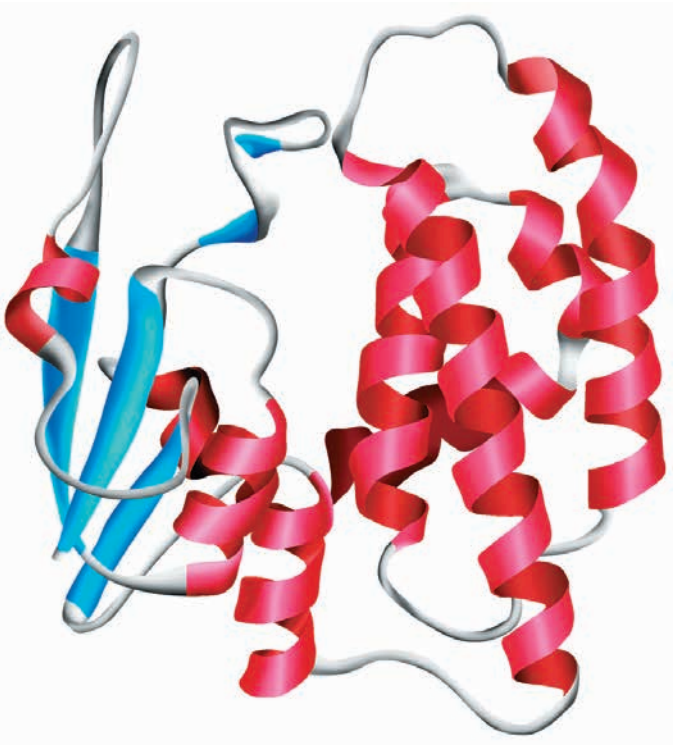
GE Healthcare
Life Sciences



GST Gene Fusion System

Handbook

Handbooks from GE Healthcare

Imaging
Principles and Methods
29-0203-01

GST Gene Fusion System
Handbook
18-1157-58

Affinity Chromatography
Principles and Methods
18-1022-29

Antibody Purification
Handbook
18-1037-46

Ion Exchange Chromatography and Chromatofocusing
Principles and Methods
11-0004-21

Cell Separation Media
Methodology and Applications
18-1115-69

Purifying Challenging Proteins
Principles and Methods
28-9095-31

Isolation of mononuclear cells
Methodology and Applications
18-1152-69

High-throughput Process Development with PreDictor Plates
Principles and Methods
28-9403-58

Protein Sample Preparation
Handbook
28-9887-41

Biacore Assay
Handbook
29-0194-00

MicroCal Calorimetry—Achieving High Quality Data
Handbook
29-0033-51

ÄKTA Laboratory-scale Chromatography Systems
Instrument Management Handbook
29-0108-31

Gel Filtration
Principles and Methods
18-1022-18

Recombinant Protein Purification Handbook
Principles and Methods
18-1142-75

Hydrophobic Interaction and Reversed Phase Chromatography
Principles and Methods
11-0012-69

2-D Electrophoresis using immobilized pH gradients
Principles and Methods
80-6429-60

Microcarrier Cell Culture
Principles and Methods
18-1140-62

Nucleic Acid Sample Preparation for Downstream Analyses
Principles and Methods
28-9624-00

Western Blotting
Principles and Methods
28-9998-97

Strategies for Protein Purification
Handbook
28-9833-31

Spectrophotometry
Handbook
29-0331-82

Biacore Sensor Surface
BR-1005-71

GST Gene Fusion System

Handbook

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

Introduction

The Glutathione S-transferase (GST) Gene Fusion System is a versatile system for the expression, purification, and detection of GST-tagged proteins produced in *Eschericia coli (E. coli).* The system consists of three major components: pGEX plasmid vectors, products for GST purification, and GST detection products. A series of site-specific proteases for GST-tag cleavage complements the system. The pGEX vectors are designed for inducible, high-level intracellular expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST. Expression in *E. coli* yields tagged proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus.

GST occurs naturally as a M_r 26 000 protein that can be expressed in *E. coli* with full enzymatic activity. Tagged 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. The crystal structure of recombinant *S. japonicum* GST from pGEX vectors has been determined and matches that of the native protein. Appendix 1 shows the characteristics of GST, as determined in pGEX-1N.

Purification of GST-tagged proteins is based on the affinity of GST to the glutathione ligand coupled to a matrix. The binding of a GST-tagged protein to the ligand is reversible, and the protein can be eluted under mild, nondenaturing conditions by the addition of reduced glutathione to the elution buffer. The technique thus provides a mild purification process that does not affect a protein's native structure 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 (MCS) on the pGEX plasmids. Tagged proteins can be detected using colorimetric or immunological methods.

Selecting an expression strategy

Selecting an expression strategy begins with choosing the vector best suited for your purpose, taking note of reading frame, cloning sites, and protease cleavage sites. Correct preparation of the insert is important and must take into account the reading frame and orientation, size, and maintenance issues and anticipated expression levels. Finally, the growth conditions must be evaluated in order to optimize expression. Table 1 summarizes the choices to consider when devising a strategy for expression and purification of tagged proteins.

Table 1. Criteria for devising expression and purification strategy

Choice of	Criteria	Comments
Vector	Reading frame	Tagged 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.

continues on following page

Choice of	Criteria	Comments
Host cells	Cloning and maintenance	Choose a strain that transforms well, such as JM109, but not one carrying the recA1 allele.
	Expression	Use BL21, which is protease-deficient and designed to maximize expression of full-length tagged protein.
Growth conditions	Medium, temperature, induction conditions, aeration, positive selection, handling of inclusion bodies	Evaluate different parameters to optimize expression of tagged 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 to 3 ml culture.
		GST MultiTrap™ 4B and GST MultiTrap FF: For convenient high-throughput parallel screening. For use with robotics or manually by centrifugation or vacuum.
		GST SpinTrap™: For small-scale purification from clarified lysates and screening of cell lysates using a standard microcentrifuge.
	Batch/gravity flow	GST GraviTrap™: Provides simple purification with gravity-flow columns. No system needed.
		GST Bulk Kit: For batch purification or gravity-flow column chromatography. Includes reagents for induction, expression, and elution.
		Glutathione Sepharose 4B: For high binding capacity.
		Glutathione Sepharose 4 Fast Flow: For batch or column purification and scale-up due to good flow properties.
		Glutathione Sepharose High Performance: For high resolution and elution of a more concentrated sample (high-performance purification) of GST-tagged proteins.
	Preparative purification	GSTrap™ 4B: For high binding capacity. For use with syringe, peristaltic pump, or chromatography system.
		GSTrap FF: For scale-up due to high flow rates. For use with syringe, peristaltic pump, or chromatography system.
		GSTrap HP: For reliable, high-resolution purification at laboratory scale. For use with a peristaltic pump or chromatography system in preference over syringe.
		GSTPrep™ FF 16/10: Provides additional capacity for scale-up purification. For use with a chromatography system.
Detection method	Type of detection method	GST 96-Well Detection Module for ELISA: Uses 100 µl of sample/well. Designed 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 to 50 µl of sample. Rapid assay; well suited for screening. Gives estimate of relative level of expression.
		SDS-PAGE with Coomassie™ or silver staining: Uses 5 to 10 µl of sample. Provides information on size and percent purity. Detects tagged protein and contaminants.
		Western blot using anti-GST antibody: Uses 5 to 10 µl of sample. Highly specific; detects only GST-tagged protein. Little or no background detectable when using detection systems with optimized concentrations of secondary horseradish peroxidase (HRP)-conjugated antibody.

continues on following page

Choice of	Criteria	Comments
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: Is a GST-tagged protein itself that simplifies the GST-tag removal of the expressed protein. The GST tag can be removed and 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 tagged 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).

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 troubleshooting guides and extensive appendices.

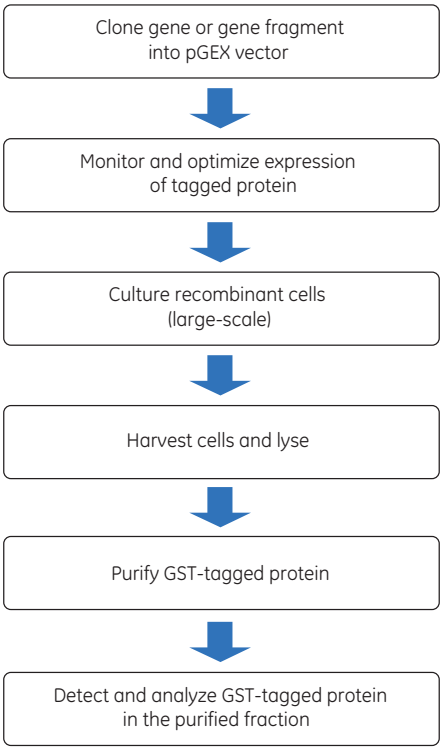


Fig 1. A typical protocol for expression and purification of GST-tagged proteins. On- or off-column cleavage of the GST tag is an option.

Common acronyms and abbreviations

A_{280}	UV absorbance at specified wavelength (in this example, 280 nanometers)
ABTS™	2',2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt
AC	affinity chromatography
AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride
BCA™	bicinchoninic acid
CDNB	1-chloro-2,4-dinitrobenzene
CV	column volume
DMSO	dimethylsulfoxide
DNase	deoxyribonuclease
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
FF	Fast Flow
Gua-HCl	guanidine-HCl
GF	gel filtration
GST	glutathione S-transferase
HP	High Performance
HRP	horseradish peroxidase
IPTG	isopropyl β-D-thiogalactoside
LMW	low molecular weight
MCS	multiple cloning site
MWCO	molecular weight cutoff
MPa	megaPascal
M_r	relative molecular weight
PBS	phosphate buffered saline
pI	isoelectric point, the pH at which a protein has zero net surface charge
psi	pounds per square inch
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene fluoride
r	recombinant, as in rGST and rBCA
RNase	ribonuclease
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMB	3, 3',5,5'-tetramethyl benzidine

Symbols used in this handbook

This symbol indicates general advice to improve procedures or recommend action under specific situations.

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

Chemicals, buffers, and equipment

Experimental protocol

Chapter 1
Cloning the gene or gene fragment into a pGEX expression vector

pGEX vectors

GST-tagged proteins are constructed by inserting a gene or gene fragment into the MCS of one of the 13 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 *lacI*^{q} gene. The *lacI*^{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.

Nine of the vectors have an expanded MCS that contains six restriction sites. The expanded MCS facilitates the unidirectional cloning of cDNA inserts obtained from libraries constructed using many available lambda vectors. pGEX-6P-1, pGEX-6P-2, and pGEX-6P-3 each encode the recognition sequence for site-specific cleavage by PreScission Protease between the GST domain and the MCS. pGEX-4T-1, pGEX-4T-2, and pGEX-4T-3 are derived from pGEX-2T and contain a thrombin recognition site. pGEX-5X-1, pGEX-5X-2, and pGEX-5X-3 are derivatives of pGEX-3X and possess a Factor Xa recognition site (see Table 1.1).

Table 1.1. 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, pGEX-1λT, pGEX-2T	Thrombin
pGEX-5X-1, pGEX-5X-2, pGEX-5X-3, pGEX-3X	Factor Xa
pGEX-2TK Allows detection of expressed proteins by direct labeling *in vitro*	Thrombin

pGEX-2TK has a different MCS from that of the other vectors. pGEX-2TK is designed to allow the detection of expressed proteins by directly labeling the tagged products *in vitro*. 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 labeled using protein kinase and [γ–^{32}P]ATP and readily detected using standard radiometric or autoradiographic techniques. pGEX-2TK is a derivative of pGEX-2T, and its tagged protein can be cleaved with thrombin.

Collectively, the pGEX vectors provide all three translational reading frames beginning with the EcoRI restriction site (Fig 1.1). pGEX-1λT, pGEX-6P-1, pGEX-4T-1, and pGEX-5X-1 can directly accept and express cDNA inserts isolated from λgt11 libraries.

Refer to Appendix 2 for a listing of the control regions of the pGEX vectors. Complete DNA sequences and restriction site data are available from GenBank™. GenBank accession numbers are listed in Appendix 2.

pGEX-1λT (28-9546-56)

Thrombin

Leu Val Pro Arg↓Gly Ser Pro Glu Phe Ile Val Thr Asp
CTG GTT CCG CGT GGA TCC CCG GAA TTC ATC GTG ACT GAC TGA CGA

BamHI EcoRI Stop codons

pGEX-2T (28-9546-53)

Thrombin

Leu Val Pro Arg↓Gly Ser Pro Gly Ile His Arg Asp
CTG GTT CCG CGT GGA TCC CCG GGA ATT CAT CGT GAC TGA CTG ACG

BamHI SmaI EcoRI Stop codons

pGEX-2TK (28-9546-46)

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

BamHI SmaI EcoRI Stop codons

pGEX-4T-1 (28-9545-49)

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

BamHI EcoRI SmaI SalI XhoI NotI Stop codons

pGEX-4T-2 (28-9545-50)

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

BamHI EcoRI SmaI SalI XhoI NotI Stop codon

pGEX-4T-3 (28-9545-52)

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

BamHI EcoRI SmaI SalI XhoI NotI Stop codons

pGEX-3X (28-9546-54)

Factor Xa

Ile Glu Gly Arg↓Gly Ile Pro Gly Asn Ser Ser
ATC GAA GGT CGT GGG ATC CCC GGG AAT TCA TCG TGA CTG ACT GAC

BamHI SmaI EcoRI Stop codons

pGEX-5X-1 (28-9545-53)

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

BamHI EcoRI SmaI SalI XhoI NotI Stop codons

pGEX-5X-2 (28-9545-54)

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

BamHI EcoRI SmaI SalI XhoI NotI Stop codon

pGEX-5X-3 (28-9545-55)

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

BamHI EcoRI SmaI SalI XhoI NotI Stop codons

pGEX-6P-1 (58-9546-48)

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

BamHI EcoRI SmaI SalI XhoI NotI

pGEX-6P-2 (28-9546-50)

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

BamHI EcoRI SmaI SalI XhoI NotI

pGEX-6P-3 (28-9546-51)

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

BamHI EcoRI SmaI SalI XhoI NotI

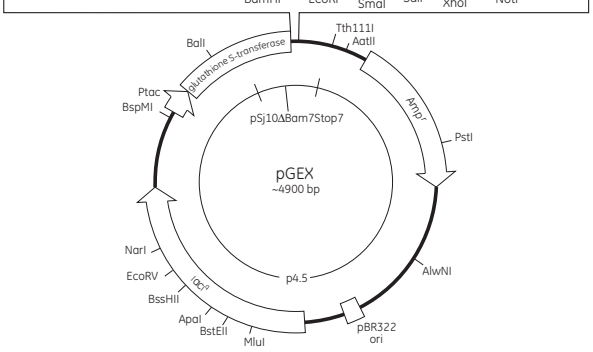


Fig 1.1. Map of the GST vectors showing the reading frames and main features. All 13 vectors have stop codons in all three reading frames downstream from the MCS (not depicted in this map). See Appendix 2 for the control regions of the 13 vectors.

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

Consider which protease and conditions for cleavage are most suitable for your target protein preparation.

pGEX-6P PreScission Protease vectors offer the most efficient method for cleavage and purification of GST-tagged 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 5.

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 tagged proteins. Strains deficient in known cytoplasmic protease gene products, such as Lon, OmpT, DegP, or HtpR, may aid in the expression of tagged proteins by minimizing the effects of proteolytic degradation by the host.

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

E. coli BL21, a strain defective in OmpT protease production, gives high levels of expression of GST-tagged proteins. It is the host of choice for expression studies with GST-tagged proteins. Details on the genotype and handling of *E. coli* BL21 are found in Appendix 1.

A lyophilized (noncompetent) culture of *E. coli* BL21 is available separately.

Use an alternative strain for cloning and maintenance of the vector (e.g., JM109, DH5α). Generally, do not use an *E. coli* strain carrying the *rec*A1 allele for propagation of pGEX plasmids to avoid 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 that follow, 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 1.2 lists the procedures described in this chapter.

Table 1.2. Procedures for cloning the gene or gene fragment into a pGEX expression vector

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

Restriction digestion of pGEX vectors

Reagents required

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

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

Procedure

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 to 10 µl of 10× One-Phor-All Buffer PLUS (OPA$^+$) or buffer supplied with enzyme
   5 to 10 µl of optional components (e.g. BSA, Triton™ X-100, NaCl, etc.)
   10 to 25 units of restriction enzyme
   Water to 50 µl
2. Incubate at the appropriate temperature for 2 to 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 (see Dephosphorylation of linearized pGEX vector, below). 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 illustra GFX PCR DNA and Gel Band Purification Kit.

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
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

Procedure

1. Dilute sufficient calf intestinal alkaline phosphatase for all dephosphorylations to be performed. When diluted, 1 to 2 µl should provide 0.1 unit to the reaction. For dilution, use 10× OPA$^+$ and water to give a final buffer concentration of 1× OPA$^+$.
2. Add 0.1 unit (1 to 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 to 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 to 20 µl of TE buffer.

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

Ligation of insert to pGEX DNA

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

Reagents required

Linearized pGEX DNA	
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	

Procedure

1. Prepare linearized pGEX DNA and insert DNA so that they will 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 to 5 units of T4 DNA ligase, and should be incubated for 1 to 4 h at 10°C.
   - For ligation of blunt ends, the final reaction mix should contain 0.1 to 1 mM ATP (diluted) and 10 to 15 units of T4 DNA ligase, and should be incubated for 2 to 16 h at 4°C to 16°C.
2. Based upon the above considerations in step 1, prepare the following reaction mixture specific for your application:
   1 to 5 µl of linearized pGEX DNA
   1 to 5 µl of insert DNA
   2 µl of 10× One-Phor-All Buffer PLUS (OPA$^+$)
   0.2 µl of 100 mM ATP
   0.5 to 15 units of T4 DNA ligase
   Water to 20 µl
3. Incubate for either 1 to 4 h at 10°C (cohesive ends) or 2 to 16 h at 4°C to 16°C (blunt ends).
4. 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.

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.* (Chung, C. T. *et al.* One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. USA* **86**, 2172–2175 [1989]).

All steps in this procedure should be carried out aseptically.

Reagents required

Use double-distilled water for preparation of all solutions.

Glycerol stock of *E. coli* host strain

LB medium and LB agar plates (prepared fresh):	Combine 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 900 ml of water. Stir to dissolve, and adjust volume to 1 l. Sterilize by autoclaving. To prepare as a solid medium, add 1.2% to 1.5% agar.
TSS (transformation and storage solution) (ice-cold):	For 100 ml: combine 1.0 g of tryptone, 0.5 g of yeast extract, 0.5 g of NaCl, 10.0 g of polyethylene glycol (M_r 3350), 5.0 ml of dimethylsulfoxide (DMSO), and 5.0 ml of $MgCl_2$ (1 M) in 70 ml of sterile distilled water. Stir until dissolved. Adjust the pH to 6.5 with HCl or NaOH. Adjust to 100 ml with sterile distilled water. Sterilize by filtering through a 0.2 µm filter. Store at 4°C. Stable for up to 6 m.
LBG medium (LB + 20 mM glucose):	Dissolve 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl in 900 ml of distilled water. Sterilize by autoclaving. After the medium has cooled to 50°C to 60°C, add 10 ml of sterile 2 M glucose. Adjust to 1 l with sterile distilled water. To prepare as a solid medium, add 1.2% to 1.5% agar.
LBGA 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% to 1.5% agar.
Ampicillin stock solution:	Dissolve 400 mg of the sodium salt of ampicillin in 4 ml of water. Sterilize by filtration and store in small aliquots at -20°C.
Glycerol:	80% in sterile distilled water

Procedure

1. Using sterile technique, streak an *E. coli* host strain (e.g., JM109, BL21, etc.) from a glycerol stock onto an LB agar plate. Incubate overnight at 37°C.
2. Isolate a single colony and inoculate 50 to 100 ml of LB broth. Incubate at 37°C with shaking at 250 rpm. Grow cells to an A_{600} of 0.4 to 0.5.

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

Prewarming 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 to 10 ml) of ice-cold TSS and place on ice.

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

Transformation of competent cells

1. For each ligation reaction, as well as for the uncut vector control and the negative control (nontransformed competent *E. coli* host cells), add 1 ml of freshly prepared competent *E. coli* host cells to separate prechilled 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 water.
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 tube containing 900 µl of LBG medium (prewarmed 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 LBGA plates. Also plate 100 µl of the nontransformed, competent *E. coli* host cells. Incubate the plates at 37°C overnight, then proceed to screening.
6. To prepare a frozen stock culture, add 100 µl of the diluted, transformed cells containing the pGEX DNA to 1 ml of LBGA 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 pipette tip. Store at -70°C.

Screening

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

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.

Screening using illustra PuReTaq Ready-To-Go PCR Beads

Reagents required

illustra PuReTaq Ready-To-Go PCR Beads

pGEX 5' Sequencing Primer (5 pmol/µl)

pGEX 3' Sequencing Primer (5 pmol/µl)

Procedure

1. Resuspend a bead in 25 µl of water 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 micropipette tip to the bacterial colony to be screened and then transfer to the resuspended PCR bead. Pipette 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 micropipette tip onto an LB agar grid plate as a source for the procedures outlined in Small-scale isolation of pGEX DNA and Large-scale isolation of pGEX DNA.

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 to 20 µl by agarose gel electrophoresis.

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 water 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)	

Procedure

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
   Water to 99.5 µl
2. Gently touch a sterile micropipette tip to the bacterial colony to be screened and transfer to the above PCR mixture. Pipette 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 micropipette tip onto an LB agar grid plate as a source for the procedures outlined in Small-scale isolation of pGEX DNA and Large-scale isolation of pGEX DNA.

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 to 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 to 40 µl by agarose gel electrophoresis.

Small-scale isolation of pGEX DNA

Rapid and phenol-free isolation of plasmid DNA is greatly simplified by the use of illustra plasmidPrep Midi Flow Kit or illustra plasmidPrep Mini Spin 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 water.
Isopropanol	
Phenol:	Redistilled phenol saturated with TE buffer containing 8-hydroxy quinoline
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

Procedure

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.

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 illustra plasmidPrep Midi Flow Kit.

Reagents required

2× YTA medium (2× YT + 100 µg/ml ampicillin):	Prepare 2× YT medium by dissolving 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl in 900 ml of distilled water. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 l with distilled water. 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).

Procedure

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 prewarmed to the growth temperature.
3. Grow with aeration to an A_{600} of 1 to 2.
4. Isolate plasmid DNA using illustra plasmidPrep Midi Flow Kit.

Troubleshooting

Problem	Probable cause	Solution
A high basal level of expression is observed	Lack of catabolic repression of the lac promoter.	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 GST-tagged protein is detected in the bacterial lysate	DNA sequence is not cloned in the proper translation frame in the vector.	Check DNA sequence. It is essential that protein-coding DNA sequence is cloned in the proper translation frame in the vector. Cloning junctions should be sequenced to verify that insert is 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 MCS for each pGEX vector is shown in Figure 1.1.

Chapter 2
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 tagged proteins. As previously noted, an alternate strain (e.g, JM109, DH5α) is recommended for maintenance of the plasmid. For all expression studies, however, *E. coli* 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 1), the next step is to optimize expression of tagged proteins. The capability to screen crude lysates from many clones is critical to this process so that optimal expression levels and growth conditions can be readily determined. Once conditions are established, one is ready to prepare large-scale bacterial lysates of the desired clones.

Various methods for the purification of tagged proteins are available (Chapter 3). 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 three purification methods for initial screening. In the first method, a crude lysate suitable for screening from 2 to 3 ml of culture is prepared, using a batch purification method with Glutathione Sepharose 4B. The second method uses GST SpinTrap columns together with a standard microcentrifuge; each column can isolate protein from up to 600 µl of lysate. To further increase the throughput, GST MultiTrap 4B or GST MultiTrap FF, prepacked 96-well filter plates, can be used for parallel purification of multiple samples containing GST-tagged proteins.

The batch method with Glutathione Sepharose 4B and the screening method using multiwell plates and GST SpinTrap columns are presented in this chapter. Additional purification procedures for use of GST SpinTrap and GST multiwell plates are presented in Chapter 3.

Various detection methods are also available for screening lysates for expression of GST-tagged proteins. More information and detailed procedures for several methods can be found in Chapter 4.

After clones expressing the tagged protein have been selected, growth conditions should be evaluated for optimal expression, for example, cell culture media, growth temperature, culture density, and induction conditions. 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). High-level expression of foreign tagged proteins in *E. coli* often results in formation of inclusion bodies, which comprise dense, insoluble aggregates that are failed folding intermediates. Formation of inclusion bodies should be monitored and possibly be avoided by optimizing expression. Handling of inclusion bodies is described in the Challenging Protein Purification Handbook (28-9095-31).

Monitor both cell density (A_{600}) and protein expression for each variable evaluated.

Yield of tagged protein is highly variable, depending on the nature of the tagged protein, the host cell, and the culture conditions. Tagged protein yields can range up to 10 mg/l. Table 2.1 can be used to approximate culture volumes based on an average yield of 2.5 mg/l.

Table 2.1. Estimate of culture volume based on average yield

Tagged 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 lysate	0.25 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 lysate (see Table 2.2). Refer to Chapter 3 for additional purification options and to Chapter 4 for additional detection options.

Table 2.2. Procedures for screening and preparing lysates of tagged proteins

Description	Comments
Screening pGEX recombinants for tagged protein expression	Prepare lysate from 2 to 3 ml of culture; use SDS-PAGE for detection of tagged protein.
Screening using GST MultiTrap 4B and GST MultiTrap FF	For convenient high-throughput parallel screening. Can load unclarified cell lysates.
Screening using GST SpinTrap	For screening of cell lysates. Use a standard microcentrifuge.
Preparation of large-scale bacterial lysate	Prepare lysate from 0.2 to 10 l of culture, then proceed to a purification method in Chapter 3.

