6), 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100. Separate $MgCl_2$ solution (25 mM) also provided.*

*‡ Supplied with: 10× PCR buffer containing 100 mM Tris-HCl (pH 9.0), 500 mM KCl, and 15 mM $MgCl_2$. Separate $MgCl_2$ solution (25 mM) also provided.*

| | | |
|---|---|---|
| *Taq* DNA Polymerase (*T. aquaticus*) | 250 units<br>4 × 250 units<br>10 × 250 units<br>1000 units<br>5000 units<br>10 000 units<br>25 000 units | 27-0799-04<br>27-0799-05<br>27-0799-06<br>27-0799-61<br>27-0799-62<br>27-0799-63<br>27-0799-64 |
| dNTP Set, 100 mM Solutions (dATP, dCTP, dGTP, dTTP) | 4 × 25 µmol<br>4 × 100 µmol<br>4 × 500 µmol | 27-2035-01<br>27-2035-02<br>27-2035-03 |
| pGEX 5' Sequencing Primer | 0.05 $A_{260}$ unit | 27-1410-01 |
| pGEX 3' Sequencing Primer | 0.05 $A_{260}$ unit | 27-1411-01 |
| FlexiPrep Kit | 1 kit | 27-9281-01 |
| GFX *Micro* Plasmid Prep Kit | 1 kit | 27-9601-01 |
| **Columns, media, and equipment** | | |
| MicroPlex 24 Vacuum | 1 system | 27-3567-01 |
| Empty Disposable PD-10 Columns | 50 | 17-0435-01 |
| HR and XK columns (see catalog or web site for range) | | |
| ÄKTAprime and other ÄKTAdesign chromatography systems (see catalog or web site for range) | | |
| MicroSpin G-25 Columns | 50 columns | 27-5325-01 |
| HiTrap Desalting | 5 × 5 ml | 17-1408-01 |
| HiPrep 26/10 Desalting | 1 × 53 ml | 17-5087-01 |
| Disposable PD-10 Desalting Columns | 30 columns | 17-0851-01 |
| CNBr-activated Sepharose 4B | 15 g | 17-0430-01 |
| Superdex Peptide HR 10/30 | 1 × 24 ml | 17-1453-01 |
| Superdex 75 HR 10/30 | 1 × 24 ml | 17-1047-01 |
| Superdex 200 HR 10/30 | 1 × 24 ml | 17-1088-01 |
| HiLoad 16/60 Superdex 30 prep grade | 1 × 120 ml | 17-1139-01 |
| HiLoad 16/60 Superdex 75 prep grade | 1 × 120 ml | 17-1068-01 |
| HiLoad 16/60 Superdex 200 prep grade | 1 × 320 ml | 17-1069-01 |

| Product | Quantity | Code No. |
|---|---|---|
| **Columns, media, and equipment (continued)** | | |
| HiLoad 26/60 Superdex 30 prep grade | 1 × 320 ml | 17-1140-01 |
| HiLoad 26/60 Superdex 75 prep grade | 1 × 120 ml | 17-1070-01 |
| HiLoad 26/60 Superdex 200 prep grade | 1 × 320 ml | 17-1071-01 |
| Ultrospec 1100 *pro* Spectrophotometer | 1 | Inquire |
| **Western blotting** | | |
| Hybond-P | 10 sheets | RPN2020F |
| Hybond ECL | 10 sheets | RPN2020D |
| ECL Western Blotting Detection Reagents | for 1000 $cm^2$ | RPN2109 |
| ECL Plus Western Blotting Detection System | for 1000 $cm^2$ | RPN2132 |

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