Screening pGEX recombinants for tagged protein expression

Sections of this procedure have been adapted with permission from Current Protocols in Molecular Biology, Vol. 2, Supplement 10, Unit 16.7. Copyright © 1990 by Current Protocols.

The following steps may be used prior to large-scale purification to check clones for expression of the desired tagged protein.

Reagents required

Preparation of the medium

Bulk Glutathione Sepharose 4B prepared to 50% slurry as described below in the procedural steps	
PBS:	140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 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 of tryptone, 10 g of yeast extract, and 5 g of NaCl in 900 ml of distilled water. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 l with distilled water. 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 water. 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 to 10 ml aliquots and store at -20°C until needed. Avoid more than five freeze/thaw cycles.

Procedure

Preparation of the medium

Glutathione Sepharose 4B is supplied preswollen in 20% ethanol. The medium is used at a final slurry concentration of 50%.

1. Determine the bed volume 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 Batch purification using Glutathione Sepharose 4B and Batch/column purification using Glutathione Sepharose 4B in Chapter 3).

2. Gently shake the bottle of Glutathione Sepharose 4B to resuspend the medium.
3. Use a pipette 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 5 ml of PBS per 1 ml of slurry (=50% slurry). Invert to mix.

Glutathione Sepharose 4B must be thoroughly washed with 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. Carefully decant the supernatant.
7. Repeat steps 5 and 6 once for a total of two washes. Add PBS to obtain a 50% slurry.

Note: The bed volume is equal to half of the volume of the 50% slurry.

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 to 0.8 (3 to 5 h) with vigorous agitation at 30°C to 37°C.

Lower temperatures, even as low as 20°C, may be used if inclusion bodies are problematic.

3. Induce tagged 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 to 2 h.
5. Transfer 1.5 ml of the liquid cultures to labeled microcentrifuge tubes.
6. Centrifuge in a microcentrifuge for 5 s and discard the supernatants.
7. Resuspend each pellet in 300 µl of ice-cold PBS. Transfer 10 µl of each cell suspension into separate labeled 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 or other mechanical methods available. Alternatively, use chemical lysis buffers for protein extraction.

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 foaming (foaming may denature proteins). Keep on ice.

9. Crude lysates can be screened for the relative level of expression of GST-tagged proteins using the GST substrate CDNB. See GST Detection Module with CDNB enzymatic assay, Chapter 4.
10. 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.
11. 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.
12. Add 100 µl of PBS, vortex briefly, and centrifuge for 5 s to sediment the Glutathione Sepharose 4B beads.
13. Discard the supernatants. Repeat this PBS wash twice for a total of three washes.
14. Elute the tagged protein by adding 10 µl of elution buffer. Suspend the Glutathione Sepharose 4B beads and incubate at room temperature for 5 min.

Centrifuge in a microcentrifuge for 5 min to sediment the Glutathione Sepharose 4B beads, then transfer the supernatants to fresh tubes for SDS-PAGE analysis.

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

Screening using GST MultiTrap FF and GST MultiTrap HP

GST MultiTrap FF and GST MultiTrap 4B are prepacked, disposable 96-well filter plates for reproducible, high-throughput screening of GST-tagged proteins. Typical applications include expression screening of different constructs, screening for solubility of proteins, and optimization of the conditions for small-scale parallel purification. These filter plates simplify the purification screening and enrichment of up to 0.5 mg of GST-tagged proteins/well. After thorough cell disruption, it is possible to apply up to 600 µl of unclarified lysate directly to the wells in the 96-well filter plate without precentrifugation and/or filtration of the sample. It is recommended to extend the duration of mechanical/chemical lysis if the sample is too viscous after lysis; alternatively, include nucleases to disrupt nucleic acids. The GST-tagged proteins are eluted under mild, nondenaturing conditions that preserve protein structure and function. Prepacked GST MultiTrap FF and GST MultiTrap 4B plates give high consistency in reproducibility well-to-well and plate-to-plate. For more information about these products, see Chapter 3.

Screening using GST SpinTrap columns

GST SpinTrap columns are designed for rapid, small-scale purification of GST-tagged proteins using conditions of mild affinity purification. Greater than 90% purity can be achieved in a single step. The columns are suitable for purification of multiple samples in parallel, for example, expression screening experiments or optimization of purification conditions.

Each microspin column contains 50 µl of Glutathione Sepharose 4B, enough to purify up to 500 µg of recombinant GST (rGST) when loading a maximum of 600 µl of sample volume. The capacity will vary with the nature of the GST-tagged protein and the binding conditions used. For more information about this product, see Chapter 3.

Preparation of large-scale bacterial lysates

Reagents required

2× YTA medium (2× YT + 100 µg/ml ampicillin):	Prepare 2× YT medium by dissolving 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl in 900 ml of distilled water. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 l with distilled water. 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 water. Filter-sterilize and store in small aliquots at -20°C.
PBS (ice-cold):	140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3. Store at 4°C.
20% Triton X-100	

Procedure

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

Lower temperatures, even as low as 20°C, may be used if inclusion bodies are problematic.

To ensure adequate aeration, fill flasks to only 20% to 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 tagged protein.

4. Add 100 mM IPTG to a final concentration of 0.1 to 1.0 mM and continue incubation for an additional 2 to 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 pipette, completely suspend the cell pellet by adding 50 µl of ice-cold 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.

Cell disruption is evidenced by partial clearing of the suspension or may be checked by microscopic examination. Avoid foaming as this may denature the tagged protein.

Detection of GST activity can be performed at this stage using one of the methods described in Chapter 4.

9. Add 20% Triton X-100 to a final concentration of 1%. Mix gently for 30 min to aid in solubilization of the tagged 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. These samples can be used to identify the fraction in which the tagged 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 3.

Troubleshooting

The troubleshooting guide below addresses common problems associated with the expression and growth of GST-tagged proteins.

Problem	Possible cause	Solution
No GST-tagged protein is detected in the bacterial lysate	The culture conditions are not optimized.	Cell strain, medium composition, incubation temperature, and induction conditions can all affect yield. Exact conditions will vary for each tagged protein expressed.
	The detection method is not sufficiently sensitive.	Check for expression by immunoblotting, which is generally more sensitive than stained gels. Some tagged 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 tagged proteins in most of these cases. Run an SDS-polyacrylamide gel of induced cells and transfer the proteins to a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. Detect tagged protein using anti-GST antibody. Alternatively, purify the extract using Glutathione Sepharose media prior to SDS-PAGE analysis.
	Experimental error.	Select a new, independently transformed isolate and check for expression.
Most of the tagged protein is in the post-sonicate pellet	Cell disruption is not sufficient during mechanical lysis.	Add lysozyme (0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris-HCl, pH 8.0) prior to sonication.
	Tagged proteins are produced as insoluble inclusion bodies.	Slow the rate of translation by altering the growth conditions: – Lower the growth temperature (within the range of 20°C to 30°C) to improve solubility. – 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. For more information on how to avoid formation of inclusion bodies, solubilization, and refolding, see the Challenging Protein Purification Handbook (28-9095-31).

Chapter 3
Purification of GST-tagged proteins

GST-tagged proteins are easily purified from bacterial lysates by affinity chromatography using glutathione immobilized to a matrix such as Sepharose (Fig 3.1). When applied to the affinity medium, proteins bind to the ligand, and impurities are removed by washing with binding buffer. Tagged proteins are then eluted from the Glutathione Sepharose under mild, nondenaturing conditions using reduced glutathione, to preserve both protein structure and function.

If separation of the cloned protein from its GST tag is desired, the tagged protein can be digested with an appropriate site-specific protease while the tagged protein is bound to Glutathione Sepharose (on-column cleavage). Alternatively, the tagged protein can be digested after elution from the medium (see Chapter 5 for both of these alternatives). Cleavage of the bound tagged protein eliminates an extra step of separating the eluted protein from the GST tag because the GST moiety remains bound to the medium while the fused protein is eluted using wash buffer.

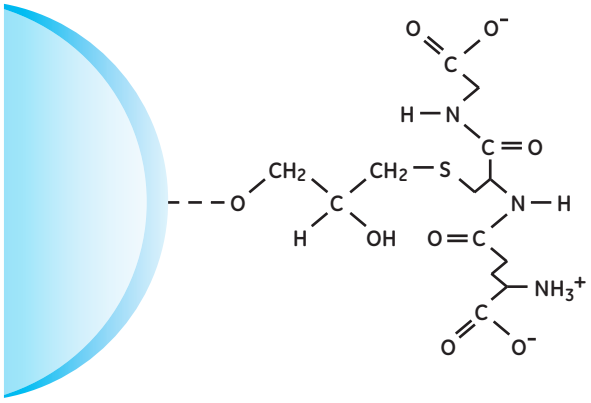


Fig 3.1. Terminal structure of Glutathione Sepharose. Glutathione is attached to Sepharose by coupling to the oxirane group using epoxy-activation. The structure of glutathione is complementary to the GST binding site.

Selecting a GST affinity chromatography product

A variety of affinity chromatography products are available from GE Healthcare that have glutathione immobilized as ligand: Sepharose High Performance (HP), Sepharose 4 Fast Flow (FF), and Sepharose 4B. The Glutathione Sepharose media are available in several formats, ranging from 96-well filter plates, spin columns, and gravity-flow columns to prepacked HiTrap™ and HiPrep™ columns. The media are also available in lab packs (media packs in sizes from 10 ml to 500 ml).

These media vary in their performance parameters, as shown in Table 3.1 and Figure 3.2. As seen in the table, a suitable medium can be selected based on which criteria are most important. For example, for highest capacity and yield choose Glutathione Sepharose 4B, for high eluate concentration choose Glutathione Sepharose HP, and for shortest purification time and for scale-up, choose Glutathione Sepharose FF, which allows a higher flow rate.

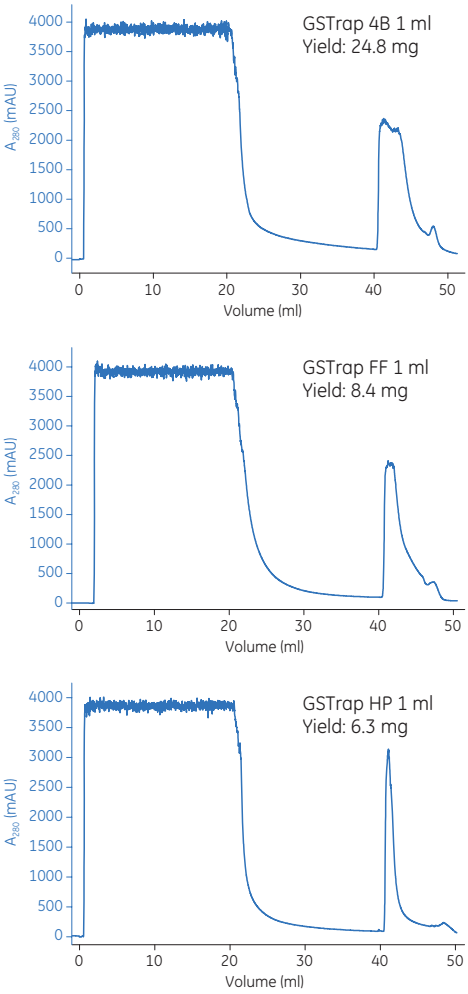


Fig 3.2. Twenty ml of clarified *E. coli* lysate spiked with GST-hippocalcin was loaded on each of three, 1 ml GSTrap columns: GSTrap 4B, GSTrap FF, and GSTrap HP, respectively. The flow rate during sample application was 0.3 ml/min, and during equilibration, wash, and elution it was 1 ml/min.

Table 3.1 summarizes the purification options when using Glutathione Sepharose products, and Figure 3.3 provides a selection guide for their use. Appendix 5 provides tables listing the key characteristics of the bulk media and column/filter plate formats.

Table 3.1. Purification options for GST-tagged proteins using Glutathione Sepharose products

Product	Format or column size	Approx. protein binding capacity[1]	Description[2]	High-throughput screening	Minipreps	Batch/Gravity flow	Syringe compatible	ÄKTA™ system compatible
Glutathione Sepharose High Performance	25 ml 100 ml	7 mg rGST/ml	For high resolution and elution of a more concentrated sample (high-performance purification).	• •				• •
GSTrap HP	5 × 1 ml 1 × 5 ml 5 × 5 ml	7 mg rGST/column 35 mg rGST/column	For reliable, high-resolution purification at laboratory scale. For use with a peristaltic pump or chromatography system in preference over syringe.					• • •
Glutathione Sepharose 4 Fast Flow	25 ml 100 ml 500 ml	10 mg rGST/ml	For batch or column purification and scale-up due to good flow properties.	• • •	• • •	• • •		• • •
GSTrap FF	2 × 1 ml 5 × 1 ml 1 × 5 ml 5 × 5 ml	10 mg rGST/column 50 mg rGST/column	For scale-up due to high flow rates. For use with syringe, peristaltic pump, or chromatography system.				• • • •	• • • •
GSTPrep FF 16/10	1 × 20 ml	200 mg rGST/column	Provides additional capacity for scale-up purification. For use with a chromatography system.					•
GST MultiTrap FF	4 × 96-well filter plate	500 µg rGST/well	For convenient high-throughput parallel screening. Can load unclarified cell lysates. Consistent performance, high reproducibility. For use with robotics or manually by centrifugation or vacuum. For high binding capacity.	•				
Glutathione Sepharose 4B	10 ml 100 ml 300 ml	25 mg horse liver GST/ml	For high binding capacity	• • •	• • •	• • •		
GSTrap 4B	5 × 1 ml 1 × 5 ml 5 × 5 ml	25 mg horse liver GST/column 125 mg horse liver GST/column	For high binding capacity. For use with syringe, peristaltic pump, or chromatography system.				• • •	• • •
GST MultiTrap 4B	4 × 96-well filter plate	500 µg horse liver GST/well	For convenient high-throughput parallel screening. Can load unclarified cell lysates. Consistent performance, high reproducibility. For use with robotics or manually by centrifugation or vacuum.	•				
GST GraviTrap	10 × 2 ml	50 mg horse liver GST/column	Simple purification with gravity-flow columns. No system needed.			•		
GST Bulk Kit	1 kit	25 mg/ml medium	Batch purification or gravity-flow column chromatography. Reagents for induction, expression, and elution of GST-tagged proteins.			•		
GST SpinTrap	50 × 50 µl	500 µg horse liver GST/column	For small-scale purification from clarified cell lysates, also suitable for screening of cell lysates. For use in a standard microcentrifuge.		•			
GST Buffer Kit	1 kit	N/A		•	•	•	•	•

[1] *The binding of GST-tagged proteins depends on size, conformation, and concentration of the protein in the sample loaded. Binding of GST to glutathione is also flow dependent, and lower flow rates often increase the binding capacity. This is important during sample loading. Protein characteristics, pH, and temperature may also affect the binding capacity.*

[2] *NOTE: In every package easy-to-follow instructions are included.*

Contains Glutathione Sepharose High Performance (HP)
Contains Glutathione Sepharose 4 Fast Flow (FF)
Contains Glutathione Sepharose 4B

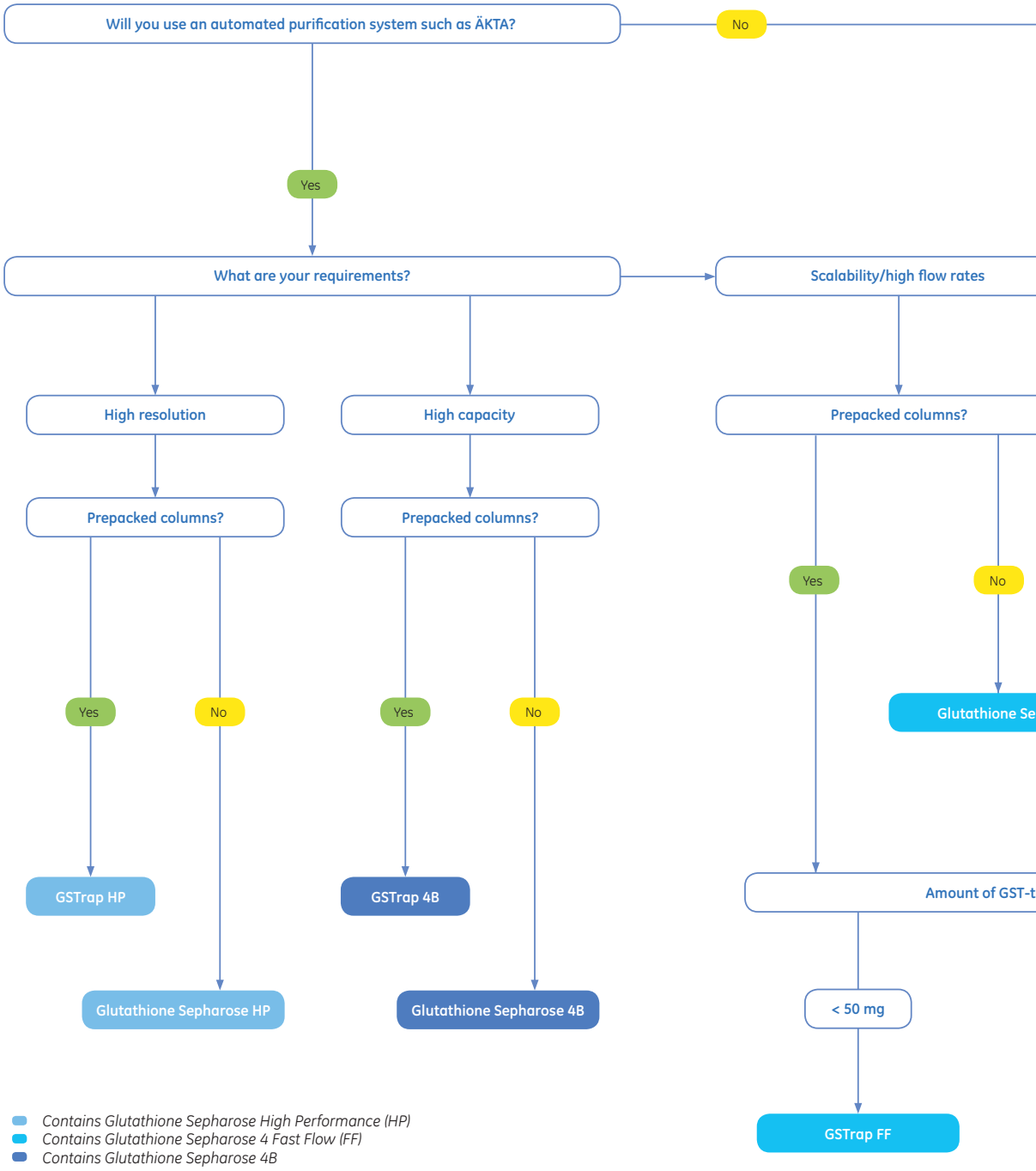
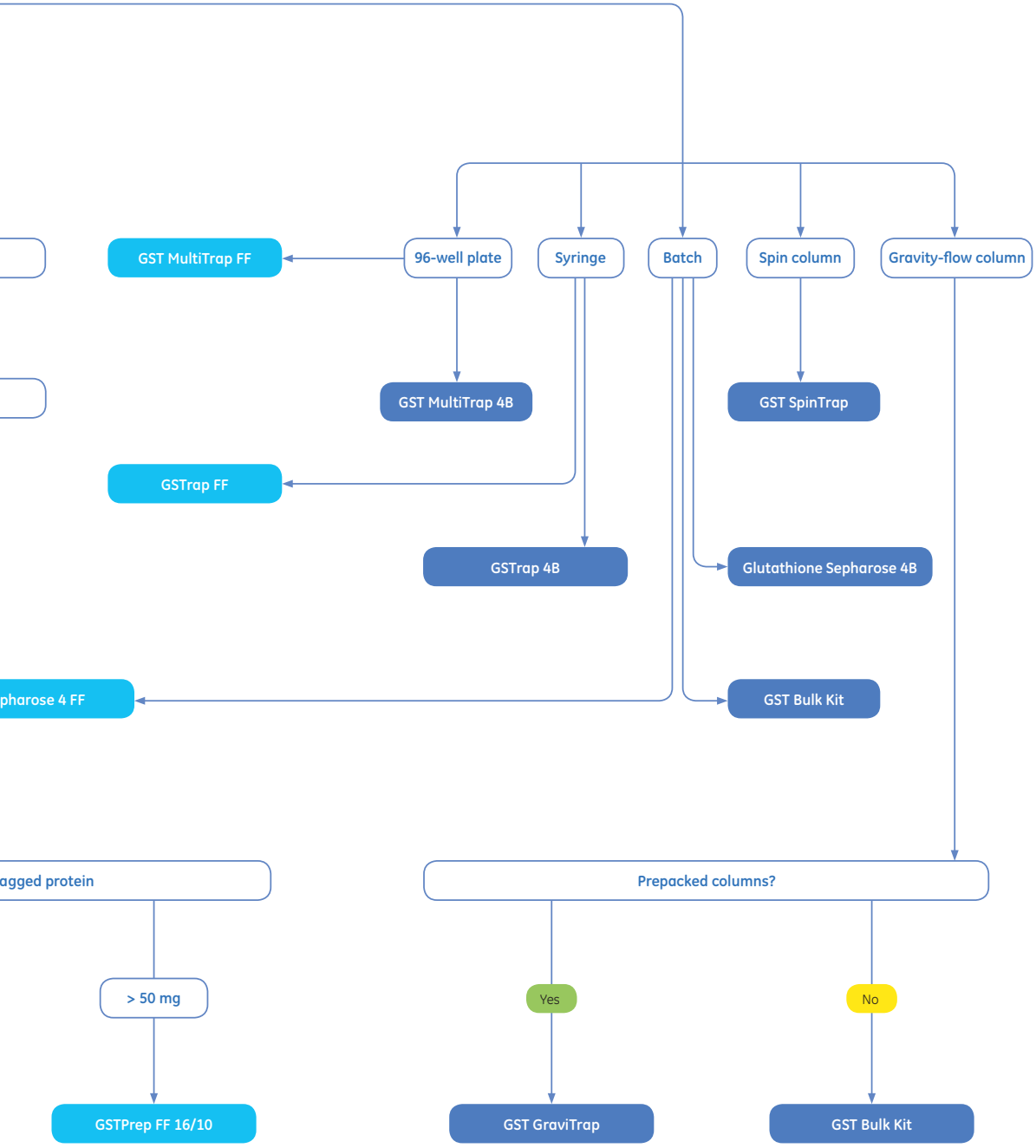


Fig 3.3. Selection guide for Glutathione Sepharose products.


GST MultiTrap FF
96-well plate
Syringe
Batch
Spin column
Gravity-flow column
GST MultiTrap 4B
GST SpinTrap
GSTrap FF
GSTrap 4B
Glutathione Sepharose 4B
GST Bulk Kit
Prepacked columns?
> 50 mg
Yes
No
GSTPrep FF 16/10
GST GraviTrap
GST Bulk Kit

General considerations for purification of GST-tagged proteins

The yield of GST-tagged proteins is highly variable, ranging from 1 mg/l to 10 mg/l. The yield depends on various parameters, such as nature of the tagged protein, the host cell, and the culture conditions used. Table 3.2 shows cell culture, medium, and buffer volumes for obtaining an average yield of 2.5 mg/l.

Table 3.2. Recommendations on reagent volumes and culture size

Tagged protein yield	50 mg	10 mg	1 mg	50 µg
Culture volume	20 l	4 l	400 ml	20 ml
Volume of lysate	1 l	200 ml	20 ml	1 ml
Glutathione Sepharose bed volume	10 ml	2 ml	200 µl	10 µl
Volume of wash buffer	300 ml	60 ml	6 ml	300 µl
Glutathione elution buffer	10 ml	2 ml	200 µl	10 µl

One of the most important parameters affecting the binding of GST-tagged proteins to Glutathione Sepharose media is the flow rate. Because 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. Washing and elution can be performed at slightly higher flow rates. For batch purification, the incubation time should be considered.

Use deionized or double-distilled water and high-grade 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 it with binding buffer to prevent clogging of the column, or perform an efficient lysis treatment, for example, by sonication and homogenization. DNase/RNase can be added to the sample to reduce the size of nucleic acid fragments.

The binding properties of the target protein can be improved by diluting the sample in binding buffer or performing a buffer exchange using a desalting column such as PD-10 Desalting Columns, HiTrap Desalting 5 ml, or HiPrep 26/10 Desalting.

Volumes and times used for elution may vary for different tagged proteins. Further elution with higher concentrations of glutathione (20 to 50 mM) may improve the yield. At concentrations above 15 mM glutathione, the buffer concentration should also be increased to maintain the pH within the range 6.5 to 8. Flowthrough, wash, and eluted fractions from the column should be monitored for detection of the GST-tagged protein using SDS-PAGE, in combination with Western blotting, or CDNB assay (see Chapter 4) if necessary.

After the elution steps, there might still be some remaining tagged protein bound to the medium. Additional elutions may be required.

If monomers are desired, the GST tag should be cleaved off. Gel filtration of the GST-tagged protein will probably give an unstable preparation of GST-tagged monomers that will immediately start to form dimers via GST-GST interactions.

Batch preparation procedures are frequently mentioned in the literature, but the availability of prepacked columns and easily packed Glutathione Sepharose provides faster and 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, giving low yields from the affinity purification step. A more convenient alternative to improve the 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 4. The GST Detection Module contains components that can be used for enzymatic or immunochemical determination of GST-tagged protein concentrations.

The yield of the purified tagged protein can also be estimated by measuring the absorbance at 280 nm or by standard chromogenic methods (e.g., Lowry, bicinchoninic acid [BCA], Bradford, etc.). The Bradford method can be performed in the presence of glutathione, but when a Lowry or BCA type method is used, the glutathione in the purified material must be removed using a desalting column or dialysis against 2000 volumes of PBS to reduce interference with the assay.

Reuse of purification columns and affinity media depends on the nature of the sample and should only be performed with identical samples to prevent possible cross-contamination.

Purification using Glutathione Sepharose High Performance, Glutathione Sepharose 4 Fast Flow, and Glutathione Sepharose 4B

These three media are all used for the purification of GST-tagged recombinant proteins and other S-transferases or glutathione-dependent proteins. They allow mild elution conditions that preserve protein structure and function. All are supplied preswollen in 20% ethanol and are also available in various prepacked formats, as described later in this chapter. See Appendix 5 for the main characteristics of all Glutathione Sepharose media.

Procedures for both batch and column purification of GST-tagged proteins follow.

Table 3.3 shows different procedures for using prepacked columns and media available from GE Healthcare for GST-tagged protein purification.

Table 3.3. Different procedures for purification of GST-tagged proteins

Description	Comments
High-throughput purification using GST MultiTrap	Suitable for expression screening of different constructs, for protein solubility screening, and for optimization of conditions for small-scale parallel purifications.
High-throughput purification using GST SpinTrap columns with a microcentrifuge	For small-scale purification of up to 600 µl of cell lysate. Yield may be increased by repeating the elution step and pooling the eluates.
Manual purification using a GSTrap column with a syringe	One-step purification with yield of up to 25 mg/1 ml column or up to 125 mg/5 ml column, depending on the chromatography medium. Columns may be used in series to increase the yield.
Manual purification using GST GraviTrap	Fast and simple purification using gravity flow, purifying 50 mg of protein/prepacked GraviTrap column.
Simple purification using a GSTrap column with any ÄKTA system	Automatic, preprogrammed application templates for purification of GST-tagged proteins.
Preparative purification using GSTPrep FF 16/10 column with ÄKTA system	One-step preparative purification of up to 200 mg of GST-tagged protein.
Batch purification using Glutathione Sepharose media	Flexible method able to accommodate 50 µl to 10 ml of Glutathione Sepharose 4B.
Batch/column purification using Glutathione Sepharose media	This is a hybrid procedure that binds the protein in batch and elutes in a column. It can be scaled up to purify from 50 µg to 50 mg of GST-tagged protein.
Column packing and purification using Glutathione Sepharose media	The media can be packed in empty columns for scaling-up experiments.

High-throughput screening using GST MultiTrap FF and GST MultiTrap 4B 96-well filter plates

GST MultiTrap FF and GST MultiTrap 4B (Fig 3.4) are prepacked, disposable 96-well filter plates for reproducible, high-throughput screening of GST-tagged proteins. The plates are filled with a defined amount of affinity medium, Glutathione Sepharose 4 Fast Flow (4% highly cross-linked agarose beads) or Glutathione Sepharose 4B (4% agarose beads). Each well contains 500 µl of 10% slurry of the medium in 20% ethanol as storage solution. Typical applications include expression screening of different constructs, screening for solubility of proteins, and optimization of the conditions for small-scale parallel purifications. These filter plates simplify the purification screening and enrichment of up to 0.5 mg of GST-tagged proteins/well. Note that binding depends on the flow and may vary between proteins. Incubation of the sample with medium is necessary, and optimization for optimal binding of the GST-tagged protein is recommended. It is also possible to apply up to 600 µl of unclarified lysate, after thorough cell disruption, directly to each of the wells without precentrifugation and/or filtration of the sample. It is recommended to extend the duration of mechanical/chemical lysis if the sample is too viscous after lysis; alternatively, include nucleases to disrupt nucleic acids. The GST-tagged proteins are eluted under mild, nondenaturing conditions to preserve protein structure and function.

The 96-well filter plates with 800 µl volume capacity per well are made of polypropylene and polyethylene. Characteristics of GST MultiTrap FF and GST MultiTrap 4B are listed in Appendix 5.

Prepacked GST MultiTrap FF and GST MultiTrap 4B plates give high reproducibility well-to-well and plate-to-plate, allowing parallel screening of chromatographic conditions. The repeatability of yield and purity of eluted protein is also high. The plates can be used in automated workflows using robotic systems, or can be operated using centrifugation or by vacuum pressure. The purification protocol included with the plates can easily be scaled for use with the different prepacked formats: GST GraviTrap, GSTrap FF, and GSTrap 4B (1 ml and 5 ml columns) and GSTPrep FF 16/10 (20 ml column) as discussed later in this chapter.

Fig 3.4. GST MultiTrap FF and GST MultiTrap 4B 96-well filter plates.

Sample preparation

Adjust the sample to the binding buffer conditions by diluting it with binding buffer or by buffer exchange.

After thorough cell disruption, it is possible to apply unclarified lysate directly to the wells without precentrifugation or filtration of the sample. The unclarified lysate should be used directly after preparation, as the lysate may precipitate. The unclarified lysate can also be frozen until use but needs to be lysed again before starting the procedure.

Lysis with commercial kits may give large cell debris particles that may interfere with drainage of the wells during purification. This problem can be solved by centrifugation or filtration of the sample before applying to the wells. The binding properties of the target protein can be improved by performing a buffer exchange using a PD MultiTrap G-25 96-well filter plate.

Before starting the procedure, refer to page 32 for general considerations for purification of GST-tagged proteins.

Reagents required

Use high-purity water and chemicals, and pass all buffers through 0.45 µm filters before use.

Binding buffer:	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

1 to 20 mM DTT can be included in the binding and elution buffers to reduce the risk of oxidation of free -SH groups of GST. Oxidation may cause aggregation of the tagged target protein, resulting in lower yield of GST-tagged protein.

Centrifugation procedure

1. Hold the 96-well filter plate horizontally over a sink and carefully peel off the bottom seal.
2. Hold the filter plate upside down and gently shake it to dislodge any medium adhering to the top seal.
3. Reposition the filter plate upright, and place it on a bench, then peel off the top seal.

Note: If the medium has dried out in one or several wells, add buffer to rehydrate. The performance of the medium is not affected.

4. Place the filter plate on top of a collection plate.

Note: Remember to change or empty the collection plate as necessary during the following steps.

5. Centrifuge the filter plates for 2 min at 500 × g, to remove the storage solution from the medium.
6. Add 500 µl of deionized water per well and centrifuge for 2 min at 500 × g.
7. Add 500 µl of binding buffer per well, and mix briefly to equilibrate the medium. Centrifuge for 2 min at 500 × g.
   Repeat entire step once.

Do not apply more than 700 × g for centrifugation.

8. Apply unclarified or clarified lysate (maximum 600 µl per well) to the wells and incubate for 3 min.

Note: For increasing the protein yield, gently shake the filter plate for an effective mixing and/or increase the incubation time.

9. Centrifuge the plate at 100 × g for 4 min or until all the wells are empty. Discard the flowthrough.
10. Add 500 µl of binding buffer per well to wash out any unbound sample. Centrifuge at 500 × g for 2 min. Repeat once or until all unbound sample is removed.

Note: Removal of unbound material can be monitored by measuring A_{280}. An A_{280} value < 0.1 indicates effective removal of the unbound sample.

11. Add 200 µl of elution buffer per well and mix for 1 min.

Note: For higher protein concentration in the eluted sample, the elution volume can be changed between 50 and 100 µl. Smaller volume may give uncertain absorbance values.

12. Change the collection plate and centrifuge at 500 × g for 2 min to collect the eluted protein. Repeat twice or until all of the target protein has been eluted, as monitored by A_{280} measurement.

Note: The collection plate can be changed and collected separately between each elution step to avoid unnecessary dilution of the target protein.

Vacuum procedure

If problems with foaming, reproducibility, or bubbles in the collection plate occur using vacuum, the centrifugation procedure should be considered.

The distance between the bottom of the filter plate and the top of the collection plate in the vacuum manifold should be about 5 mm to avoid cross-contamination in the collection plate. Place an appropriate spacer block into the lower chamber of the vacuum manifold to reduce the distance between the plates.

If a robotic system is used for purification, the vacuum must be adjusted according to methods applicable to the system.

Do not apply a pressure higher than -0.5 bar during vacuum operation.

1. Hold the 96-well filter plate horizontally over a sink and carefully peel off the bottom seal.
2. Hold the filter plate upside down and gently shake it to dislodge any medium adhering to the top seal.
3. Reposition the filter plate upright, and place it on a bench, then peel off the top seal.

Note: If the medium has dried out in one or several wells, add buffer to rehydrate. The performance of the medium is not affected.

4. Place the filter plate on top of a collection plate.

Note: Remember to change or empty the collection plate as necessary during the following steps.

5. Set the vacuum to -0.15 bar. Place the filter plate and collection plate on the vacuum manifold to remove the storage solution from the wells. Turn off the vacuum as soon as all the solution is removed, to avoid cross-contamination in the collection plate.
6. Add 500 µl of deionized water to each well and apply vacuum. Maintain vacuum until all liquid passes through the wells.
7. Add 500 µl of binding buffer to each well to equilibrate the medium. Apply vacuum as in step 5. Repeat once. The filter plate is now ready to use.
8. Apply unclarified or clarified lysate (maximum 600 µl per well) to the wells of the filter plate and incubate for 3 min.

Note: For increasing the protein yield, gently shake the plate and/or increase the incubation time.

9. Vacuum (-0.15 bar) until all liquid passes through the filter plate and until all the wells are empty. Slowly increase the vacuum to -0.30 bar and turn off the vacuum after approximately 5 s. Discard the flowthrough.

Increasing the vacuum too quickly can result in foaming under the filter plate with subsequent cross-well contamination as the consequence.

10. Add 500 µl of binding buffer per well to wash out any unbound sample. Apply vacuum of -0.15 bar as in step 9. Repeat once or until all unbound sample is removed.

Note: Removal of unbound material can be monitored by measuring A_{280}. An A_{280} value < 0.1 indicates effective removal of the unbound sample.

11. Add 200 µl of elution buffer per well and mix for 1 min.

Note: The volume of elution buffer can be varied (50 µl to 100 µl per well), depending on the required concentration of target protein. Smaller volumes may give uncertain absorbance values when measuring A_{280}.

12. Change the collection plate and apply vacuum of -0.15 bar to collect the eluted protein. Repeat twice or until all of the target protein has been eluted, as monitored by measuring A_{280}.

Note: The collection plate can be changed and collected separately between each elution step to avoid unnecessary dilution of the target protein.

Application example

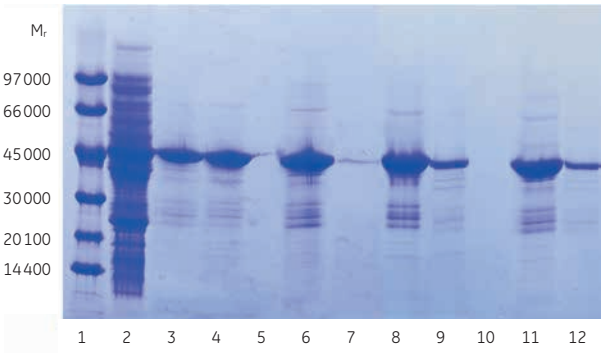
Screening and purification of GST-hippocalcin using GST MultiTrap FF

In this example, the conditions of the binding buffer were optimized for purification of GST-hippocalcin using GST MultiTrap FF. A buffer-screening study to determine optimal buffer conditions for the purification was designed including pH, sodium chloride, glycerol, DTT, and glutathione amount. A comparison between sonication and use of a commercial cell lysis kit was also performed. Factorial design (design-of-experiments) and statistical analysis were performed using MODDE™ software. The different buffer conditions and sample preparation methods were randomly applied and tested on the filter plate.

The screening results showed that the optimal buffer conditions for purifying GST-hippocalcin with the highest yield and purity were: 10 to 20 mM sodium phosphate, 140 to 400 mM NaCl, pH 6.2 to 7.0 (data not shown). Moreover, the results showed that either the commercial cell lysis kit or sonication can be used to lyse *E. coli* without any significant changes in the purification result (Fig 3.5).

The presence of glutathione in the sample and binding buffer (also used as wash buffer) decreased the yield of purified GST-hippocalcin significantly, while the different types of buffer had no effect. Low pH improved the yield whereas high pH (8.0) affected the yield negatively. No significant effect on purity (Fig 3.5) was seen with changing the pH. Additives such as DTT, glycerol, and NaCl did not significantly affect the yield or purity of this particular protein.

Sample:	Unclarified *E. coli* BL21 lysate containing GST-tagged hippocalcin, M_r 43 000
Sample preparation:	Lysis using a commercial cell lysis kit or sonication. Both methods were performed according to standard protocols.
Sample volume:	500 µl
Elution volume:	3 × 200 µl
Binding buffer:	10 to 20 mM PBS; 50 to 100 mM Tris-HCl; pH 6.2 to 8.0; 140 to 400 mM NaCl; 0 to 5 mM DTT; 0% to 5% glycerol; and 0 to 2 mM glutathione. These buffer conditions were randomly tested.
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Elution method:	Centrifugation
Purification protocol:	According to GST MultiTrap instructions, 28-4070-75
Data evaluation:	MODDE software, UV-spectrometry (A_{280}), and SDS-PAGE
96-well filter plate:	GST MultiTrap FF



Lanes

1. LMW markers
2. Start material
3. Sonication, 10 mM PBS, 140 mM NaCl, pH 7.4
4. Cell lysis kit, 10 mM PBS, 140 mM NaCl, pH 7.4
5. Cell lysis kit, 10 mM PBS, 400 mM NaCl, 2 mM glutathione, 5% glycerol, pH 8
6. Sonication, 20 mM PBS, 400 mM NaCl, 5% glycerol, pH 6.2
7. Sonication, 20 mM PBS, 400 mM NaCl, 2 mM glutathione, pH 8
8. Sonication, 50 mM Tris-HCl, 400 mM NaCl, 5% glycerol, pH 6.2
9. Sonication, 50 mM Tris-HCl, pH 8
10. Sonication, 50 mM Tris-HCl, 140 mM NaCl, 2 mM glutathione, 5 mM DTT, 5% glycerol, pH 8
11. Sonication, 100 mM Tris-HCl, 140 mM NaCl, 5 mM DTT, pH 6.2
12. Sonication, 100 mM Tris-HCl, 270 mM NaCl, 1 mM glutathione, 2.5 mM DTT, 2.5% glycerol, pH 7.4

Fig 3.5. Coomassie-stained SDS-polyacrylamide gel (ExcelGel™ 8–18%) of collected eluted GST-hippocalcin fractions from some of the GST MultiTrap FF filter plate wells.

Purification using GST SpinTrap

GST SpinTrap columns are designed for rapid, small-scale purification of GST-tagged proteins using mild conditions of affinity purification. More than 90% purity can be achieved in a single step. The columns are suitable for purification of multiple samples in parallel, for screening experiments, or for optimization of purification conditions.

Each microspin column contains 50 µl of Glutathione Sepharose 4B, enough to purify up to 500 µg of recombinant GST (rGST). The capacity varies with the nature of the GST-tagged protein and the binding conditions used. Refer to Appendix 5 for the main characteristics of GST SpinTrap.

Fig. 3.6. GST SpinTrap is a single-use column for rapid, small-scale purification of GST-tagged proteins.

Sample preparation

For small-scale cultures, freeze/thaw or chemical lysis with commercial kits is recommended for cell lysis. GE Healthcare provides lysis kits for different expression systems: Mammalian Protein Extraction Buffer for mammalian expression systems and Yeast Protein Extraction Buffer Kit for yeast expression systems. For bacteria, several chemical lysis kits are available on the market.

- Adjust the sample to the composition and pH of the binding buffer by additions from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange.
- Pass the sample through a 0.22 µm or a 0.45 µm filter, and/or centrifuge it immediately before sample application. If the sample is too viscous, dilute it with binding buffer to prevent clogging; increase lysis treatment (sonication, homogenization); or add DNase/RNase to reduce the size of nucleic acid fragments. The binding properties of the target protein can be improved by performing a buffer exchange using a desalting column, for example, a PD SpinTrap G-25 column.

Note: Cell culture lysates may also be directly applied to the column without prior clarification.

- Before starting the procedure, refer to page 32 for general considerations for purification of GST-tagged proteins.

The capacity of each SpinTrap column is 500 µg of GST-tagged protein. The following procedure is designed for lysates prepared from 2 to 12 ml of culture, which represents roughly 100 to 600 µl of lysate.

Perform purifications on GST SpinTrap using a standard microcentrifuge. Place the column in a 2 ml microcentrifuge tube to collect the liquid during centrifugation. Use a new 2 ml tube for every step (steps 1 to 5).

Recommended buffers can easily be prepared from the GST Buffer Kit.

Reagents required

Binding buffer:	10 mM PBS, pH 7.4 (10 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4)
Elution buffer:	50 mM Tris-HCl, 10 to 20 mM reduced glutathione, pH 8.0

1 to 20 mM DTT may be included in the binding and elution buffers to reduce the risk of oxidation of free -SH groups on GST, which may cause aggregation of the tagged target protein, resulting in lower yield of GST-tagged protein.

Procedure

1. Invert and shake the column repeatedly to resuspend the medium.
2. Loosen the top cap one-quarter of a turn and twist/break off the bottom closure.
3. Place the column in a 2 ml microcentrifuge tube and centrifuge for 30 s at 100 × g (approx. 1500 rpm in an Eppendorf™ 5415R, 24-position fixed-angle rotor) to remove the storage liquid.
4. Remove and discard the top cap. Equilibrate the column by adding 600 µl of binding buffer. Centrifuge for 30 s at 100 × g.
5. Add the sample (see Sample preparation). A suitable sample volume is 600 µl per column.
6. Mix gently at room temperature for 5 to 10 min to ensure optimal binding of GST-tagged proteins to the Glutathione Sepharose 4B medium. Centrifuge for 30 s at 100 × g.
7. Wash with 600 µl of binding buffer. Centrifuge for 30 s at 100 × g. Repeat the wash step once.
8. Elute the target protein twice with 200 µl of elution buffer. Centrifuge for 30 s at 100 × g, and collect the purified sample. The first 200 µl will contain the majority of the target protein.

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

It is possible to make several sample applications as long as the binding capacity of the column is not exceeded.

Application example

Rapid screening of GST-tagged proteins using GST SpinTrap

E. coli transformants containing cDNA expressing a GST-tagged human myoglobin were randomly selected, expressed, and purified using GST SpinTrap columns. A human myoglobin cDNA was ligated to linearized pGEX-5X-1 and used to transform *E. coli* BL21 cells. Twenty-four randomly selected colonies were used to inoculate 3 ml cultures, which were grown overnight. Expression was induced with IPTG for 2 h. Lysates were prepared from 1.5 ml aliquots of each culture by a freeze-thaw procedure and applied to GST SpinTrap columns. Aliquots of each reduced glutathione eluate were applied to an SDS gel for analysis by SDS-PAGE (Fig 3.7). The results showed that 7 of the 24 transformants expressed the GST- tagged myoglobin.

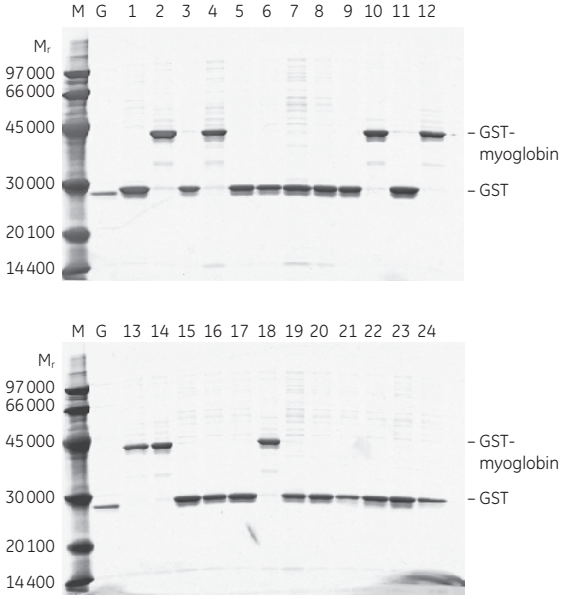


Fig 3.7. SDS-PAGE analysis of eluates from a screening of 24 randomly selected *E. coli* transformants containing cDNA expressing GST-tagged human myoglobin. M = LMW-SDS Marker Kit. G = purified rGST. Lanes 1 to 24 contain products eluted from the GST SpinTrap columns using reduced glutathione.

Gravity-flow purification using GST GraviTrap

GST GraviTrap is designed for fast and simple purification of GST-tagged proteins using gravity flow. The column is prepacked with 2 ml of Glutathione Sepharose 4B, enough to purify up to 50 mg of GST. The capacity varies with the nature of the tagged protein and the binding conditions used. Refer to Appendix 5 for the main characteristics of GST GraviTrap.

GST GraviTrap columns are delivered in a package that can be converted into a column stand (Workmate). The plastic tray in this package can be used to collect liquid waste. When handling volumes above 10 ml, connecting LabMate reservoir to the column increases the loading capacity to approximately 35 ml. For optimal performance, use GST GraviTrap with buffers prepared from GST Buffer Kit.

Fig 3.8. Purifying GST-tagged proteins with GST GraviTrap is a simple four-step procedure.

Sample preparation

Before starting the procedure, refer to page 32 for general considerations for purification of GST-tagged proteins.

Adjust the sample to the binding buffer conditions by diluting the sample with binding buffer or by buffer exchange.

Pass the sample through a 0.22 µm or a 0.45 µm filter, and/or centrifuge it immediately before sample application. If the sample is too viscous, dilute it with binding buffer to prevent clogging; improve lysis treatment by sonication and/or homogenization; or add DNase/RNase to reduce the size of nucleic acid fragments. The binding properties of the target protein can be improved by diluting the sample in binding buffer or performing a buffer exchange using a desalting column such as PD-10 Desalting Columns, PD MidiTrap columns, or PD MiniTrap columns.

Note: Cell culture lysates may also be directly applied to the column without prior clarification.

Reagents required

Recommended buffers can easily be prepared from GST Buffer Kit.

Binding buffer:	10 mM PBS, pH 7.4 (10 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4)
Elution buffer:	50 mM Tris-HCl, 10 to 20 mM reduced glutathione, pH 8.0

1 to 20 mM DTT may be included in the binding and elution buffers to reduce the risk of oxidation of free -SH groups on GST. The oxidation may cause aggregation of the target protein, resulting in lower yield of the GST-tagged protein.

Procedure

1. Cut off the bottom tip. Remove the top cap and pour off excess liquid. Place the column in the Workmate column stand. If needed, mount LabMate (funnel) on the top of the column.
2. Equilibrate the column with 10 to 20 ml of binding buffer to remove the storage solution.
3. Apply the sample to the column.
4. Wash the column with 2 to 10 ml of binding buffer.
5. Elute the bound material with 10 ml of elution buffer, and collect in 1 to 2 ml fractions.

Purification using GST HiTrap 1 ml and 5 ml columns: GSTrap 4B, GSTrap FF, and GSTrap HP

GSTrap affinity columns are specially designed 1 ml and 5 ml HiTrap columns packed with Glutathione Sepharose High Performance, Glutathione Sepharose 4 Fast Flow, or Glutathione Sepharose 4B media. Refer to the selection guide in Table 3.1 for purification options using these columns and to Appendix 5 for a summary of their characteristics.

Sample application, washing, and elution can be performed using a syringe with a supplied connector, a peristaltic pump, or a liquid chromatography system such as ÄKTA (see Table 3.4 for equipment choices). For easy scale-up, two to three columns can be connected to each other in series simply by screwing the end of one column into the top of the next. Figure 3.9 shows a schematic representation of the simple steps needed for successful purification using GSTrap columns.

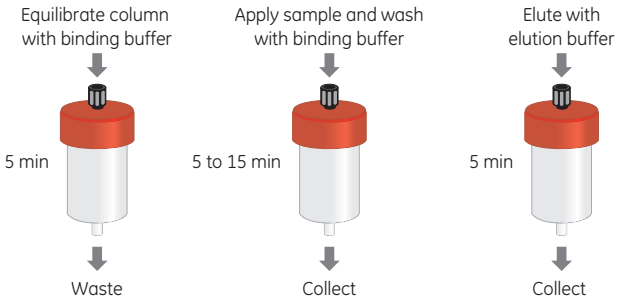


Fig 3.9. Simple purification of GST-tagged proteins using a GSTrap column.

GSTrap columns are made of polypropylene, which is biocompatible and noninteractive with biomolecules. The top and bottom frits are manufactured from porous polyethylene. The columns are delivered with a stopper on the inlet and a snap-off end on the outlet. Each package includes all necessary components for connection of the columns to different types of equipment. Note that GSTrap columns cannot be opened or refilled.

GSTrap columns are directly compatible with existing purification protocols for GST-tagged proteins, including on-column proteolytic cleavage methods. If removal of the GST moiety is required, the tagged protein can be digested with an appropriate site-specific protease while it is bound to the medium or, alternatively, after the elution (see Chapter 5).

One of the GST media, Glutathione Sepharose 4 Fast Flow, is also available in prepacked 20 ml GSTPrep FF 16/10 columns (see Preparative purification using GSTPrep FF 16/10 column, page 51). All three GST media are available in bulk packs (varying from 10 to 500 ml) for packing in empty column of the user's choice.

The chromatography media are very stable and the purification process very reproducible. This can be seen from the results of an experiment in which *E. coli* homogenates containing GST-hippocalcin (M_r 43 000) were repeatedly purified 10 times on the same column without cleaning between runs. Figure 3.10 shows the results of these experiments. The 10 overlaid chromatograms (Fig 3.10A) show a near perfect match, with little or no variation in binding capacity and indicating stability of the medium. SDS-PAGE analysis (Fig 3.10B) confirmed no changes in purity or binding capacity after these 10 runs.

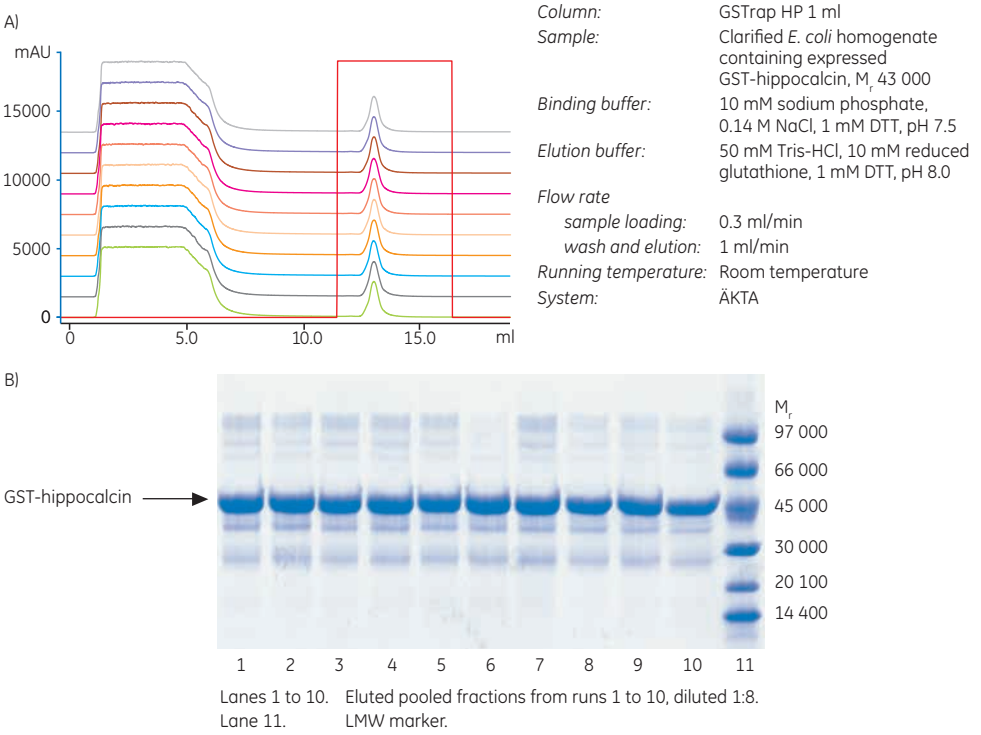


Fig 3.10. (A) Confirmation of the stability of Glutathione Sepharose High Performance prepacked in 1 ml GSTrap HP columns. Chromatographic overlay of 10 repetitive purifications. (B) Coomassie-stained nonreduced SDS-polyacrylamide gel (ExcelGel SDS Gradient 8–18%) of pooled fractions from repetitive purification runs shown in (A).

Before starting the procedure, refer to page 32 for general considerations for purification of GST-tagged proteins.

Reagents required

Binding buffer:	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

1 to 20 mM DTT may be included in the binding and elution buffers to reduce the risk of oxidation of free -SH groups on GST, which may cause aggregation of the tagged target protein, resulting in lower yield of GST-tagged protein.

Procedure

1. Fill the syringe or pump tubing with binding buffer. Remove the stopper and connect the column to the syringe (use the connector supplied), laboratory pump, or chromatographic system "drop to drop" to avoid introducing air into the column.
2. Remove the snap-off end at the column outlet.
3. Equilibrate the column with 5 column volumes of binding buffer.
4. Apply the pretreated sample using a syringe fitted to the Luer connector or by pumping it onto the column. For best results, use a flow rate of 0.2 to 1 ml/min (1 ml column) and 0.5 to 5 ml/min (5 ml column) during sample application[1].
5. Wash with 5 to 10 column volumes of binding buffer or until no material appears in the effluent. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for washing.

Optional: Collect the flowthrough (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 check for any loss of unbound target protein.

6. Elute with 5 to 10 column volumes of elution buffer. Maintain a flow rate of 0.2 to 1 ml/min (1 ml column) and 0.5 to 5 ml/min (5 ml column) for elution.
7. After elution, regenerate the column by washing it with 3 to 5 column volumes of binding buffer. The column is now ready for a new purification.

[1] *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.*

Fig 3.11. Using a GSTrap column with a syringe. A) Prepare buffers and sample. Remove the column's top cap and snap off the end. B) Load the sample and begin collecting fractions. C) Wash and elute, continuing to collect fractions.

Volumes and times used for elution may vary among tagged proteins. Additional elutions with higher concentrations of glutathione may be required. Flowthrough, wash, and eluted material from the column should be monitored for GST-tagged proteins using SDS-PAGE in combination with Western blotting, if necessary.

Flow rate will affect the binding and elution of GST-tagged proteins to the chromatography medium. Due to the relatively slow binding kinetics between GST and glutathione, it is important to keep the flow rate low for maximum binding capacity. Protein characteristics, pH, and temperature are other factors that may affect the binding capacity. However, when working with sensitive proteins, higher flow rates are recommended to minimize purification time. Combining two or three columns in tandem would increase residence time for sample passing the column, thus allowing higher flow rates to be used.

The reuse of GSTrap HP, FF, or 4B columns depends on the nature of the sample and should only be performed with identical samples to prevent cross-contamination.

Simple purification using a GSTrap column with ÄKTAprime™

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

The preprogrammed method template for purification of GST-tagged proteins using a GSTrap FF column is shown in Figure 3.12. The method provides a standard purification protocol that can be followed exactly or modified as required. Typical results are shown in Figure 3.14.

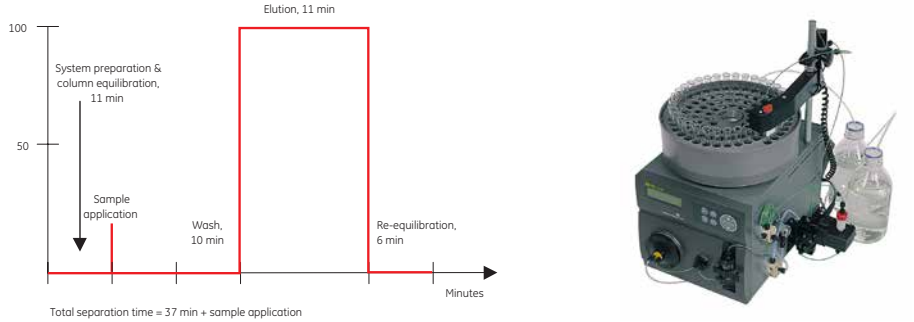


Fig 3.12. Purification of GST-tagged proteins using a GSTrap FF column and ÄKTAprime.

Before starting the procedure, refer to page 32 for general considerations for purification of GST-tagged proteins.

Reagents required

Binding buffer:	20 mM sodium phosphate, 0.15 M NaCl, pH 7.3 (or PBS, the buffer used in the manual purification procedure, page 44)
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

Procedure

Prepare at least 500 ml of each buffer.

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

Connecting the column.

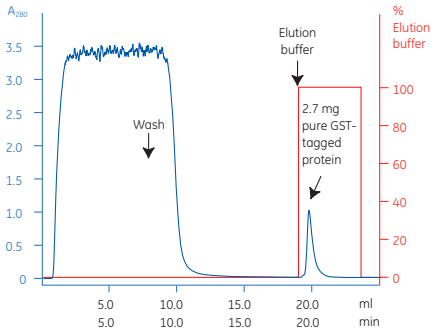
Preparing the fraction collector.

Fig 3.13. Typical procedures when using ÄKTAprime.

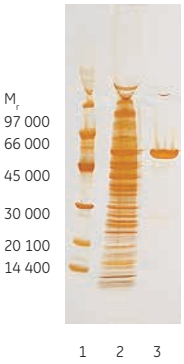
Typical results using ÄKTAprime for purification of GST-tagged protein are shown in Figure 3.14.

A)

Column:	GSTrap FF 1 ml
Sample:	8 ml of cytoplasmic extract (0.8 g of cell paste) from *E. coli* expressing a GST-tagged 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 column volumes (CV) of binding buffer, 8 ml of sample, 10 CV of binding buffer, 5 CV of elution buffer, 5 CV of binding buffer



B)



Lanes

1. Low Molecular Weight (LMW) Calibration kit
2. Start material (5 µl of sample + 35 µl of sample loading buffer, 10 µl applied)
3. Eluted GST-tagged protein (5 µl of sample + 35 µl of sample loading buffer, ≥ 10 µl applied)

Fig 3.14. Purification of GST-tagged protein on a GSTrap FF 1 ml column A) Chromatogram. B) SDS-PAGE on ExcelGel SDS Gradient 8–18% using Multiphor™ II (GE Healthcare) followed by silver staining.

Application examples

1. One-step purification of GST-hippocalcin using 1 ml and 5 ml GSTrap HP columns

In this study, 5 ml and 25 ml of *E. coli* homogenate containing GST-hippocalcin was loaded on GSTrap HP 1 ml and 5 ml columns, respectively. Figure 3.15A–B shows the chromatograms from the two runs. The amount of protein in the eluted peaks was calculated as 6.5 mg and 39.7 mg, respectively.

The SDS-PAGE analysis also showed that free GST has been expressed.

Columns:	GSTrap HP 1 ml and GSTrap HP 5 ml
Sample:	Clarified *E. coli* homogenate containing expressed GST-hippocalcin, M_r 43 000
Sample volumes:	GSTrap HP 1 ml: 5 ml 5 ml: 25 ml
Binding buffer:	10 mM sodium phosphate, 0.14 M NaCl, pH 7.4
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Flow rate *sample loading:*	GSTrap HP 1 ml: 0.3 ml/min 5 ml: 1.6 ml/min
wash and elution:	GSTrap HP 1 ml: 1 ml/min 5 ml: 4 ml/min
System:	ÄKTA

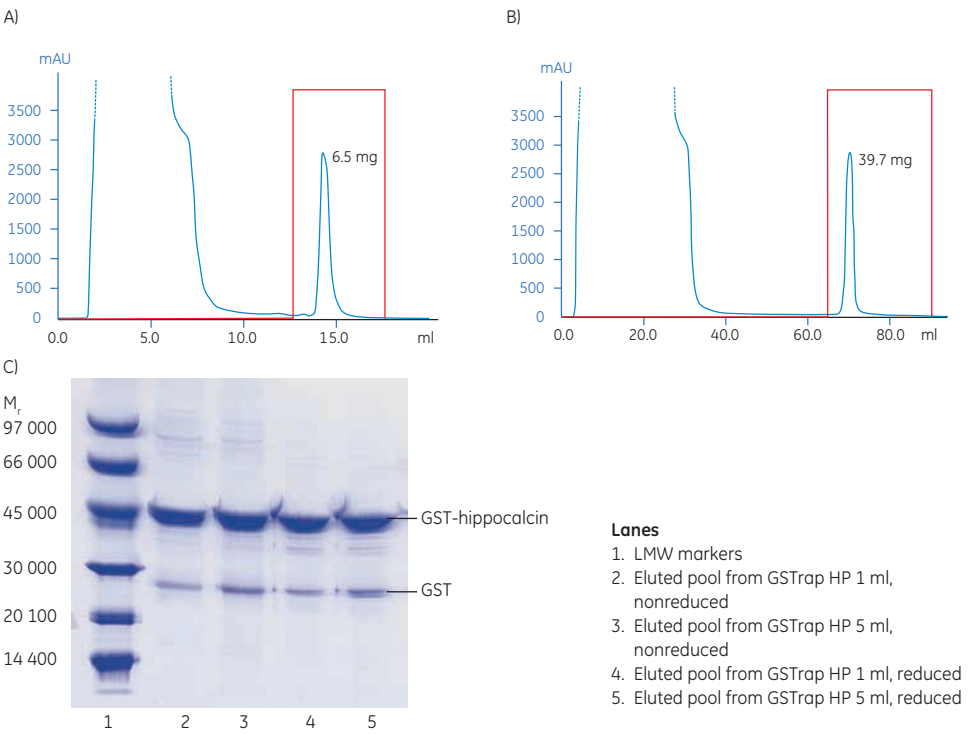


Fig 3.15. Scale-up from (A) GSTrap HP 1 ml to (B) GSTrap HP 5 ml. (C) Coomassie-stained reduced and nonreduced SDS-polyacrylamide gel (ExcelGel 8–18%) of fractions.

2. Fast purification using GSTrap FF 1 ml and 5 ml columns

A GST-tagged protein was purified from 8 ml and 40 ml of a clarified cell lysate using GSTrap FF 1 ml and 5 ml columns, respectively, on an ÄKTA system. Samples were applied to columns pre-equilibrated with PBS (pH 7.3). After washing the columns with 10 column volumes of PBS, GST-tagged protein was eluted using reduced glutathione (Fig 3.16). Each run was completed in 25 min. Analysis by SDS-PAGE indicated the isolation of highly pure GST-tagged protein (not shown). Yields of tagged proteins were 2.7 mg from GSTrap FF 1 ml and 13.4 mg from GSTrap FF 5 ml.

Sample:	8 ml of clarified *E. coli* lysate
Column:	GSTrap FF 1 ml
Binding buffer:	PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.3)
Elution buffer:	10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0
Flow rate:	1 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
System:	ÄKTA

Sample:	40 ml of clarified *E. coli* lysate
Column:	GSTrap FF 5 ml
Binding buffer:	PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.3)
Elution buffer:	10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0
Flow rate:	5 ml/min
Chromatographic procedure:	4 column volumes (CV) binding buffer, 40 ml sample 10 CV binding buffer, 5 CV elution buffer, 5 CV binding buffer
System:	ÄKTA

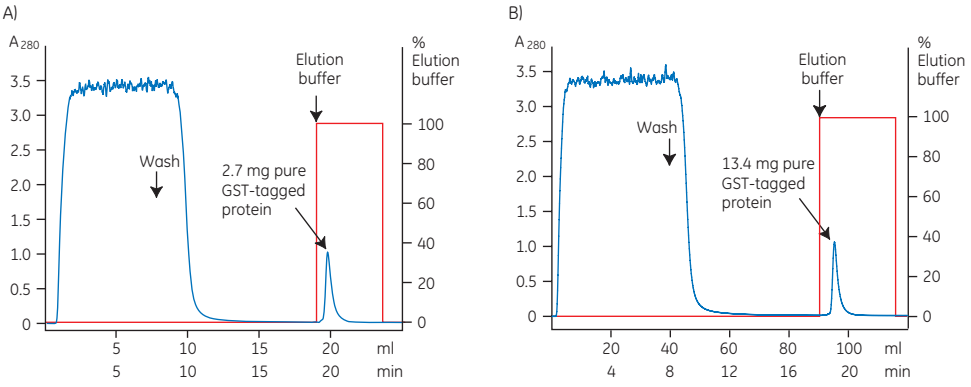


Fig 3.16. Purification of a GST-tagged protein using GSTrap FF 1 ml and 5 ml columns. Cytoplasmic extract (8 and 40 ml) from *E. coli* expressing a GST-tagged protein were applied to GSTrap FF 1 ml (A) and GSTrap FF 5 ml (B), respectively.

3. Two-step, automated purification using GSTrap 4B 1 ml and ÄKTAxpress™

A two-step, automated purification of GST-hippocalcin from clarified *E. coli* lysate was performed on ÄKTAxpress. In the first step, a GSTrap 4B 1 ml column was used for an affinity chromatography capture step, and in the second step a HiLoad™ 16/60 Superdex™ 200 pg column was used for the gel filtration chromatography polishing step.

Lysis of *E. coli* containing GST-hippocalcin was performed enzymatically, followed by sonication. The lysate was clarified by centrifugation and filtration, and 5 ml of the clarified lysate was loaded on the 1 ml GSTrap 4B column. Reducing agent (DTT) was included in both sample solution and buffers. ÄKTAxpress enabled automated loading of eluted fractions of the target protein from the capture step (GSTrap 4B) onto the HiLoad column. Chromatograms from the automated two-step purification and SDS-PAGE of the eluted pool of target protein are shown in Figure 3.17. Two peaks were obtained after the gel filtration step: one small and one large. Both peaks contained GST-hippocalcin. The large peak seemed to be the dimer of GST-hippocalcin. The small peak is possibly a larger aggregate of GST-hippocalcin. The high purity of the GST-hippocalcin in the major fraction is shown in Figure 3.17 C.

Yield of the eluted GST-hippocalcin, determined by absorbance at 280 nm (read from the chromatograph), was 6.4 mg.

The results of this application show the benefit of using an automated two-step purification for increasing the purity of GST-hippocalcin.

Columns:	GSTrap 4B, 1 ml HiLoad 16/60 Superdex 200 pg, 120 ml
Sample:	Clarified *E. coli* lysate containing expressed GST-hippocalcin, M_r 43 000
Sample volume:	5 ml loaded on GSTrap 4B
Binding buffer:	10 mM sodium phosphate, 140 mM NaCl, 20 mM DTT, pH 7.4
Elution buffer:	50 mM Tris-HCl, 20 mM glutathione, 20 mM DTT, pH 8.0
Buffer for GF:	10 mM sodium phosphate, 140 mM NaCl, 20 mM DTT, pH 7.4
Flow rate:	Sample loading, 0.3 ml/min, wash and elution, 1 ml/min (GSTrap 4B), 1.5 ml/min (HiLoad 16/60 Superdex 200 pg)
System:	ÄKTAxpress

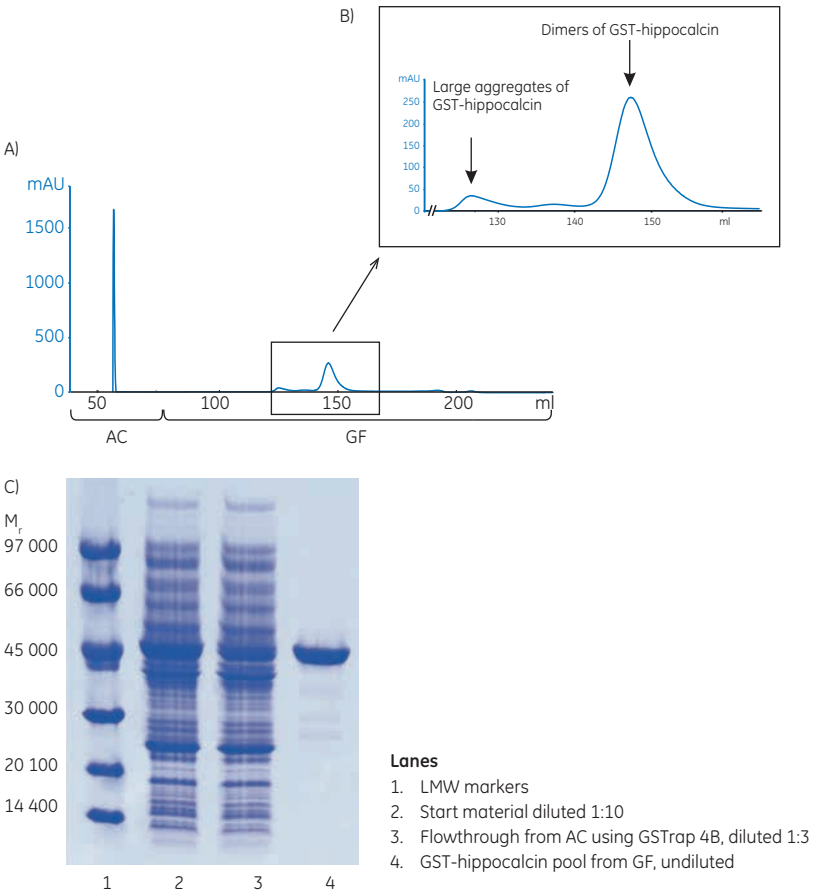


Fig 3.17. (A) Purification of GST-hippocalcin from *E. coli* lysate using an automated two-step purification on ÄKTAxpress. AC = affinity chromatography. GF = gel filtration. (B) Enlargement of the peak from the GF step showing large aggregates and dimers of purified GST-hippocalcin. (C) SDS-PAGE (ExcelGel 8–18%) showing final purity check of GST-hippocalcin.

Preparative purification using GSTPrep FF 16/10 column

GSTPrep FF 16/10 columns are based on the 20 ml HiPrep column design, ready to use for easy, one-step preparative purification of GST-tagged 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, the columns can be connected in series.

Fig 3.18. GSTPrep FF 16/10 column.

The column is made of polypropylene, which is biocompatible and noninteractive with biomolecules. Purification can be easily performed using a chromatography system such as ÄKTA. Refer to Table 3.4 for a selection guide to purification equipment, Table 3.1 for a summary of GSTPrep FF 16/10 purification options, and Appendix 5 for a list of column characteristics. Glutathione Sepharose 4 Fast Flow is also available as prepacked 1 ml and 5 ml GSTrap FF columns, as prepacked 96-well filter plates, GST MultiTrap FF, and as a bulk medium in bulk packs (25, 100, and 500 ml) for packing columns or batch purifications. Note that GSTPrep FF 16/10 columns cannot be opened or refilled.

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

Reagents required

Binding buffer:	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

1 to 20 mM DTT may be included in the binding and elution buffers to reduce the risk of oxidation of free -SH groups on GST, which may cause aggregation of the tagged target protein, resulting in lower yield of GST-tagged protein.

Sample preparation

Before starting the procedure, refer to page 32 for general considerations for purification of GST-tagged proteins.

Adjust the sample to the composition and pH of the binding buffer by additions from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange.

Pass the sample through a 0.22 µm or a 0.45 µm filter and/or centrifuge it immediately before sample application. If the sample is too viscous, dilute it with binding buffer to prevent it from clogging; increase lysis treatment (sonication, homogenization); or add DNase/RNase to reduce the size of nucleic acid fragments.

Procedure

1. Apply the centrifuged and/or filtered sample (in binding buffer) to the column at a flow rate of 1 to 5 ml/min (30 to 150 cm/h).

Note: Collect the flowthrough and save 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.

2. Wash the column with 100 to 200 ml of binding buffer at 2 to 10 ml/min (60 to 300 cm/h).
3. Elute the bound protein with 100 to 200 ml of elution buffer at a flow rate of 2 to 10 ml/min (60 to 300 cm/h).
4. Equilibrate the column with 60 to 100 ml of binding buffer at a flow rate of 2 to 10 ml/min (60 to 300 cm/h). The column is now ready for a new purification.

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.

Application example

Purification and scale-up of two GST-tagged proteins using 1 ml and 5 ml GSTrap FF columns and GSTPrep FF 16/10 column

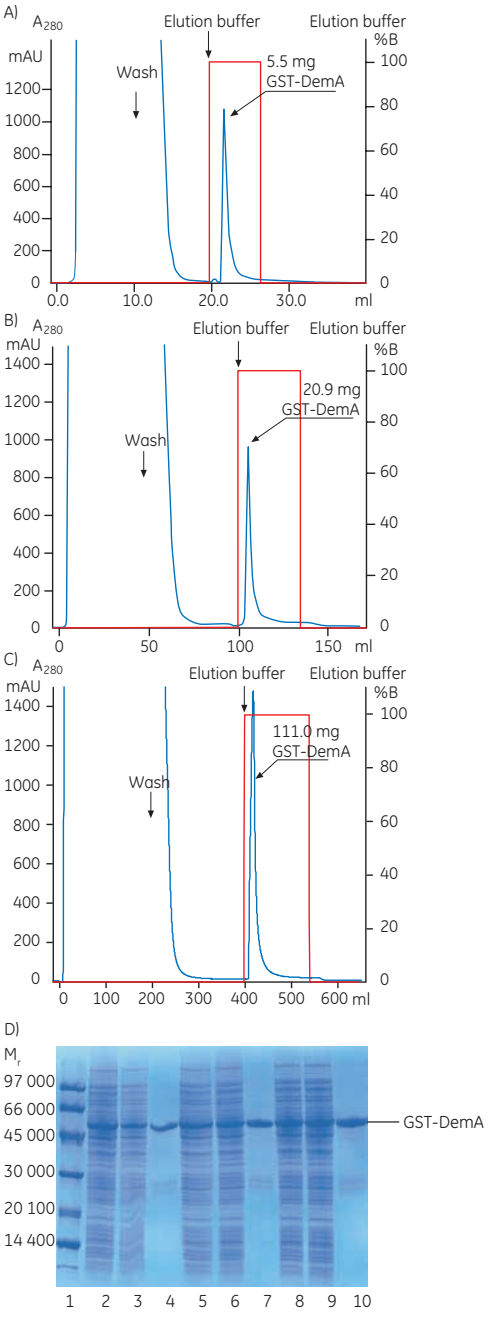
Glutathione Sepharose 4 Fast Flow is easy to use for one-step purification of GST-tagged proteins. Figures 3.19A-C and 3.20A-C show scale-up studies on GSTrap FF 1 ml, GSTrap FF 5 ml, and GSTPrep FF 16/10. Two different GST-tagged proteins were purified: GST-DemA and GST-Purα. The gene encoding for DemA was isolated from *Streptococcus dysgalactiae*. DemA is a fibrinogen-binding protein that shows both plasma protein binding properties and sequence similarities with the M and M-like proteins of other streptococcal species. Purα has been shown to be involved in transcriptional regulation.

E. coli expressing the GST-tagged proteins was resuspended (1 g/5 ml) in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) supplemented with 1 mM PMSF, 1 mM DTT, 100 mM $MgCl_2$, 1 U/ml RNase A, and 13 U/ml DNase I. The cells were lysed by sonication with a Vibracell™ ultrasonic processor for 3 min, amplitude 50%. The cell extract was kept on ice during the sonication. Cell debris was removed by centrifugation at 48 000 × g, 4°C for 30 min. After passage through a 0.45 µm filter, the supernatant was applied to the column.

The following purification procedures were performed using an ÄKTA chromatography system. The columns, GSTrap FF 1 ml, GSTrap FF 5 ml, and GSTPrep FF 16/10 were equilibrated with 5 column volumes of PBS, pH 7.4, and the prepared sample was applied to the columns.

The columns were washed with 10 column volumes of PBS (GST-DemA) and 20 column volumes of PBS (GST-Purα) and eluted using 7 column volumes of Tris-HCl, pH 8.0 including 10 mM reduced glutathione. The purity of eluted proteins was analyzed by SDS-PAGE (see Figs 3.19D and 3.20D).

The main parameter in this scale-up study was the residence time (i.e., the period of time the sample is in contact with the chromatography medium). The residence time was the same for the GSTrap FF 1 ml and 5 ml columns whereas it was twice as long for the GSTPrep FF 16/10 column (20 ml column volume) due to the difference in column length and column diameter. The amount of protein bound differed between GST-DemA and GST-Purα due to the different binding characteristics of these proteins. Some of the applied protein was found in the flowthrough as an effect of the slow binding kinetics of GST. The amount of eluted GST-tagged proteins increased proportionally with increased column volume and sample load.



A) GSTrap FF 1 ml

Column:	GSTrap FF 1 ml
Sample:	10 ml extract from *E. coli* expressing GST-DemA
Binding buffer:	PBS
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 0.5 ml/min Washing and elution: 1 ml/min
Chromatographic procedure:	5 CV (CV = column volume) binding buffer, 10 ml sample, 10 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTA

B) GSTrap FF 5 ml

Column:	GSTrap FF 5 ml
Sample:	50 ml extract from *E. coli* expressing GST-DemA
Binding buffer:	PBS
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 2.5 ml/min Washing and elution: 5 ml/min
Chromatographic procedure:	5 CV binding buffer, 50 ml sample, 10 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTA

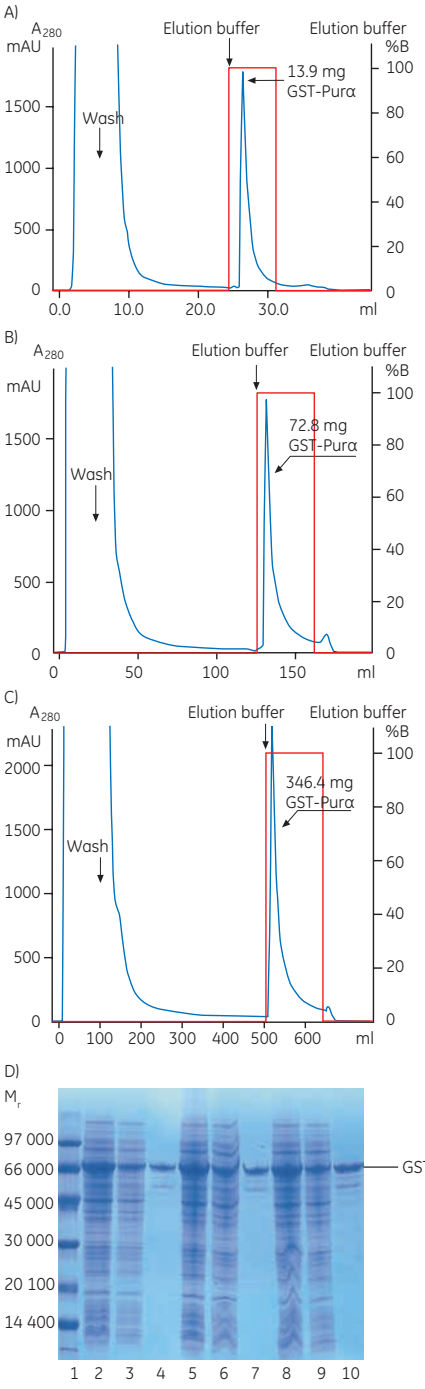
C) GSTPrep FF 16/10 (20 ml column volume)

Column:	GSTPrep FF 16/10 (20 ml column volume)
Sample:	200 ml extract from *E. coli* expressing GST-DemA
Binding buffer:	PBS
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 5 ml/min Washing and elution: 10 ml/min
Chromatographic procedure:	5 CV binding buffer, 200 ml sample, 10 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTA

Lanes

1. LMW markers
2. Extract of *E. coli* expressing GST-DemA, 1 g cell paste/5 ml
3. Flowthrough from GSTrap FF 1 ml
4. GST-DemA eluted from GSTrap FF 1 ml
5. Extract of *E. coli* expressing GST-DemA, 1 g cell paste/5 ml
6. Flowthrough from GSTrap FF 5 ml
7. GST-DemA eluted from GSTrap FF 5 ml
8. Extract of *E. coli* expressing GST-DemA, 1 g cell paste/5 ml
9. Flowthrough from GSTPrep FF 16/10
10. GST-DemA eluted from GSTPrep FF 16/10

Fig 3.19. Purification and scale-up of GST-DemA on (A) GSTrap FF 1 ml, (B) GSTrap FF 5 ml, and (C) GSTPrep FF 16/10. (D) SDS-PAGE analysis of GST-DemA on ExcelGel 12.5% followed by Coomassie staining. Due to the relatively slow binding kinetics of GST and the rather high load, some of the applied protein was found in the flowthrough.



A) GSTrap FF 1 ml

Column:	GSTrap FF 1 ml
Sample:	5 ml extract from *E. coli* expressing GST-Purα
Binding buffer:	PBS
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 0.5 ml/min Washing and elution: 1 ml/min
Chromatographic procedure:	5 CV binding buffer, 5 ml sample, 20 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTA

B) GSTrap FF 5 ml

Column:	GSTrap FF 5 ml
Sample:	25 ml extract from *E. coli* expressing GST-Purα
Binding buffer:	PBS
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 2.5 ml/min Washing and elution: 5 ml/min
Chromatographic procedure:	5 CV binding buffer, 25 ml sample, 20 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTA

C) GSTPrep FF 16/10 (20 ml column volume)

Column:	GSTPrep FF 16/10 (20 ml column volume)
Sample:	100 ml extract from *E. coli* expressing GST-Purα
Binding buffer:	PBS
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 5 ml/min Washing and elution: 10 ml/min
Chromatographic procedure:	5 CV binding buffer, 100 ml sample, 20 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTA

Lanes

1. LMW markers
2. Extract of *E. coli* expressing GST-Purα, 1 g cell paste/5 ml
3. Flowthrough from GSTrap FF 1 ml
4. GST-Purα eluted from GSTrap FF 1 ml
5. Extract of *E. coli* expressing GST-Purα, 1 g cell paste/5 ml
6. Flowthrough from GSTrap FF 5 ml
7. GST-Purα eluted from GSTrap FF 5 ml
8. Extract of *E. coli* expressing GST-Purα, 1 g cell paste/5 ml
9. Flowthrough from GSTPrep FF 16/10
10. GST-Purα eluted from GSTPrep FF 16/10

Fig 3.20. Purification and scale-up of GST-Purα on (A) GSTrap FF 1 ml, (B) GSTrap FF 5 ml, and (C) GSTPrep FF 16/10. (D) SDS-PAGE analysis of GST-Purα on ExcelGel 12.5% using Multiphor II followed by Coomassie staining. Due to the slow binding kinetics of GST, some of the applied protein was found in the flowthrough.

Purification using bulk GST media

Glutathione Sepharose 4B, FF, and HP are available in lab packs, varying from 10 to 500 ml, for affinity purification of GST-tagged proteins using batch- or column-based methods. Glutathione Sepharose media are supplied preswollen in 20% ethanol. The media are used at a final slurry concentration of 50%.

Sample preparation

Before starting either the batch or column purification procedure, refer to page 32 for general considerations for purification of GST-tagged proteins.

Adjust the sample to the composition and pH of the binding buffer by additions from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange.

Pass the sample through a 0.22 µm or a 0.45 µm filter and/or centrifuge it immediately before sample application. If the sample is too viscous, dilute it with binding buffer to prevent it from clogging; increase lysis treatment (sonication, homogenization); or add DNase/RNase to reduce the size of nucleic acid fragments.

Reagents required

Binding buffer:	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.

1 to 20 mM DTT may be included in the binding and elution buffers to reduce the risk of oxidation of free -SH groups on GST, which may cause aggregation of the tagged target protein, resulting in lower yield of GST-tagged protein.

Preparation of medium

1. Determine the bed volume of Glutathione Sepharose required for your purification.
2. Gently shake the bottle to resuspend the slurry.
3. Use a pipette or measuring cylinder to remove sufficient slurry for use and transfer to an appropriate container/tube.
4. Sediment the chromatography medium by centrifugation at 500 × g for 5 min. Carefully decant the supernatant.
5. Wash the Glutathione Sepharose HP, FF, or 4B by adding 5 ml of PBS (see Binding buffer recipe above) per 1 ml of slurry (= 50% slurry).

Glutathione Sepharose media must be thoroughly washed with PBS to remove the ethanol storage solution because residual ethanol may interfere with subsequent procedures.

6. Sediment the chromatography medium by centrifugation at 500 × g for 5 min. Carefully decant the supernatant.
7. Repeat steps 5 and 6 once for a total of two washes.

Note: The medium is now equilibrated with PBS.

The bed volume is equal to half of the volume of the 50% slurry.

Batch purification procedure

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

1. Add the cell lysate to the prepared Glutathione Sepharose medium and incubate for at least 30 min at room temperature, using gentle agitation such as end-over-end rotation.

Note: Save the flowthrough and wash fractions until the procedure has been successfully completed. Check a sample from each step by SDS-PAGE or CDNB assay to measure the efficiency of protein binding to the medium.

2. Use a pipette or cylinder to transfer the mixture to an appropriate container/tube.
3. Sediment the medium by centrifuging at 500 × g for 5 min. Carefully decant the supernatant (= flowthrough) and save it for SDS-PAGE analysis.
4. Wash the medium by adding 5 ml of PBS per 1 ml of slurry (= 50% slurry). Invert to mix.
5. Sediment the medium by centrifuging at 500 × g for 5 min. Carefully decant the supernatant (= wash) and save it for SDS-PAGE analysis.
6. Repeat steps 4 and 5 twice for a total of three washes.

The bound tagged protein can be eluted directly at this stage using elution buffer, or an on-column cleavage can be performed (see Chapter 5 for details).

7. Elute the bound protein from the sedimented medium by adding 0.5 ml of elution buffer per 1 ml of slurry of Glutathione Sepharose medium.
8. Mix gently to resuspend the medium. Incubate at room temperature for 5 to 10 min using 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) and collect it into a fresh centrifuge tube.
10. Repeat steps 7 to 9 twice for a total of three elutions. Check the eluates for purified protein and pool those eluates containing protein.

Column purification procedure

Before starting the procedure, refer to page 32 for general considerations for purification of GST-tagged proteins.

See instructions supplied with the products or refer to Appendix 6 for general guidelines for column packing. For recommended flow velocities, see Appendix 5, Table A5.1.

Prepare a 50% slurry as described in Preparation of medium steps 1 to 7.

1. Equilibrate the column with approximately 5 column volumes of binding buffer.
2. Apply the pretreated sample at low flow rate (approximately one-third of the flow rate used during wash and elution).
3. Wash the column with 5 to 10 column volumes of binding buffer or until no material appears in the flowthrough. Save the flowthrough for SDS-PAGE analysis to check for any loss of unbound target protein.
4. Elute the bound protein with 5 to 10 column volumes of elution buffer. Collect the fractions and check separately for purified protein. Pool those fractions containing the GST-tagged target protein.

GST Bulk Kit

GST Bulk Kit contains a 10 ml bulk pack of Glutathione Sepharose 4B medium, five gravity-flow columns, IPTG, and GST Buffer Kit. It is designed for small-scale manual purification of GST-tagged proteins in batch experiments or with packed columns using a gravity-flow column format. GST Bulk Kit facilitates optimization studies of expression, solubility conditions, and purification parameters of a GST-tagged protein. One kit is suited for purification of GST-tagged proteins from 2 ml to 20 l of culture in up to five columns in parallel (up to 100 mg of purified GST-tagged proteins).

Fig 3.21. GST Bulk Kit is designed for small-scale manual purification of GST-tagged proteins using either column chromatography or batch method.

Selecting equipment for purification

The choice of equipment is dependent on the specific purification. Many purification procedures can be carried out using simple equipment and methods, for example, in a step-gradient elution method using a syringe in combination with a prepacked HiTrap column. For more complex elution methods, such as linear gradients, a dedicated system can be used. A system can also be used when the same column is intended to be used for several runs in series. Table 3.4 shows appropriate equipment for different purification processes. See also ÄKTA Laboratory-scale Chromatography Systems: Instrument Management Handbook, GE Healthcare, 29-0108-31.

Table 3.4. Ways of working with standard ÄKTA systems

Way of working	ÄKTAprime plus	ÄKTApurifier™	ÄKTAxpress	ÄKTA avant	ÄKTAmicro™	ÄKTA pure
Scale						
Laboratory scale	•	•	•	•	•	•
Process development	–	–	–	•	–	–
Regulatory demands						
System control and data handling for regulatory requirements	–	•	•	•	•	•
Type of work						
Method development	–	(•)	–	•	–	•
Generic methods	•	•	•	•	•	•
Micropreparative and analysis	–	–	–	–	•	(•)
Automation						
Buffer preparation function	–	(•)	–	•	–	–
pH scouting	–	(•)	–	•	–	(•)
Media or column scouting	–	(•)	–	•	–	(•)
Multistep purification	(•)	–	•	–	–	(•)
Software						
UNICORN™	–	•	•	•	•	•
PrimeView™	•	–	–	–	–	–

• *Recommended*
(•) *Optional*
– *Not recommended or not applicable*

ÄKTAprime plus

ÄKTApurifier

ÄKTAxpress (one module)

ÄKTA avant

ÄKTAmicro

ÄKTA pure

Fig 3.22. The standard ÄKTA system configurations.

Troubleshooting

This troubleshooting guide addresses the common problems associated with the majority of purification methods using the different Glutathione Sepharose media.

Problem	Possible cause	Solution
GST-tagged protein does not bind or binds poorly to the medium.	The flow rate used during sample loading is too high.	Decrease the flow rate during sample loading or, when manual methods are used, incubate the sample after loading. One of the most important parameters affecting the binding of GST-tagged proteins to Glutathione Sepharose is the flow rate. Due to the relatively slow binding kinetics between GST and glutathione, it is important to keep the flow rate low during sample loading for maximum binding capacity.
	GST-tagged protein denatured by mechanical lysis (e.g., sonication). Too extensive lysis can denature the tagged protein and prevent it from binding.	Use mild mechanical/chemical lysis conditions during cell lysis. Conditions for lysis must be empirically determined.
	GST-tagged proteins have aggregated in the sample, causing precipitation.	Add DTT to the sample prior to cell lysis and also add DTT to the buffers. Adding DTT to a final concentration of 1 to 20 mM may significantly increase the yield of some GST-tagged proteins.

continues on following page

Problem	Possible cause	Solution
	Concentration of tagged protein is too low.	Concentrate the sample using Vivaspin™ concentration devices. The binding kinetics are concentration dependent. Proteins with low expression may not bind as efficiently as highly expressed proteins; therefore, concentrating the sample may improve binding.
	The tagged protein may have altered the conformation of GST, thereby reducing the affinity for the GST-tagged protein.	Test the binding of GST from parental pGEX: Prepare a lysate of cells harboring the parental pGEX plasmid and check binding to the medium. If GST produced from the parental plasmid binds with high affinity, the tagged protein may have altered the conformation of GST, thereby reducing the affinity for the GST-tagged protein. Adequate results may be obtained by reducing the temperature used for binding to 4°C, and by limiting washing.
	Equilibration time is too short.	Ensure that the column has been equilibrated with at least 5 column volumes of a buffer pH 6.5 to 8.0 (e.g., PBS).
	Binding of GST-tagged proteins is not efficient at pH less than 6.5 or greater than 8.	Equilibrate with a buffer pH 6.5 to 8.0 (e.g., PBS) before the clarified cell lysate is applied. Make sure that lysis is done at pH 6.5 to 8.0. Check that the sample has been adjusted to the binding buffer conditions.
	GSTrap column: Column needs cleaning.	Clean the column according to the standard cleaning protocol. If the GSTrap column has already been used several times, it may be necessary to replace it with a new one.
	Glutathione Sepharose medium has been used too many times.	Use fresh Glutathione Sepharose medium.
	GSTrap columns. The column or ÄKTA system is clogged, leading to high back pressure and no binding.	Clogged column: Clean the column according to instructions. Make sure the sample has been centrifuged and/ or filtered through a 0.45 µm filter before loading. Clogged system: clean system according to manual.
GST-tagged protein is not eluted efficiently.	The volume of elution buffer is insufficient.	Increase the volume of elution buffer. In some cases, especially after on-column cleavage of a tagged protein, a larger volume of buffer may be necessary to elute the tagged protein.
	The time for elution is insufficient.	Increase the elution duration by decreasing the flow rate during elution. For GSTrap columns, use a flow rate of 0.2 to 1 ml/min (1 ml HiTrap column) and 0.5 to 5 ml/min (5 ml HiTrap column) during sample application. For centrifugation methods, decrease the centrifugation speed during elution.

continues on following page

Problem	Possible cause	Solution
	The concentration of glutathione is insufficient.	Increase the concentration of glutathione in the elution buffer: The recommended 10 mM should be sufficient for most applications, but occasionally this concentration needs to be increased. Try 50 mM Tris-HCl, 20 to 40 mM reduced glutathione, pH 8.0 as elution buffer.
	The pH of the elution buffer is too low.	Increase the pH of the elution buffer: Increasing the pH to 8 to 9 may improve the elution without requiring an increase in the concentration of the glutathione used for elution.
	The ionic strength of the elution buffer is too low.	Increase the ionic strength of the elution buffer: Adding 0.1 to 0.2 M NaCl to the elution buffer may also improve the results.
	The glutathione in the elution buffer is oxidized.	Use fresh elution buffer or add DTT.
	Nonspecific hydrophobic interactions with the medium cause protein aggregation, preventing solubilization and elution of tagged proteins.	Add a nonionic detergent to the elution buffer: Adding 0.1% Triton X-100 or 2% n-octylglucoside can significantly improve elution of some types of GST-tagged proteins.
Multiple bands are observed after electrophoresis/ Western blot analysis of eluted target protein.	M_r 70 000 protein copurifies with the GST-tagged protein.	The M_r 70 000 protein is probably a protein product of the *E. coli* gene dnaK. This protein is involved in protein folding in *E. coli*. It has been reported that such association can be disrupted by incubating the tagged protein in 50 mM Tris-HCl, 2 mM ATP, 10 mM $MgSO_4$, pH 7.4 for 10 min at 37°C prior to loading. Alternatively, the DnaK protein can be removed by passing the tagged protein solution through ATP-agarose or a similar purification medium, or by performing ion exchange.
	Tagged proteins may have been partially degraded by proteases.	Add a protease inhibitor: Multiple bands may be a result of partial degradation of tagged proteins by proteases. Adding 1 mM PMSF to the lysis solution may improve results. A nontoxic, water-soluble alternative to PMSF is 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), commercially available as Pefabloc™ SC from Roche Biochemicals. *Note:* Serine protease inhibitors must be removed prior to cleavage by thrombin or Factor Xa. PreScission Protease is not a consensus serine protease and is insensitive to many of the protease inhibitors tested at GE Healthcare. PMSF is toxic, with acute effects. Use Pefabloc whenever possible.
	Proteolysis may have occurred in the host bacteria.	Use a protease-deficient host: Multiple bands may be the result of proteolysis in the host bacteria. If this is the case, the use of a host-deficient *E. coli* strain may be required (e.g., lon- or ompT). *E. coli* BL21 is defective in OmpT and Lon protease production.

continues on following page

Problem	Possible cause	Solution
	Cell disruption may have been too extensive during mechanical lysis.	Decrease lysis time: Cell disruption is apparent by partial clearing of the suspension and can be checked by microscopic examination. Adding lysozyme (0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris-HCl, pH 8.0) prior to mechanical lysis may improve the results. Avoid foaming as this may denature the tagged protein. Over-lysis can also lead to the copurification of some of the host proteins with the GST-tagged protein.
	Chaperones may have copurified.	Include an additional purification step: Additional bands may be caused by the copurification of a variety of proteins known as chaperones, which are involved in the correct folding of nascent proteins in *E. coli*. These include, but may not be limited to: DnaK (M_r ~ 70 000), DnaJ (M_r ~ 37 000), GrpE (M_r ~ 40 000), GroEL (M_r ~ 57 000), and GroES (M_r ~10 000). Several methods for purifying GST-tagged proteins from these copurifying proteins have been described.
	Antibodies that react with various *E. coli* proteins may be present in the tagged protein sample.	Cross-adsorb antibody with *E. coli* proteins: Depending on the source of the anti-GST antibody, it may contain antibodies that react with various *E. coli* proteins that may be present in the tagged protein sample. Cross-adsorb the antibody with an *E. coli* lysate to remove anti-*E. coli* antibodies from the preparation. Anti-GST antibody from GE Healthcare has been cross-adsorbed against *E. coli* proteins and tested for its lack of nonspecific background binding in Western blots.

Chapter 4
Detection of GST-tagged proteins

Several methods are available for detection of GST-tagged proteins, and can be selected based on the experimental situation. Functional assays based on the properties of the protein of interest (and not the GST tag) are useful, but are beyond the scope of this handbook. See Table 4.1 for a description of the procedures that follow.

Table 4.1. Detection methods for GST-tagged proteins

Detection method	Comments
GST 96-Well Detection Module for ELISA	Designed 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	Rapid assay; well suited for screening. Gives estimate of relative level of expression.
SDS-PAGE with Coomassie or silver staining	Provides information on size and percent purity. Detects tagged protein and contaminants.
Western blot using anti-GST antibody	Highly specific, detects only GST-tagged protein. Little or no background detectable when using detection systems with optimized concentrations of secondary HRP-conjugated antibody. Amersham™ ECL™ detection systems enhance detection in Western blots. ECL provides adequate sensitivity for most recombinant expression applications. Provides information on size.

GST 96-Well Detection Module for ELISA

The GST 96-Well Detection Module provides a highly sensitive enzyme-linked immunosorbent assay (ELISA) for testing clarified lysates and intermediate purification fractions for the presence of GST-tagged proteins (see Figs 4.1 and 4.2). Samples are applied directly into the wells of the plates, and GST-tagged proteins are captured by specific binding to anti-GST antibody that is immobilized on the walls of each well. Captured GST-tagged proteins are then detected with HRP/Anti-GST conjugate provided in the module. Standard curves for quantitation of tagged 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-tagged partner to screen and identify clones expressing the desired GST-tagged protein.

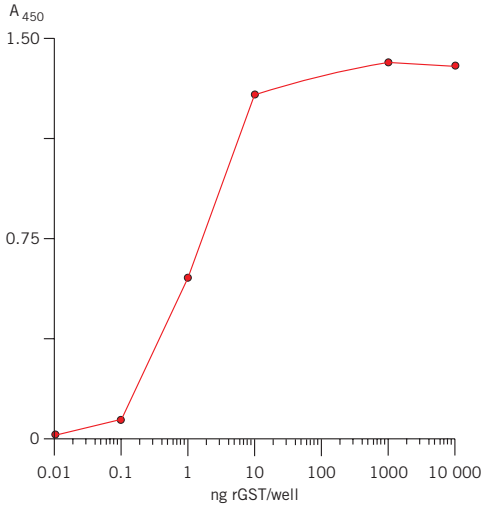


Fig 4.1. Sensitive detection of recombinant GST using the GST 96-Well Detection Module. Recombinant GST protein was prepared in 1× blocking buffer, and 100 µl volumes were applied directly to the wells of a GST 96-well capture plate. After 1 h binding at room temperature, the wells were washed in wash 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.

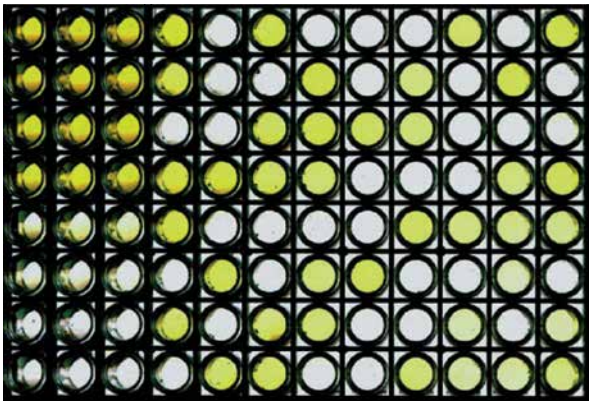


Fig 4.2. Screening of bacterial lysates for GST-tagged protein expression using the GST 96-Well Detection Module.

Each tagged protein is captured uniquely; therefore, if quantitation is required, prepare standards of recombinant GST protein and the tagged target 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 GST standard protein

Additional reagents required for ELISA

PBS:	140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3
Wash buffer:	0.05% Tween™ 20 in PBS (500 ml/96-well plate). Store at room temperature until needed.
Blocking buffer (1×):	3% nonfat dry milk in PBS with 0.05% Tween 20 (10 ml/96-well plate)
Blocking buffer (2×):	6% nonfat dry milk in PBS with 0.1% Tween 20 (5 ml/96-well plate)
Substrate	

Procedure

1. Bring each test sample to a final volume of 50 µl with 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 tagged 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[1] to each well and incubate according to the supplier's instructions.

[1] *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.

GST Detection Module with CDNB enzymatic assay

GST-tagged proteins produced using pGEX vectors can be detected enzymatically using the GST substrate CDNB, 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. Assay results are available in less than 10 min for crude bacterial lysates, column eluates, or purified GST-tagged protein. Figure 4.3 shows typical results from a CDNB assay. Each GST Detection Module contains reagents sufficient for 50 assays.

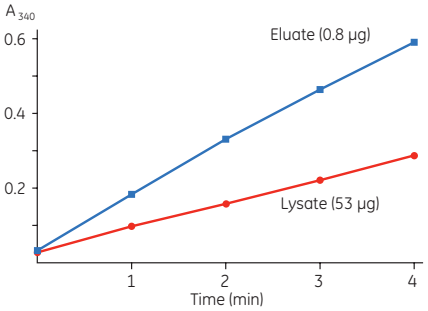


Fig 4.3. Typical results of a CDNB assay for GST-tagged proteins. 53 µg of total protein from an *E. coli* TG1/pGEX-4T-Luc lysate and 0.8 µg of total protein eluted from Glutathione Sepharose medium were assayed according to instructions included with the GST Detection Module.

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 glutathione in sterile distilled water. 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.

Procedure

1. In a microcentrifuge tube, combine the following:

Distilled water	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 labeled sample and blank. Add sample (5 to 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

ΔA_{340}/min/ml values can be used as a relative comparison of GST-tagged protein content between samples of a given tagged protein.

Adapt the assay to give absolute concentrations of tagged proteins by constructing a standard curve of ΔA_{340}/min versus amount of tagged protein. Purified sample of the tagged protein is required to construct the curve.

The assay detects active GST. Additional inactive GST-tagged protein may be present.

SDS-PAGE

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

Reagents required

2x sample loading buffer:	0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, 200 mM DTT. Store in aliquots at -20°C.

DTT should be freshly prepared and added to the sample loading buffer just before adding the sample loading buffer to the samples. β-mercaptoethanol (500 µl per 10 ml) can be used as an alternative to DTT.

Gel electrophorersis

1. Add 1 volume of 2x sample loading buffer to 1 volume of supernatant from crude extracts, cell lysates, or purified fractions, as appropriate.
2. Vortex briefly and heat for 5 min at 95°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 using preferred staining method.

Western blot

Expression and purification of GST-tagged proteins can be monitored by Western blot analysis, using Amersham ECL, Amersham ECL Prime, or Amersham ECL Select detection systems to enhance sensitivity.

Reagents required

Anti-GST antibody (goat polyclonal)	
Blocking/incubation buffer:	5% (w/v) nonfat dry milk and 0.1% (v/v) Tween 20 in 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 PBS (as above), referred to as PBS-Tween in the protocol below OR 0.1% (v/v) Tween 20 in 1x TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4 to 8), referred to as TBS-Tween in the protocol below
Secondary antibody to detect the anti-GST antibody (such as anti-goat IgG HRP conjugate)	
Appropriate membrane, such as Amersham Hybond™ ECL (nitrocellulose) or Amersham Hybond-P (PVDF)	

Electrophoretic separation of proteins

1. Separate the protein samples by SDS-PAGE.

Although anti-GST antibody from GE Healthcare has been cross-adsorbed with *E. coli* proteins, low levels of cross-reacting antibodies may remain. Samples of *E. coli* lysates 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 the membrane.

Electrophoresis and protein transfer can be accomplished using a variety of equipment and reagents. For further details, refer to the Western Blotting Handbook (28-9998-97) from GE Healthcare.

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 to 200 ml of blocking/incubation buffer to the container.
3. Incubate with agitation for 1 h at room temperature, or at 37°C if the background is persistently and unacceptably high. Alternatively, membranes may be left in the blocking solution overnight at 2°C to 8°C, if more convenient.
4. Decant and discard the buffer.
5. Briefly rinse the membrane in wash buffer.

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

Chemiluminescence detection with Amersham ECL, Amersham ECL Prime, and Amersham ECL Select

1. Dilute the primary antibody in PBS-Tween or TBS-Tween.
2. Place the membrane (protein side up) in the primary antibody solution and incubate with agitation for 1 h at room temperature or overnight at 4°C. Always refer to manufacturers' recommendations.
3. Wash the membrane three to six times in PBS-Tween or TBS-Tween for 5 min per wash or according to manufacturers' recommendations.
4. Place the membrane in the secondary antibody diluted in PBS-Tween or TBS-Tween and incubate with agitation for 1 h at room temperature or overnight at 4°C.
5. Place the membrane in washing solution and wash four to six times for 5 min per wash.
6. Continue with detection as recommended for the selected detection reagent and imaging system.

Refer to GE Healthcare Western Blotting Handbook (28-9998-97) for further information on optimization of antibody concentration for Western blotting.

Amersham ECL, Amersham ECL Prime, and Amersham ECL Select 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.

Figure 4.4 shows typical Western blot results using Anti-GST Antibody.

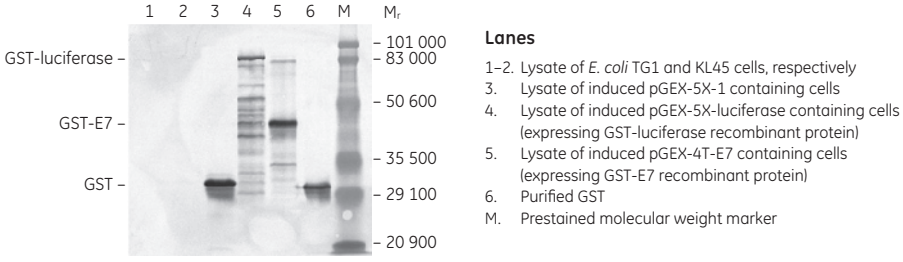


Fig 4.4. Western blot of *E. coli* lysates containing GST-tagged proteins. For detection, Anti-GST Antibody, anti-goat IgG alkaline phosphatase conjugate, and CDNB/nitro-blue tetrazolium chloride (NBT) enzyme substrate were used.

Troubleshooting of detection methods

The troubleshooting guide below addresses problems common to the majority of detection methods as well as problems specific to a particular method. In the latter case, the relevant method is indicated.

Problem	Possible cause	Solution
Poor results with the GST Detection Module	The reaction rate is nonlinear.	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-tagged protein to maintain a linear reaction rate.
	The target protein has inhibited the folding of the GST tag.	The tagged protein may have inhibited the correct folding of the GST moiety. The GST-tagged proteins will thus 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 tagged protein expression.
	There is baseline drift.	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 ΔA_{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. Alternatively, get the slope directly from the spectrophotometer software. The slope will be the same as long as the spontaneous reaction is limited.
Poor results with the GST 96-Well Detection Module	Low absorbance is seen in the assay.	Check that host cells were sufficiently induced, that the samples were sufficiently lysed, and that inclusion bodies have not been formed. (See Troubleshooting purification methods.)
	Concentration of blocking buffer is inadequate.	If clarified lysate is being tested, mix the initial GST sample with 2× blocking buffer to give a final concentration of 1× blocking buffer.
	There is poor day-to-day reproducibility.	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.
No signal in Western blotting	Proteins are not transferred during Western blotting.	Stain gel and membrane with total protein stain to check transfer efficiency. Optimize gel acrylamide concentration, time for transfer, and current.
		Ensure gel and membrane make proper contact during blotting and are orientated correctly with respect to the anode.
		Check that excess temperatures are not reached during electroblotting, producing bubbles or membrane distortion.
	Proteins are not retained on membrane.	Assess transfer of proteins (as above). Use a fresh supply of membrane.

continues on following page

Problem	Possible cause	Solution
	There are problems with detection reagents.	Ensure reagents are being used correctly. Prepare reagents freshly each time. Store reagents at correct temperature.
Weak signal in Western blotting	Protein transfer efficiency is poor.	Check transfer efficiency as above.
	Insufficient protein has been loaded.	Load more protein on gel.
	Exposure time is too short.	Increase exposure time.
Excessive diffuse signal in Western blotting	Too much protein has been loaded.	Reduce the amount of protein loaded.
High backgrounds in Western blotting	Washing is inadequate.	Ensure post-conjugate washes are performed for a sufficient amount of time with an adequate volume of wash buffer (> 4 ml/cm^2 membrane).
	Blocking is inadequate.	Check the blocking buffer has been made correctly. Use freshly prepared blocking buffer each time. Increase the concentration of blocking reagent—try 10%. Use alternative blocking agent (e.g., 1% to 10% BSA, 0.5% to 3% gelatin). Increase incubation time with blocking buffer.
	Blotting equipment or buffers are contaminated.	Clean equipment. Prepare fresh buffers.
Multiple bands are seen in Western blotting	Conjugate is binding non-specifically to other proteins.	Include a negative control of expression host not containing expression vector to determine nonspecific binding.
	GST-tagged protein may have been degraded.	Include protease inhibitors during purification. Reduce purification time and temperature. Add a second purification step to remove incomplete target protein.

Chapter 5
Removal of GST tag by enzymatic cleavage

Removal of the GST tag is often necessary to be able to perform functional or structural studies of the target protein. Tagged 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. Cleavage releases the target protein from the column and allows elution using the binding buffer. The GST moiety remains bound to the medium.

PreScission Protease itself has a GST tag and therefore will bind to Glutathione Sepharose; it will thus not co-elute and contaminate the cleaved target protein. Cleavage with PreScission Protease is very specific, and maximum cleavage is obtained in the cold (the protein is most active at 4°C), thus improving the stability of the target protein (Fig 5.1).

If thrombin or Factor Xa are used for cleavage of the tag, a convenient way to remove these enzymes is to connect in series one GSTrap FF column and one HiTrap Benzamidine FF (high sub) column. During the elution, the cleaved product passes directly from the GSTrap into the HiTrap Benzamidine FF (high sub). The cleaved target protein passes through the HiTrap Benzamidine FF (high sub) column but the proteases bind. Thus in a single step the enzymes are removed and a pure cleaved target protein is achieved (Fig 5.2). Note, however, that thrombin and Factor Xa may produce a less specific cleavage than PreScission Protease and that sometimes the target protein can be fragmented itself.

Table 5.1. Proteases used for cleavage of GST tag

Protease	Molecular weight	Pack size	Capacity	Application
PreScission Protease	46 000	500 units	One unit cleaves > 90% of 100 µg of a test GST-tagged protein when incubated in 1 mM EDTA, 1 mM DTT, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.0) at 5°C for 16 h.	For specific, low-temperature cleavage between Gln and Gly residues in the sequence Leu-Glu- Val-Leu-Phe-Gln-Gly-Pro. A tagged protein consisting of human rhinovirus protease and GST. Can be used for tag cleavage when the PreScission Protease recognition sequence occurs between the tag sequence and the protein of interest, e.g., the GST tag from proteins expressed using the pGEX-6P vector.
Bovine thrombin	37 000	500 units	One unit cleaves > 90% of 100 µg of a test GST-tagged protein when incubated in 1x PBS at 22°C for 16 h.	Serine protease for specific cleavage at the recognition sequence for thrombin. Can be used for tag cleavage when the thrombin recognition sequence occurs between the tag sequence and the protein of interest, e.g., the GST tag from proteins expressed using the pGEX-T vectors.
Bovine Factor Xa	48 000	400 units	One unit cleaves > 90% of 100 µg of a test GST-tagged protein when incubated in 1 mM $CaCl_2$, 100 mM NaCl, and 50 mM Tris-HCl (pH 8.0) at 22°C for 16 h.	Serine protease for specific cleavage following the tetrapeptide Ile-Glu-Gly-Arg. Can be used for tag cleavage when the Factor Xa recognition sequence occurs between the tag sequence and the protein of interest, e.g., the GST tag from proteins expressed using pGEX-X vectors.

Cleavage of GST tag using PreScission Protease

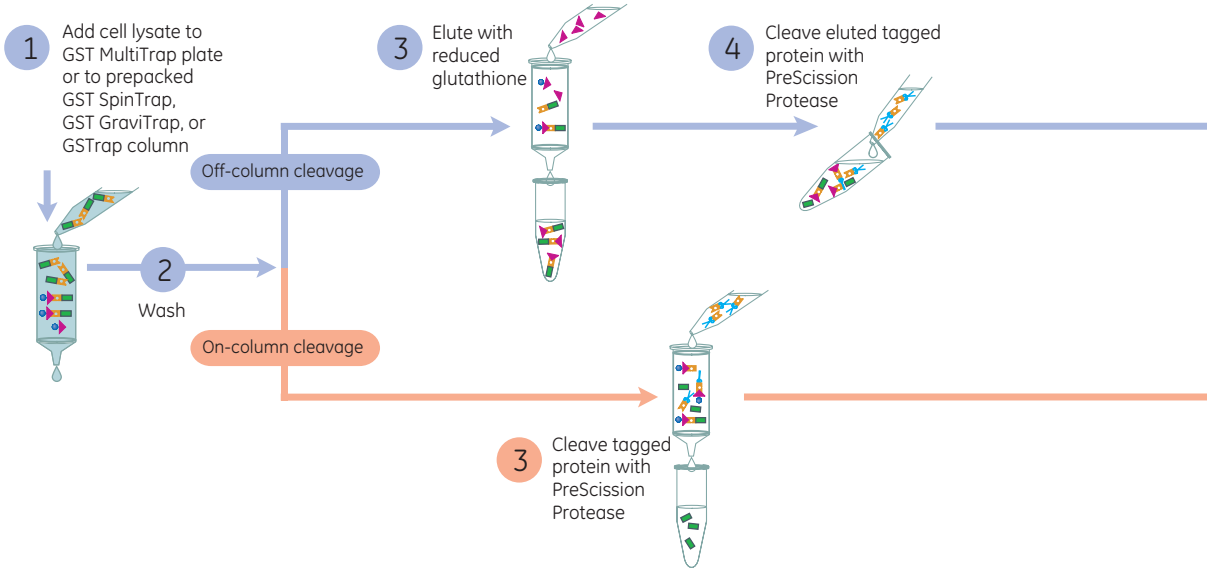


Fig 5.1. Flow chart of the affinity purification procedure and PreScission Protease cleavage of GST-tagged proteins.

Cleavage of GST tag using thrombin or Factor Xa

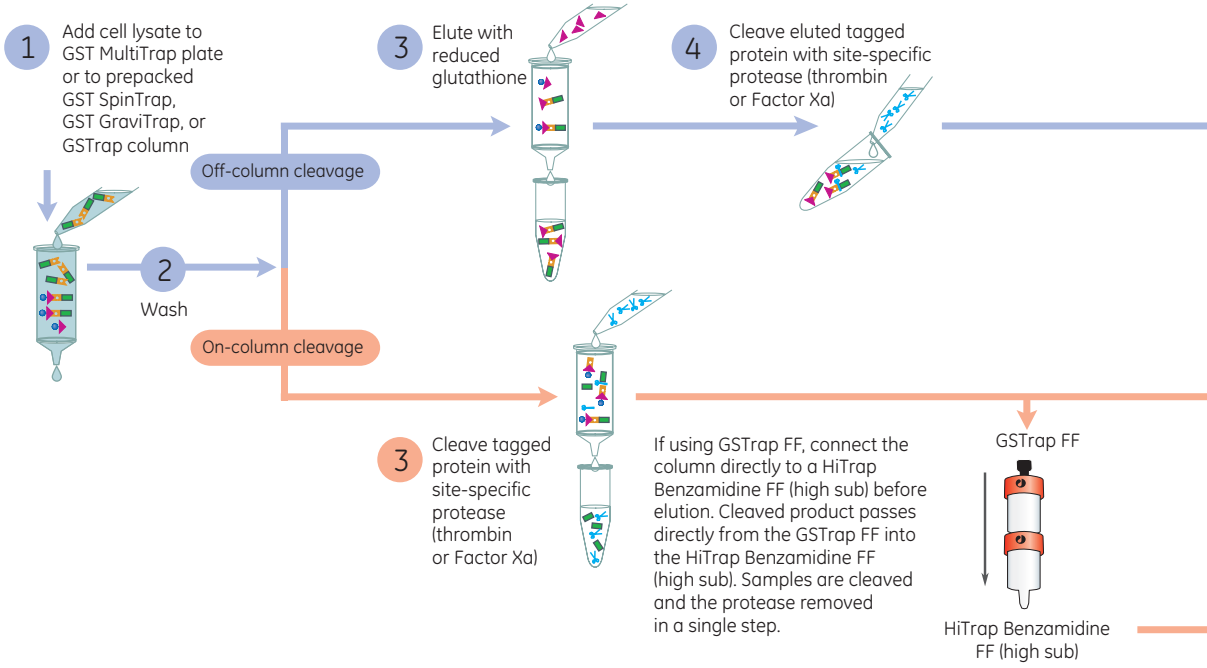
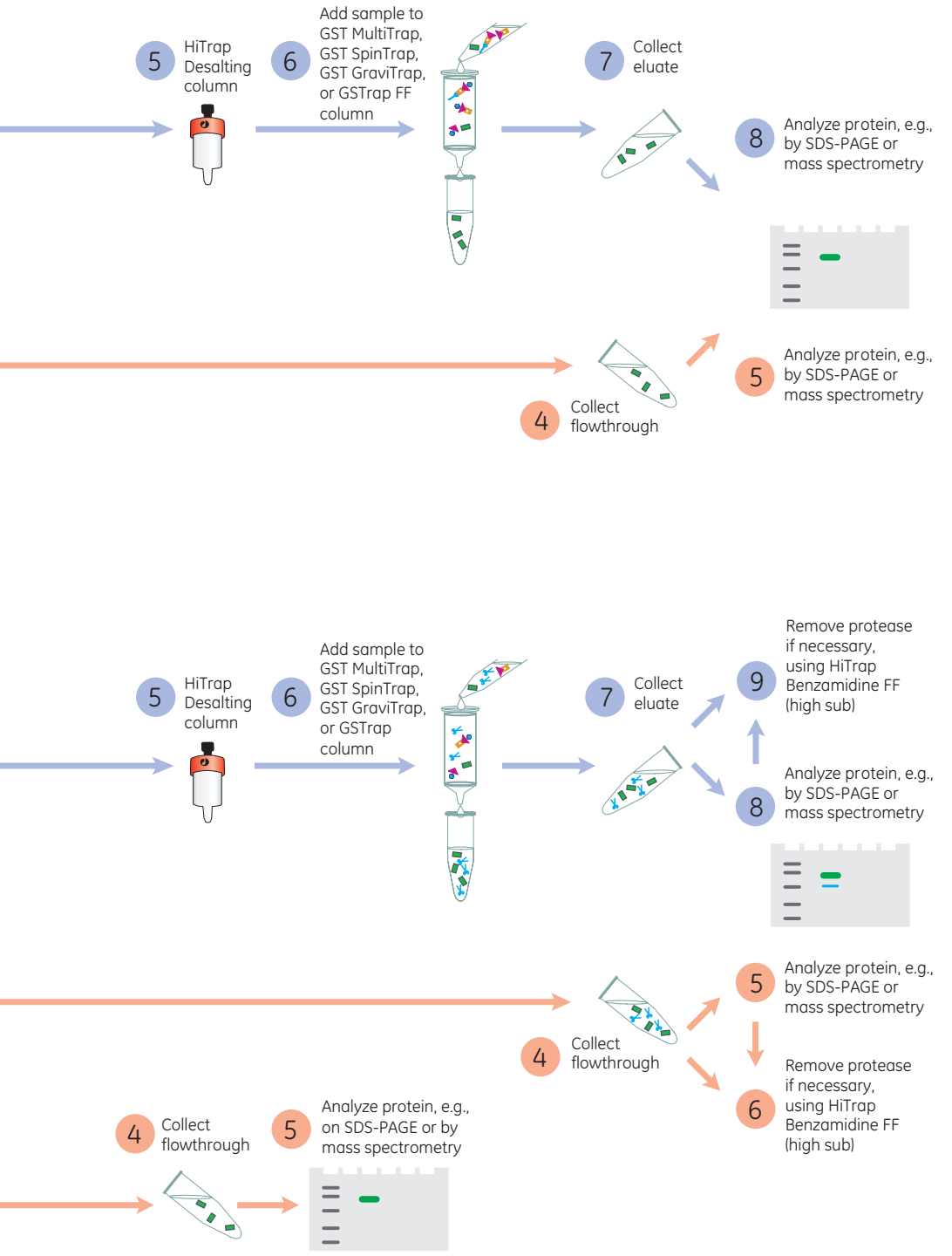


Fig 5.2. Flow chart of the affinity purification procedure and thrombin or Factor Xa cleavage of GST-tagged proteins.


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

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

If protease inhibitors (see Table 5.2) 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 flowthrough when sample is loaded onto a GSTrap column.)

Table 5.2. Inhibitors of the various proteases

Enzyme	Inhibitor
PreScission Protease	100 mM $ZnCl_2$ (> 50% inhibition)
	100 µM chymostatin
	4 mM Pefabloc
Factor Xa and thrombin	AEBSF, APMSF, antithrombin III, Antipain, a1-antitrypsin, aprotinin, chymostatin, hirudin, leupeptin, PMSF
Factor Xa only	Pefabloc FXa
Thrombin only	Pefabloc TH Benzamidine

Cleavage of tagged proteins is most commonly performed on milligram quantities of tagged protein suitable for purification on GSTrap or GST GraviTrap columns. Protocols that follow describe manual cleavage and purification using a syringe and a 1 ml or 5 ml GSTrap column. The protocols can be adapted for use with GST GraviTrap columns, or for use with GST MultiTrap 96-well plates or GST SpinTrap columns to work at smaller scales.

Cleavage and purification of GST-tagged protein bound to GSTrap

Recommended buffers

Binding buffer: PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3

For PreScission Protease cleavage:

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

Cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0

PreScission Protease

For thrombin cleavage:

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

Cleavage buffer: 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 PBS prechilled to 4°C. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.

For Factor Xa cleavage:

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

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 water to give a final solution of 1 unit/µl. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.

Purification and cleavage

The protocol below is an example optimized for 8 mg of target protein. It is worth estimating how much target protein is applied to the column, as this allows one to minimize the amount of protease added.

1. Fill the syringe or pump tubing with distilled water. Remove the stopper and connect the column to the syringe (use the connector supplied), laboratory pump, or chromatography system "drop to drop" to avoid introducing air into the system.
2. Remove the snap-off end at the column outlet.
3. Wash out the ethanol with 3 to 5 column volumes of distilled water.
4. Equilibrate the column with at least 5 column volumes of binding buffer. Recommended flow rates are 1 ml/min (1 ml column) and 5 ml/min (5 ml column).
5. Apply the pretreated sample using a syringe fitted to the Luer connector or by pumping it onto the column. For best results, use a flow rate of 0.2 to 1 ml/min (1 ml column) and 0.5 to 5 ml/min (5 ml column) during sample application.
6. Wash with binding buffer (generally at least 5 to 10 column volumes) until the absorbance reaches a steady baseline or no material remains in the effluent. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for washing.

7a. For PreScission Protease and Factor Xa, wash the column with 10 column volumes of cleavage buffer.

7b. For thrombin, proceed to step 8b.

7c. For Factor Xa, proceed to step 8c.

8a. 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.

8b. Prepare the thrombin mix:

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

– For GSTrap FF 5 ml columns, mix 400 µl (400 units) of thrombin solution with 4.6 ml of PBS.

8c. 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 protease mix onto the column using a syringe and the connector supplied. Seal the column with the top cap and the stopper supplied.

10a. For PreScission Protease, incubate the column at 5°C for 4 h.

10b. For thrombin and Factor Xa, incubate the column at room temperature (22°C to 25°C) for 2 to 16 h.

The incubation times are starting points and may need to be changed for an optimal yield of cleaved target protein.

11. Fill a syringe with 3 ml (1 ml column) or 15 ml (5 ml column) of cleavage buffer. Remove the top cap and stopper from the column and attach the syringe. Avoid introducing air into the column.
12. Begin elution of the cleaved target protein. Maintain flow rates of 1 to 2 ml/min (1 ml column) or 5 to 10 ml/min (5 ml column), and collect the eluate (0.5 to 1 ml/tube for 1 ml column, 1 to 2 ml/tube for 5 ml column).

For PreScission Protease: The eluate will contain the protein of interest, while the GST moiety of the tagged protein and the PreScission Protease (also GST-tagged) will remain bound to the Glutathione Sepharose column. This means that the protein of interest will not be contaminated with protease and thus no additional purification will be required to purify the target protein from the protease.

For thrombin and Factor Xa: The eluate will contain the protein of interest and thrombin or Factor Xa, respectively, while the GST moiety of the tagged protein will remain bound to the Glutathione Sepharose column. Thrombin or Factor Xa can be removed from the protein of interest in one step using a HiTrap Benzamidine FF (high sub) column in series after the GSTrap column. In this process, the cleaved, tagged protein and thrombin or Factor Xa is washed from the GSTrap column onto the HiTrap Benzamidine FF (high sub) column. This second column captures the thrombin or Factor Xa, thus enabling the collection of free protein in the eluent. Refer to the application on page 87 for an example of the purification and on-column cleavage of GST-tagged SH2 domain using thrombin and GSTrap FF, with sample cleanup accomplished using HiTrap Benzamidine FF (high sub) column in series with GSTrap FF.

Application examples

1. 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 neuron-specific calcium-binding protein family, was cloned into a pGEX vector containing a PreScission Protease site adjacent to the GST tag. The expressed tagged 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-tagged protein, or free GST tag that could co-elute with the sample during the additional wash with binding buffer (Fig 5.3). For every gram of wet *E. coli* cells, 10 mg of pure, untagged hippocalcin was obtained.

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

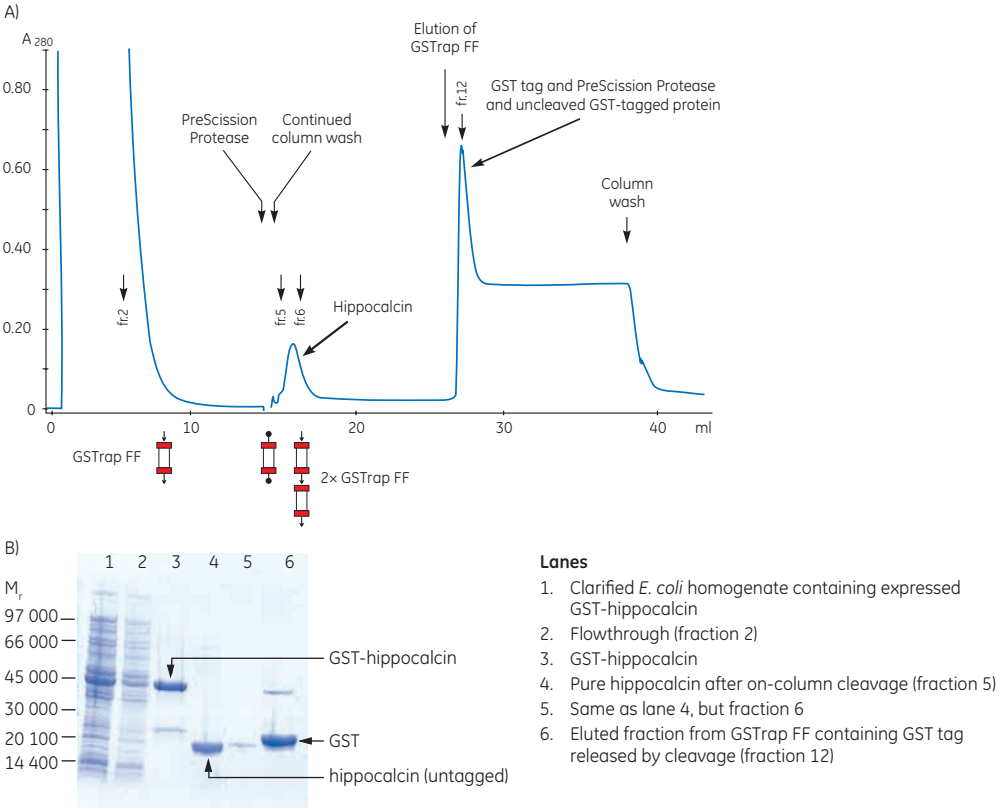


Fig 5.3. Purification of human hippocalcin-GST-tagged protein with on-column cleavage and post-cleavage removal of PreScission Protease using GSTrap FF columns. A) Chromatogram showing purification of hippocalcin. B) SDS-PAGE analysis of various sample processing steps. ExcelGel SDS Gradient, 8–18%, Coomassie blue staining.

2. Automatic removal of the GST tag with PreScission Protease

This example of automated tag removal uses ÄKTAxpress. All multistep purification protocols in ÄKTAxpress can be combined with automated on-column tag cleavage. Tag cleavage is always performed on the affinity column prior to further purification steps. When the cleaved protein has been eluted, the affinity column is regenerated and affinity tag, tagged protease, and remaining uncleaved protein are collected in a separate outlet. The procedure involves binding the tagged protein, injection of protease, incubation, elution of cleaved protein, and collection in capillary loop(s), followed by further purification steps.

The example in Figure 5.4 shows purification results for a GST-tagged protein, GST-purα (M_r 61 600), expressed in *E. coli*. The M_r of the cleaved product is 35 200. After harvest, cell lysis was performed by sonication. The samples were clarified by centrifugation prior to sample loading.

Affinity chromatography and gel filtration were performed on ÄKTAxpress using columns as indicated in the figure. The purity of each sample was analyzed by SDS-PAGE (Coomassie staining). The reduced samples were applied on an ExcelGel SDS-polyacrylamide gel.

Sample:	GST-pura, M_r 61 600 (cleaved product M_r 35 200)
Columns:	AC: GSTrap HP 5 ml GF: HiLoad 16/60 Superdex 75 pg, 120 ml
AC binding and cleavage buffer:	20 units of PreScission Protease/mg protein, 8 h incubation time in cold room 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5
AC elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
GF buffer:	50 mM Tris-HCl, 150 mM NaCl, pH 7.5
System:	ÄKTAxpress

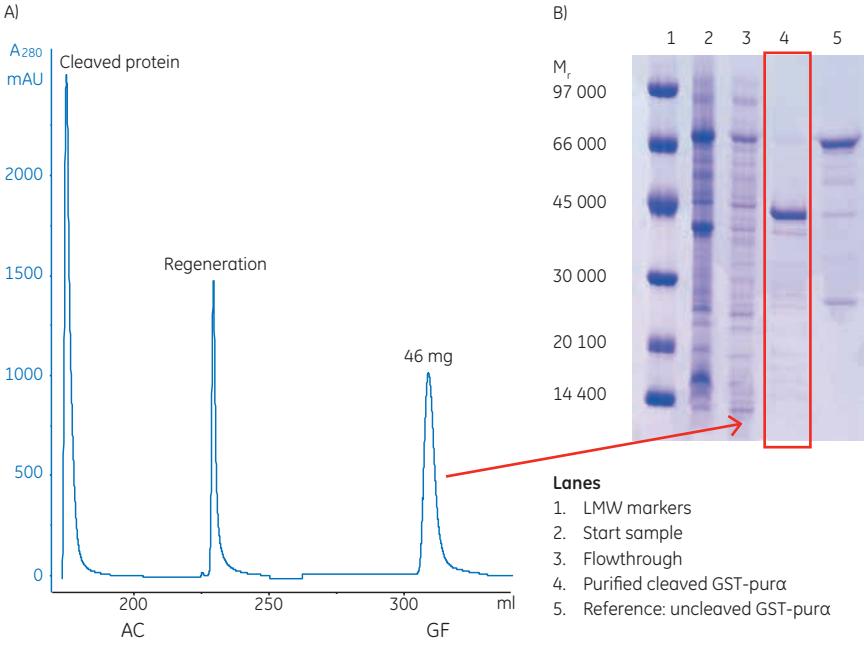


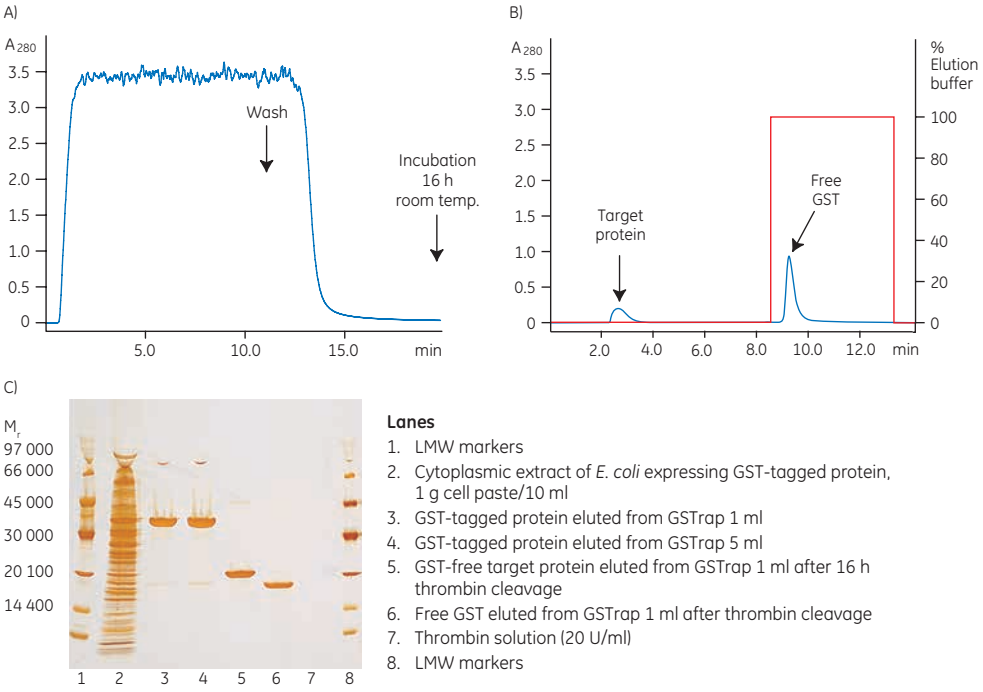
Fig 5.4. (A) Two-step protocol for automatic GST-tagged protein cleavage with PreScission Protease and purification. AC = affinity chromatography. GF = gel filtration. (B) Analysis by SDS-polyacrylamide gel (Coomassie staining) of the untagged target protein after purification and cleavage.

3. On-column cleavage of a GST-tagged protein using thrombin on a GSTrap FF column

To demonstrate the efficiency of on-column cleavage in conjunction with purification, a GST-tagged 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 PBS, pH 7.3) and sealed using the supplied connectors. After incubation for 16 h at room temperature, the target protein minus the GST moiety was eluted using PBS, pH 7.3, and the bound GST was subsequently eluted using elution buffer (Fig 5.5). The cleavage reaction yield was 100%. Intact GST-tagged protein was not detected in the eluate by SDS-PAGE and silver staining (see Fig 5.5C, lane 5).

Sample:	10 ml of clarified cytoplasmic extract from *E. coli* expressing a GST-tagged protein
Column:	GSTrap FF 1 ml
Binding buffer:	PBS, pH 7.3 (150 mM NaCl, 20 mM phosphate buffer)
Flow rate:	1 ml/min
Chromatographic procedure:	4 column volumes (CV) binding buffer, 10 ml sample, 10 CV binding buffer, fill column with 1 ml thrombin protease solution using a syringe
System:	ÄKTA

Column:	GSTrap FF 1 ml column after 16 h incubation with thrombin protease
Binding buffer:	PBS, pH 7.3 (150 mM NaCl, 20 mM phosphate buffer)
Elution buffer:	10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0
Flow rate:	1 ml/min
Chromatographic procedure:	8 column volumes (CV) binding buffer (elution of cleaved target protein), 5 CV elution buffer (elution of free GST and noncleaved GST-tagged protein), 5 CV binding buffer
System:	ÄKTA



Lanes

1. LMW markers
2. Cytoplasmic extract of *E. coli* expressing GST-tagged protein, 1 g cell paste/10 ml
3. GST-tagged protein eluted from GSTrap 1 ml
4. GST-tagged protein eluted from GSTrap 5 ml
5. GST-free target protein eluted from GSTrap 1 ml after 16 h thrombin cleavage
6. Free GST eluted from GSTrap 1 ml after thrombin cleavage
7. Thrombin solution (20 U/ml)
8. LMW markers

Fig 5.5. On-column thrombin cleavage of a GST-tagged protein. (A) Equilibration, sample application, and washing of a GST-tagged protein on GSTrap FF 1 ml were performed using ÄKTA chromatography system. After washing, the column was filled by syringe with 1 ml of thrombin (20 U/ml) and incubated for 16 h at room temperature. (B) GST-free target protein was eluted using PBS, pH 7.3. GST was eluted using 10 mM reduced glutathione. (C) SDS-PAGE followed by silver staining. The GST-free target protein fraction also contained a small amount of thrombin not detectable by SDS-PAGE (lane 6). The thrombin can be removed using a HiTrap Benzamidine FF (high sub) column.

4. Purification and on-column cleavage of TLP40-GST-tagged 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, and the GST-tagged proteins were purified from clarified lysates using two GSTrap FF 5 ml columns connected in series. After washing with PBS and equilibration with 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0, buffer flow was stopped.

For PreScission Protease digestion, 2 units of enzyme/100 µg of bound GST-tagged protein (diluted in 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0) was manually injected into the columns. Following injection, the columns were closed, sealed, and incubated for 12 to 16 h at 4°C.

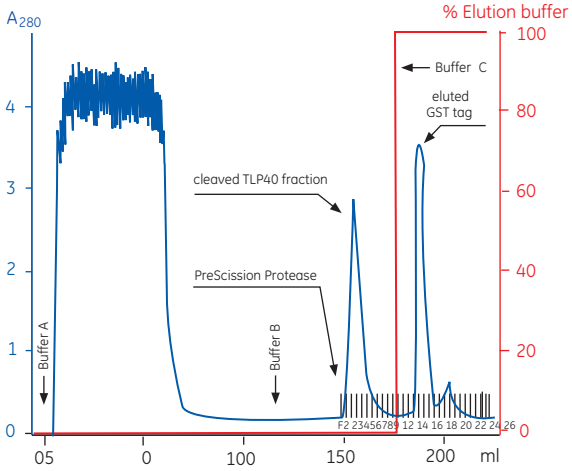
Prior to elution, a 1 ml GSTrap FF column was connected downstream to the GSTrap FF proteolytic cleavage columns to capture any released GST, uncleaved GST-tagged protein, and unbound PreScission Protease, whereas the cleaved protein was directly eluted.

After the target protein had been eluted, GST, unbound GST-tagged protein, and PreScission Protease were eluted with reduced glutathione (Fig. 5.6A). SDS-PAGE analysis of various fractions showed isolation of highly pure TLP40 after on-column cleavage (Fig 5.6B).

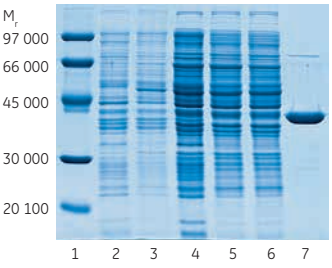
This application is reproduced with kind permission of Dr. Darcy Birse, University of Stockholm, Sweden.

A)

Sample:	50 ml of clarified lysate from *E. coli* containing TLP40-GST-tagged protein
Column:	2× GSTrap FF 5 ml columns connected in series
Binding buffer (A):	PBS, pH 7.4
Wash buffer (B):	50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0
Elution buffer (C):	Reduced glutathione buffer, pH 8.0
Instrument:	ÄKTA



B)



Lane 1: LMW markers
Lane 2: Total protein extract of noninduced culture
Lane 3: Total protein extract of induced culture
Lane 4: Supernatant after 70 000 × g centrifugation step
Lane 5: Supernatant after 300 000 × g centrifugation step
Lane 6: Flowthrough from GSTrap FF column
Lane 7: Native TLP40 cleavage product eluted after on-column digestion with PreScission Protease

Fig 5.6. Purification and SDS-PAGE analysis of TLP40-GST-tagged protein. A) Purification and on-column cleavage of tagged protein using GSTrap FF 5 ml and PreScission Protease in combination with ÄKTA chromatography system. The flow rate for sample loading and injecting the protease were 1 ml/min and 5 to 7 ml/min, respectively. B) Fractions from the purification steps were analyzed by SDS-PAGE using a 3.5% to 12% polyacrylamide gel. The gel was stained with Coomassie blue.

Cleavage and purification of GST-tagged protein eluted from GSTrap

Recommended buffers

Binding buffer: PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3

For PreScission Protease cleavage:

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

Cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0

PreScission Protease

For thrombin cleavage:

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

Cleavage buffer: 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 PBS prechilled to 4°C. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.

For Factor Xa cleavage:

Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
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 water to give a final solution of 1 unit/µl. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.

Purification and cleavage

The protocol below is an example optimized for 8 mg of target protein. It is worth estimating how much target protein is applied to the column, as this allows one to minimize the amount of protease added.

1. Fill the syringe or pump tubing with distilled water. Remove the stopper and connect the column to the syringe (use the connector supplied), laboratory pump, or chromatography system "drop to drop" to avoid introducing air into the system.
2. Remove the snap-off end at the column outlet.
3. Wash out the ethanol with 3 to 5 column volumes of distilled water.
4. Equilibrate the column with at least 5 column volumes of binding buffer. Recommended flow rates are 1 ml/min (1 ml column) and 5 ml/min (5 ml column).
5. Apply the pretreated sample using a syringe fitted to the Luer connector or by pumping it onto the column. For best results, use a flow rate of 0.2 to 1 ml/min (1 ml column) and 0.5 to 5 ml/min (5 ml column) during sample application.
6. Wash with binding buffer (generally at least 5 to 10 column volumes) until the absorbance reaches a steady baseline or no material remains in the effluent. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for washing.
7. Elute the GST-tagged protein with 5 to 10 column volumes of elution buffer. Maintain flow rates of 1 to 2 ml/min (1 ml column) or 1 to 5 ml/min (5 ml column). Collect the eluate (0.5 to 1 ml/tube for 1 ml column, 1 to 2 ml/tube for 5 ml column). Pool fractions containing the GST-tagged protein (monitored by UV absorption at A_{280}).
8. Remove the free reduced glutathione from the eluate using a quick buffer exchange on a desalting column, depending on the sample volume.

9a. For PreScission Protease, add 1 µl (2 units) of PreScission Protease for each 100 µg of tagged protein in the buffer-exchanged eluate.

9b. For thrombin and Factor Xa, add 10 µl (10 units) of thrombin or Factor Xa solution for each mg of tagged protein in the buffer-exchanged eluate.

10a. For PreScission Protease, incubate at 5°C for 4 h.

10b. For thrombin and Factor Xa, incubate at room temperature (22°C to 25°C) for 2 to 16 h.

The incubation times are starting points and may need to be changed for an optimal yield of cleaved target protein.

11. Once digestion is complete, apply the sample to an equilibrated GSTrap column as described above (steps 1 to 6) to remove the GST moiety of the tagged protein.

For PreScission Protease: The flowthrough will contain the protein of interest, while the GST moiety of the tagged protein and the PreScission Protease will remain bound to the Glutathione Sepharose column. This means that the protein of interest will not be contaminated with protease and thus no additional purification will be required to purify the target protein from the protease.

For thrombin and Factor Xa: The flowthrough will contain the protein of interest and thrombin or Factor Xa, respectively, while the GST moiety of the tagged protein will remain bound to the Glutathione Sepharose column. The thrombin or Factor Xa can be removed from the protein of interest in one step using a HiTrap Benzamidine FF (high sub) column in series after a GSTrap column. In this process, the cleaved, tagged protein and thrombin or Factor Xa is washed from the GSTrap column onto the HiTrap Benzamidine FF (high sub) column. This second column captures the thrombin or Factor Xa, thus enabling the collection of pure protease-free protein in the eluent. If GST GraviTrap is used, the eluted fraction is loaded with a syringe onto the HiTrap Benzamidine FF (high sub) column.

Cleavage and purification of GST-tagged protein bound to Glutathione Sepharose in batch mode

Glutathione Sepharose High Performance, Glutathione Sepharose 4 Fast Flow, and Glutathione Sepharose 4B can all be used for cleavage and purification of GST-tagged proteins in batch.

Recommended buffers

Binding buffer:	PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3
For PreScission Protease cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Cleavage buffer:	50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0
PreScission Protease	
For thrombin cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Cleavage buffer:	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 PBS prechilled to 4°C. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.
For Factor Xa cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
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 distilled water to give a final solution of 1 unit/µl. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.

Preparation of Glutathione Sepharose media and binding of protein

Glutathione Sepharose media are supplied in 20% ethanol. The media are used at a final slurry concentration of 50%.

1. Determine the bed volume of Glutathione Sepharose required for your purification.
2. Gently shake the bottle to resuspend the slurry.
3. Use a pipette or measuring cylinder to remove sufficient slurry for use and transfer to an appropriate container/tube.
4. Sediment the chromatography medium by centrifugation at 500 × g for 5 min. Carefully decant the supernatant.
5. Wash the Glutathione Sepharose by adding 5 ml of PBS per 1 ml of 50% slurry.

Glutathione Sepharose must be thoroughly washed with PBS to remove the ethanol storage solution because residual ethanol may interfere with subsequent procedures.

6. Sediment the chromatography medium by centrifugation at 500 × g for 5 min. Carefully decant the supernatant.
7. Repeat steps 5 and 6 once for a total of two washes.
8. Add the cell lysate to the prepared Glutathione Sepharose and incubate for at least 30 min at room temperature, using gentle agitation such as end-over-end rotation.

Purification and cleavage

Assume 8 mg of GST-tagged protein bound per ml of chromatography medium.

1. Wash the tagged-protein-bound Glutathione Sepharose with 10 bed volumes of cleavage buffer. Bed volume is equal to 0.5× the volume of the 50% Glutathione Sepharose slurry used.

2a. 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 cleavage buffer at 5°C.

2b. 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 cleavage buffer.

2c. 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 cleavage buffer.

3. Add the mixture to the Glutathione Sepharose. Gently shake or rotate the suspension end-over-end.

4a. For PreScission Protease, incubate at 5°C for 4 h.

4b. For thrombin or Factor Xa, incubate at room temperature (22°C to 25°C) for 2 to 16 h.

The incubation times in steps 4a and 4b are starting points and may need to be changed for an optimal yield of cleaved target protein.

5. Following incubation, wash out the untagged protein with approximately three bed volumes of cleavage buffer. Centrifuge the suspension at 500 × g for 5 min to pellet the Glutathione Sepharose. Carefully transfer the eluate to a tube.

For PreScission Protease: The eluate will contain the protein of interest, while the GST moiety of the tagged protein and the PreScission Protease will remain bound to the Glutathione Sepharose. This means that the protein of interest will not be contaminated with protease and thus no additional purification will be required to purify the target protein from the protease.

For thrombin and Factor Xa: The eluate will contain the protein of interest and thrombin or Factor Xa, respectively, while the GST moiety of the tagged protein will remain bound to the Glutathione Sepharose. The thrombin or Factor Xa can be removed from the protein of interest using HiTrap Benzamidine FF (high sub). This column captures the thrombin or Factor Xa, thus enabling the collection of pure protease-free protein in the eluent.

Removal of thrombin and Factor Xa using HiTrap Benzamidine FF (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.

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 such as ÄKTA. See Table 5.3 for a selection guide of purification options.

Characteristics of HiTrap Benzamidine FF (high sub) are summarized in Appendix 5.

Table 5.3. 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

Reagents required

Binding buffer:	0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4
Elution buffer alternatives for eluting the protease:	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-HCl (Gua-HCl) (denaturing solutions)

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

1. Fill the syringe or pump tubing with distilled water. Remove the stopper and connect the column to the syringe (use the connector supplied), laboratory pump, or chromatographic system "drop to drop" to avoid introducing air into the column.
2. Remove the snap-off end.
3. Wash the column with 5 column volumes of distilled water to remove the storage buffer (0.05 M acetate buffer, pH 4, containing 20% ethanol).
4. Equilibrate the column with 5 column volumes of binding buffer.
5. Apply the sample using a syringe fitted to the Luer connector 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 the flowthrough and reserve. It contains the protease-depleted material to be saved. Apply a small volume of extra binding buffer to collect all desired material from the column.
6. Wash the column with 5 to 10 column volumes of binding buffer, collecting fractions (0.5 to 1 ml fractions for 1 ml column and 1 to 3 ml fractions for 5 ml column) until no material appears in the effluent (monitored by UV absorption at 280 nm).

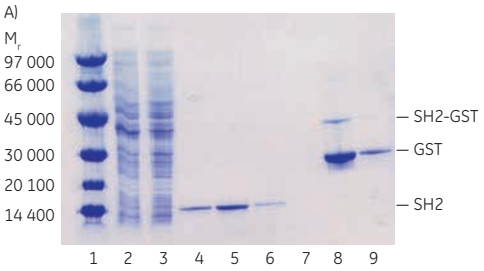
7. Pool fractions from flowthrough and/or wash that contain the thrombin- or Factor Xa-free material (monitored by UV absorption at 280 nm).
8. For reuse of column, elute the bound protease with 5 to 10 column volumes of the elution buffer of choice. If the eluted thrombin or Factor Xa is to be retained for reuse, buffer exchange the fractions containing the protease using a 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 reuse.
10. For longer-term storage, store in a buffer containing 20% ethanol in 0.05 M acetate buffer, pH 4, at 4°C to 8°C.

Thrombin activity can be followed by taking aliquots of the fractions and measuring at 405 nm using S-2238 (Chromogenix, Haemochrom Diagnostica AB; supplier in US is DiaPharma) as substrate.

Application example

Purification and on-column cleavage of GST-tagged SH2 domain using thrombin and GSTrap FF. Direct removal of thrombin using HiTrap Benzamidine FF (high sub) column in series with GSTrap FF

The following application describes the purification of GST-SH2 (M_r 37 000) on a GSTrap FF 1 ml column, followed by on-column cleavage with thrombin (Fig 5.7). After the thrombin incubation step, a HiTrap Benzamidine FF (high sub) 1 ml column was placed in series after the GSTrap FF column. As the columns were washed with binding buffer and later with high-salt buffer, the cleaved SH2-tagged 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 untagged target protein in the eluent (Fig 5.7A). 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 5.7B). This entire procedure could be completed in less than one day.



Lanes

1. LMW markers
2. Clarified *E. coli* homogenate containing SH2-GST-tagged protein with a thrombin protease cleavage site
3. Flowthrough from GSTrap FF (fraction 2)
4. SH2 domain (GST tag cleaved off), washed out with binding buffer through both columns (fraction 6)
5. Same as lane 4 (fraction 7)
6. Same as lane 4 (fraction 8)
7. Elution of thrombin protease from HiTrap Benzamidine FF (high sub)
8. Elution of GST tag and some noncleaved SH2-GST from GSTrap FF (fraction 21)
9. Same as lane 8 (fraction 22)

Sample:	2 ml of clarified *E. coli* homogenate containing GST-SH2 (M_r 37 000) with a thrombin protease 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 (GE Healthcare) for 2 h at room temperature
Thrombin protease activity:	Measured at 405 nm using S-2238 (Chromogenix, Haemochrom Diagnostica AB; supplier in US is DiaPharma) as substrate

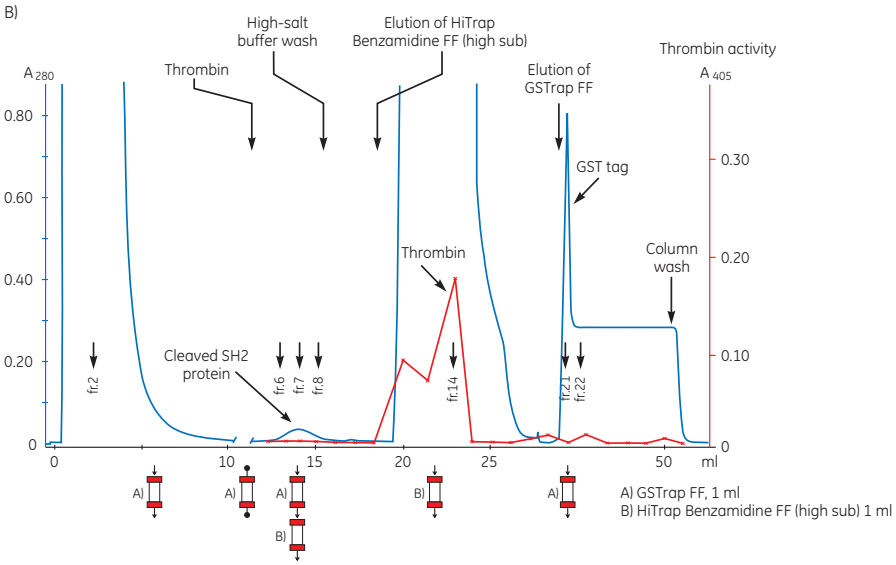


Fig 5.7. Purification of GST-SH2 GST-tagged protein with on-column cleavage and post-cleavage removal of thrombin using GSTrap FF and HiTrap Benzamidine FF (high sub) columns. (A) SDS-PAGE analysis of various sample processing steps. ExcelGel SDS Gradient 8–18%, Coomassie blue staining. (B) Chromatogram (blue: absorbance at 280 nm) and thrombin activity curve (red) demonstrating all steps in the purification of the SH2 domain.

Troubleshooting of cleavage methods

The troubleshooting guide below addresses problems common to the majority of cleavage methods as well as problems specific to a particular method. In the latter case, the relevant method is indicated.

Problem	Possible cause	Solution
GST-tagged proteins are not cleaved completely.	The ratios of PreScission Protease, thrombin, or Factor Xa to GST-tagged protein are not optimal.	For PreScission Protease and thrombin, use at least 10 units/mg of tagged protein. For Factor Xa, use an amount equivalent to at least 1% (w/w) of the weight of tagged protein. For some tagged proteins, up to 5% Factor Xa can be used. The optimal amount must be determined empirically. In some cases, optimal results have been achieved with a tagged protein concentration of 1 mg/ml. The addition of ~0.5% SDS (w/v) to the reaction buffer can significantly improve Factor Xa cleavage with some tagged proteins. Various concentrations of SDS should be tested to determine the optimal concentration.
	The incubation time is not sufficient for complete cleavage of the protein from the GST tag.	Increase the incubation time for the cleavage reaction. Increasing the reaction time to 20 h or more should improve cleavage as long as the tagged protein is not degraded by the extended incubation period.
	Specific cleavage sites for the proteases have been altered during cloning of the tagged protein.	Verify the presence of specific enzyme cleavage sites. Check the DNA sequence of the construct and compare it with a known sequence to verify that the cleavage sites have not been altered.
	The presence of cleavage enzyme inhibitors is interfering with the cleavage reaction.	Remove any enzyme inhibitors that may interfere with the cleavage reaction. Prior to cleavage with PreScission Protease, buffer exchange or dialyze the tagged protein against 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5. Prior to cleavage with Factor Xa, buffer exchange the tagged protein on a desalting column, or dialyze against 50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5.
	Factor Xa is not properly activated.	Factor Xa protease is preactivated. If using a protease from another source, activate Factor Xa protease with Russell´s viper venom to generate functional enzyme. For activation of Factor Xa protease, incubate Russell´s viper venom with Factor Xa protease at a ratio of 1% in 8 mM Tris-HCl, 70 mM NaCl, 8 mM $CaCl_2$, pH 8.0. Incubate at 37°C for 5 min.
	The first amino acid after the Factor Xa protease recognition sequence is Arg or Pro.	Check the sequence of the tagged protein to verify that the first three nucleotides after the Factor Xa protease recognition sequence do not code for Arg or Pro.
Multiple bands are observed after electrophoresis/ Western blotting analysis of the cleaved target protein	Proteolysis is occurring in the host bacteria prior to the cleavage reaction.	Determine when the extra bands appear. Verify that additional bands are not present prior to PreScission Protease, thrombin, or Factor Xa protease cleavage.
	The tagged protein itself contains recognition sequences for PreScission Protease, thrombin protease, or Factor Xa protease.	Check the sequence of the tagged protein to determine if it contains recognition sequences for the cleavage enzymes.
The tagged partner is contaminated with protease after purification	Glutathione Sepharose may have been saturated with GST-tagged protein during purification.	Pass the sample over a new GSTrap column or fresh Glutathione Sepharose to remove residual PreScission Protease, or over a HiTrap Benzamidine (high sub) column in the case of thrombin protease or Factor Xa protease.

Appendix 1
Characteristics of GST and of host bacterial strain

Table A1.1. Properties of GST

GST 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.

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

Table A1.2. Properties and handling of *E. coli* BL21

Genotype	F-, ompT, hsdS (rB-, mB-), gal, dcm.
Growth conditions	Resuspend lyophilized cultures in 1 ml of LB medium[1]. 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 to a final concentration of 15% 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 for expression of GST-tagged proteins. Use an alternate strain for cloning and maintenance of the vector (such as JM109 or other recA- strains).

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

Table A2.1. Selection Guide – pGEX Vector Control Regions

	pGEX-1λT EcoRI/BAP 28-9546-56	pGEX-2T 28-9546-53	pGEX-2TK 28-9546-46	pGEX-4T-1 28-9545-49	pGEX-4T-2 28-9545-50	pGEX-4T-3 28-9545-52	pGEX-3X 28-9546-54	pGEX-5X-1 28-9545-53	pGEX-5X-2 28-9545-54	pGEX-5X-3 28-9545-55	pGEX-6P-1 28-9546-48	pGEX-6P-2 28-9546-50	pGEX-6P-3 28-9546-51
GST Region													
tac promoter													
–10	205-211	205-211	205–211	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	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	217–237	217–237	217–237
Ribosome binding site for GST	244	244	244	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	258	258	258
Coding region for thrombin cleavage	918-935	918-935	918–935	918–935	918–935	918–935	NA	NA	NA	NA	NA	NA	NA
Coding region for Factor Xa cleavage	NA	NA	NA	NA	NA	NA	921-932	921–932	921–932	921–932	NA	NA	NA
Coding region for PreScission Protease cleavage	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	918–938	918–938	918–938
Coding for kinase recognition site	NA	NA	936–950	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Multiple Cloning Site	930-944	930-945	951–966	930–966	930–967	930–965	934-949	934–969	934–970	934–971	945–981	945–982	945–980
β-lactamase (Amp^r) Gene Region													
Promoter													
–10	1308-1313	1309-1314	1330–1335	1330–1335	1331–1336	1329–1334	1313-1318	1333–1338	1334–1339	1335–1340	1345–1350	1346–1351	1344–1349
–35	1285-1290	1286-1291	1307–1312	1307–1312	1308–1313	1306–1311	1290-1295	1310–1315	1311–1316	1312–1317	1322–1327	1323–1328	1321–1326
Start codon (ATG)	1355	1356	1377	1377	1378	1376	1360	1380	1381	1382	1392	1393	1391
Stop codon (TAA)	2213	2214	2235	2235	2236	2234	2218	2238	2239	2240	2250	2251	2249
***LacI^q* Gene Region**													
Start codon (GTG)	3296	3297	3318	3318	3319	3317	3301	3321	3322	3323	3333	3334	3332
Stop codon (TGA)	4376	4377	4398	4398	4399	4397	4381	4401	4402	4403	4413	4414	4412
Plasmid Replication Region													
Site of replication initiation	2973	2974	2995	2995	2996	2994	2978	2998	2999	3000	3010	3011	3009
Region necessary for replication	2280-2976	2281-2977	2302–2998	2302–2998	2303–2999	2301–2997	2285-2981	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	869–891	869–891	869–891
3′ pGEX Sequencing Primer binding	1019-997	1020-998	1041–1019	1041–1019	1042–1020	1040–1018	1024-1002	1044–1022	1045–1023	1046–1024	1056–1034	1057–1035	1055–1033
GenBank Accession Number	**U13849**	**U13850**	**U13851**	**U13853**	**U13854**	**U13855**	**U13852**	**U13856**	**U13857**	**U13858**	**U78872**	**U78873**	**U78874**

Complete DNA sequences and restriction site data are available at the GE Healthcare web site (http:// www.gelifesciences.com).

Appendix 3
Electroporation

Preparation of cells

Reagents required

2× YT medium:	Dissolve 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl in 900 ml of distilled water. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 l with distilled water. Sterilize by autoclaving for 20 min. To prepare as a solid medium, add 1.2% to 1.5% agar.
1 mM HEPES:	0.26 g of HEPES, sodium salt. Dissolve in 900 ml of distilled water. Adjust the pH to 7.0. Adjust the volume to 1 l with distilled water. Sterilize by autoclaving.
10% glycerol in 1 mM HEPES, pH 7.0:	Aseptically add 10 ml of sterile 100% glycerol to 90 ml of sterile 1 mM HEPES, pH 7.0.
10% glycerol in distilled water:	Add 10 ml of 100% glycerol to 90 ml of distilled water. 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
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%	

Procedure

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 of an overnight culture of host cells. Incubate for 2 to 2.5 h at 37°C with shaking at 250 rpm until an A_{600} of 0.5 to 0.7 is achieved.
3. Place the flask on ice for 15 to 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 to 3 ml of sterile 10% glycerol in distilled water.
9. Dispense in 50 to 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 water. Alternatively, the DNA can be gel band-purified.

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

Electroporation

One nanogram of uncut (supercoiled) vector DNA is recommended to be transformed in parallel with insert/pGEX ligations to determine the efficiency of each competent cell preparation. For more information about electroporation protocols, see the instructions for the selected electroporation system.

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 13 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-1λT EcoRI/BAP	1019-997
pGEX-2T	1024-998
pGEX-2TK	1041–1019
pGEX-4T-1	1041–1019
pGEX-4T-2	1042–1020
pGEX-4T-3	1040–1018
pGEX-3X	1024-1002
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
Characteristics of Glutathione Sepharose and HiTrap Benzamidine FF (high sub) media and columns

Glutathione Sepharose High Performance is recommended for high-resolution purification of GST-tagged proteins, providing sharp peaks and concentrated eluent. Glutathione Sepharose 4 Fast Flow is excellent for scaling up. Glutathione Sepharose 4B has high capacity and is recommended for packing small columns and other formats including batch purifications.

Table A5.1 summarizes key characteristics of these three Glutathione Sepharose media, and Tables A5.2 to A5.6 summarize the characteristics of these media prepacked in columns and in 96-well filter plates. Table A5.7 summarizes key characteristics of HiTrap Benzamidine Fast Flow (high sub).

Table A5.1. Characteristics of Glutathione Sepharose High Performance, Glutathione Sepharose 4 Fast Flow, and Glutathione Sepharose 4B

Characteristics	Glutathione Sepharose High Performance	Glutathione Sepharose 4 Fast Flow	Glutathione Sepharose 4B
Matrix	Highly cross-linked 6% agarose	Highly cross-linked 4% agarose	4% agarose
Average particle size	34 µm	90 µm	90 µm
Ligand concentration	1.5–3.5 mg glutathione/ml medium (based on Gly)	120–320 µmol glutathione/ml medium	200–400 µmol glutathione/g washed and dried medium
Binding capacity[1]	> 7 mg recombinant GST/ml medium	> 10 mg recombinant GST/ml medium	> 25 mg horse liver GST/ml medium
Recommended flow velocity[2]	< 150 cm/h	50–300 cm/h	< 75 cm/h
Chemical stability	Stable to all commonly used aqueous buffers, e.g., 1 M acetate, pH 4.0 and 6 M Gua-HCl for 1 h at room temperature	Stable to all commonly used aqueous buffers, e.g., 1 M acetate, pH 4.0, and 6 M Gua-HCl for 1 h at room temperature	Stable to all commonly used aqueous buffers, e.g., 0.1 M NaOH, 70% ethanol, or 6 M Gua-HCl for 2 h at room temperature or exposure to 1% (w/v) SDS for 14 d.
pH stability	3–12	3–12	4–13
Storage temperature	4°C to 30°C	4°C to 30°C	4°C to 30°C
Storage buffer	20% ethanol	20% ethanol	20% ethanol

[1] *The binding of GST-tagged proteins depends on size, conformation, and concentration of the protein in the sample loaded. Binding of GST to glutathione is also flow dependent, and lower flow rates often increase the binding capacity. This is important during sample loading. Protein characteristics, pH, temperature, and the media used may also affect the binding capacity.*

[2] *When using water at room temperature.*

Table A5.2. Characteristics of GST MultiTrap FF and GST MultiTrap 4B

Chromatography media	GST MultiTrap FF: Glutathione Sepharose 4 Fast Flow GST MultiTrap 4B: Glutathione Sepharose 4B
Filter plate size[1]	127.8 × 85.5 × 30.6 mm
Filter plate material	Polypropylene and polyethylene
Binding capacity	GST MultiTrap FF: Up to 0.5 mg GST-tagged protein/well GST MultiTrap 4B: Up to 0.5 mg GST-tagged protein/well
Reproducibility between wells[2]	+/- 10%
Volume packed medium/well	50 µl (500 µl of 10% slurry)
Number of wells	96
Centrifugation speed:	Depends on sample pretreatment and sample properties
Recommended	100–500 × g
Maximum	700 × g
Vacuum pressure:	Depends on sample pretreatment and sample properties
Recommended	-0.1 to -0.3 bar
Maximum	-0.5 bar
pH stability	Glutathione Sepharose 4 Fast Flow: 3–12 Glutathione Sepharose 4B: 4–13
Storage	20% ethanol
Storage temperature	4°C to 30°C

[1] *According to American National Standards Institute (ANSI) and Society for Biomolecular Screening (SBS). 1-2004, 3-2004, and 4-2004 standards.*

[2] *The amount of eluted target proteins/well does not differ more than +/- 10% from the average amount/well for the entire filter plate.*

Table A5.3. Characteristics of prepacked GSTrap HP, GSTrap FF, and GSTrap 4B columns

Characteristics	**GSTrap HP**	**GSTrap FF**	**GSTrap 4B**
Chromatography media	Glutathione Sepharose High Performance	Glutathione Sepharose 4 Fast Flow	Glutathione Sepharose 4B
Average particle size	34 µm	90 µm	90 µm
Dynamic binding capacity[1,2]	Approx. 7 mg rGST/ml medium	Approx. 10 mg rGST/ml medium	Approx. 25 mg horse liver GST/ml medium
Max. back pressure[3]	0.3 MPa, 3 bar	0.3 MPa, 3 bar	0.3 MPa, 3 bar
Recommended flow rate[3]	Sample loading: 0.2–1 ml/min (1 ml) and 1–5 ml (5 ml) Washing and elution: 1–2 ml/min (1 ml column) and 5–10 ml/min (5 ml column)	Sample loading: 0.2–1 ml/min (1 ml) and 1–5 ml (5 ml) Washing and elution: 1–2 ml/min (1 ml column) and 5–10 ml/min (5 ml column)	Sample loading: 0.2–1 ml/min (1 ml) and 0.5–5 ml/min (5 ml) Washing and elution: 1 ml/min (1 ml column) and 5 ml/min (5 ml column)
Chemical stability	Stable to all commonly used aqueous buffers, e.g. 1 M acetate, pH 4.0 and 6 M Gua-HCl for 1 h at room temperature	Stable to all commonly used aqueous buffers, e.g. 1 M acetate, pH 6.0, and 6 M Gua-HCl for 1 h at room temperature	Stable to all commonly used aqueous buffers. Exposure to 0.1 M NaOH, 70% ethanol, or 6 M Gua-HCl for 2 h at room temperature or to 1% (w/v) SDS for 14 d causes no significant loss of activity.

continues on following page

Table A5.3. Characteristics of prepacked GSTrap HP, GSTrap FF, and GSTrap 4B columns *(continued)*

Characteristics	GSTrap HP	GSTrap FF	GSTrap 4B
pH stability	3–12	3–12	4–13
Storage temperature	4°C to 30°C	4°C to 30°C	4°C to 30°C
Storage buffer	20% ethanol	20% ethanol	20% ethanol

The column dimensions are identical for all three GSTrap columns (0.7 × 2.5 cm for the 1 ml column and 1.6 × 2.5 cm for the 5 ml column). Column volumes are 1 ml and 5 ml.

[1] *The binding of GST-tagged proteins depends on size, conformation, and concentration of the protein in the sample loaded. Binding of GST to glutathione is also flow dependent, and lower flow rates often increase the binding capacity. This is important during sample loading. Protein characteristics, pH, temperature, and the media used may also affect the binding capacity.*

[2] *Dynamic binding capacity conditions (60% breakthrough):*
Sample: *1 mg/ml pure GST-tagged protein in binding buffer*
Column volume: *0.4 ml*
Flow rate: *0.2 ml/min (60 cm/h)*
Binding buffer: *10 mM sodium phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4*
Elution buffer: *50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0*

[3] *When using water at room temperature.*

Table A5.4. Characteristics of GSTPrep FF 16/10

Chromatography medium	Glutathione Sepharose 4 Fast Flow
Column volume	20 ml
Column dimensions	1.6 × 10 cm
Dynamic binding capacity[1,2]	Approx. 200 mg rGST/column
Recommended flow rate[3]	1–10 ml/min (30–300 cm/h)
Max. flow rate[3]	10 ml/min (300 cm/h)
Max. pressure over the packed bed during operation[3]	1.5 bar (0.15 MPa, 22 psi)
Column hardware pressure limit	5 bar (0.5 MPa, 73 psi)
Storage	20% ethanol
Storage temperature	4°C to 30°C

[1] *The binding of GST-tagged proteins depends on size, conformation, and concentration of the protein in the sample loaded. Binding of GST to glutathione is also flow dependent, and lower flow rates often increase the binding capacity. This is important during sample loading. Protein characteristics, pH, temperature, and the media used may also affect the binding capacity.*

[2] *Dynamic binding capacity conditions (60% breakthrough):*
Sample: *1 mg/ml pure GST-tagged protein in binding buffer*
Column volume: *0.4 ml*
Flow rate: *0.2 ml/min (60 cm/h)*
Binding buffer: *10 mM sodium phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4*
Elution buffer: *50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0*

[3] *When using water at room temperature.*

Table A5.5. Characteristics of GST GraviTrap prepacked columns

Column material frits	Polypropylene barrel, polyethylene
Column volume	13 ml
Medium	Glutathione Sepharose 4B
Average bead size	90 µm
Ligand	Glutathione and 10-carbon linker arm
Ligand concentration	7–15 µmol glutathione/ml medium
Protein binding capacity[1]	Approx. 50 mg horse liver GST/column
Bed volume	2 ml
Compatibility during use	All commonly used aqueous buffers
Chemical stability	No significant loss of the capacity is detected when Glutathione Sepharose 4B is exposed to 0.1 M citrate (pH 4.0), 0.1 M NaOH, 70% ethanol, or 6 M Gua-HCl[2] for 2 h at room temperature. No significant loss of binding capacity is observed after exposure to 1% SDS for 14 d.
Storage solution	20% ethanol
pH stability	4–13
Storage temperature	4°C to 30°C

Note: It is not recommended to autoclave the columns.

[1] *Binding capacity is protein dependent. The binding of GST-tagged proteins depends on size, conformation, and concentration of the protein in the sample. Binding of GST to glutathione is also flow dependent, and lower flow rates often increase the binding capacity. This is important during sample loading. Protein characteristics, pH, and temperature may also affect the binding capacity.*

[2] *Exposing the sample to 6 M Gua-HCl will denature the GST tag. It is therefore important to remove all Gua-HCl before use.*

Table A5.6. Characteristics of GST SpinTrap columns

Column material	Polypropylene barrel, polyethylene frits
Column volume	900 µl
Medium	Glutathione Sepharose 4B
Average bead size	90 µm
Ligand	Glutathione and 10-carbon linker arm
Ligand concentration	7–15 µmol glutathione/ml medium
Protein binding capacity[1]	Approx. 500 µg horse liver GST/column
Bed volume	50 µl
Compatibility during use	All commonly used aqueous buffers
Chemical stability	No significant loss of the capacity is detected when Glutathione Sepharose 4B is exposed to 0.1 M citrate (pH 4.0), 0.1 M NaOH, 70% ethanol or 6 M Gua-HCl[2] for 2 h at room temperature. No significant loss of binding capacity is observed after exposure to 1% SDS for 14 d.
Storage solution	PBS and 0.05% Kathon™ CG/ICP Biocide
pH stability	4–13
Storage temperature	4°C to 30°C

[1] *Binding capacity is protein dependent. The binding of GST-tagged proteins depends on size, conformation, and concentration of the protein in the sample loaded. Binding of GST to glutathione is also flow dependent, and lower flow rates often increase the binding capacity. This is important during sample loading. Protein characteristics, pH, and temperature may also affect the binding capacity.*

[2] *Exposing the sample to 6 M Gua-HCl will denature the GST tag. It is therefore important to remove all Gua-HCl before use.*

Table A5.7. Characteristics of HiTrap Benzamidine FF (high sub)

Column dimensions (i.d. × h)	0.7 × 2.5 cm (1 ml column) and 1.6 × 2.5 cm (5 ml column)
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
Average 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[1]	pH 1–9
pH stability long term[1]	pH 2–8
Storage temperature	4°C to 8°C
Storage buffer	20% ethanol in 0.05 M acetate buffer, pH 4

[1] *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.*

Appendix 6
Column packing and preparation

This appendix provides column packing guidelines for affinity chromatography media. Efficient column packing is essential for a good separation, especially when using gradient elution. A poorly packed column gives rise to poor and uneven flow, peak broadening, and loss of resolution. Prepacked GST columns from GE Healthcare will ensure reproducible results and the highest performance. If column packing is required, the manufacturer's instructions should be consulted. The following guidelines apply at all scales of operation. The following parameters should be chosen before packing a column.

Selecting column size

When using an affinity binding technique, or when using a low flow velocity, select short, wide columns (typically 5 to 15 cm bed height) for rapid purification.

Selecting amount of medium

The amount of chromatography medium required depends on the medium's binding capacity and the amount of sample to be purified. GST media are supplied with instructions detailing the binding capacity per ml of medium. Estimate the amount of medium required to bind the target protein and use two to five times this amount to pack the column. An excess of medium is used to ensure a maximum binding of the sample, since the binding capacity is influenced by the nature of the sample and may differ between different samples.

GST media can be packed in Tricorn™, XK, or HiScale™ columns available from GE Healthcare. A step-by-step demonstration of column packing can be seen in "Column Packing — The Movie," available in CD format (see Ordering information).

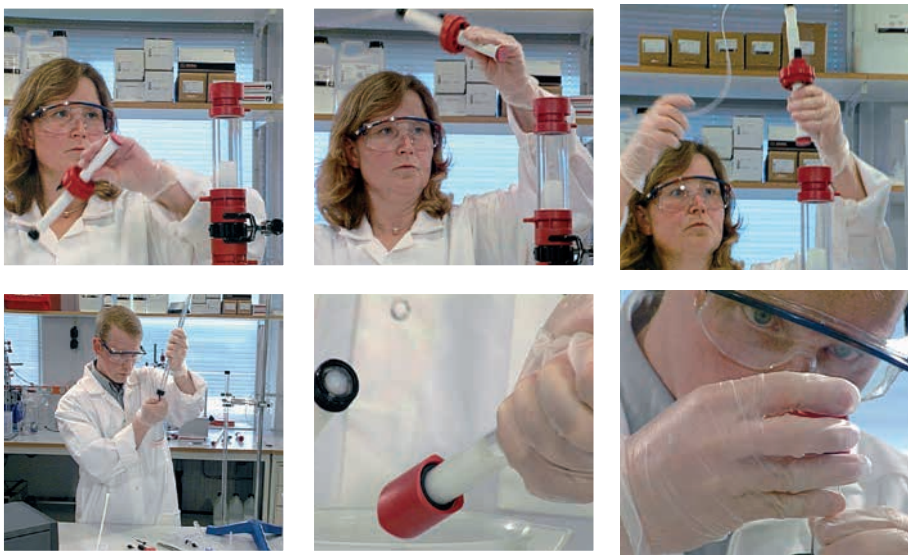


Fig A6.1. "Column Packing — The Movie" provides a step-by-step demonstration of column packing.

Selecting column type

Tricorn, XK, and HiScale columns are fully compatible with the high flow rates allowed with GST media, and a broad range of column dimensions are available (see Table A6.1). In most cases the binding capacity of the medium and the amount of sample to be purified determine the column size required. Also, empty disposable PD-10 columns are available for single-use applications using gravity flow. For a complete listing of available columns, refer to www.gelifesciences.com/protein-purification.

Table A6.1. Column bed volumes and heights[1]

	Column size			
	i.d. (mm)	**Length (cm)**	**Bed volume (ml)**	**Bed height (cm)**
Tricorn 5/20	5	2	0.10–0.57	0.5–2.9
Tricorn 5/50	5	5	0.69–1.16	3.5–5.9
Tricorn 5/100	5	10	1.67–2.14	8.5–10.9
Tricorn 10/20	10	2	0–2.29	0–2.9
Tricorn 10/50	10	5	2.29–4.64	2.9–5.9
Tricorn 10/100	10	10	7.54–8.48	7.9–10.9
XK 16/20	16	20	5–31	2.5–15.5
XK 16/40	16	40	45–70	22.5–35
XK 26/20	26	20	5.3–66	1–12.5
XK 26/40	26	40	122–186	23–35
XK 50/20	50	20	0–274	0–14
XK 50/30	50	30	265–559	14–28
HiScale 16/20	16	20	0–40	0–20
HiScale 16/40	16	40	16–80	8–40
HiScale 26/20	26	20	0–106	0–20
HiScale 26/40	26	40	69–212	13–40
HiScale 50/20	50	20	0–393	0–20
HiScale 50/40	50	40	274–785	14–40
Empty Disposable PD-10[2]	15	7.4	8.3	4.8–5.0

[1] *All Tricorn and XK column specifications are valid when one adapter is used. All HiScale specifications are valid when two adapters are used.*

[2] *For gravity-flow applications. Together with LabMate Buffer Reservoir (see Ordering information), up to 25 ml of buffer and/or sample can be applied.*

Column packing procedure

This procedure describes column packing in 20% ethanol. Packing can also be performed in water or buffers. However, the advantage of packing in 20% ethanol is that no decanting and exchange of storage solution is needed (simplified handling). In addition, the column can be stored in the 20% ethanol until use.

The medium is supplied preswollen in 20% ethanol and has a slurry concentration of 75% (settled medium).

1. Equilibrate all materials to the temperature at which the packing is to be performed.
2. Eliminate air by flushing the column end pieces with 20% ethanol. Ensure that no air is trapped under the column net. Close the column outlet leaving 1 to 2 cm filled with 20% ethanol in the column.
3. Gently resuspend the medium. (Avoid using magnetic stirrers because they may damage the matrix.)
4. Estimate the amount of slurry (resuspended medium) required. Example for a bed volume of 5 ml: use 6.7 ml of slurry (5 ml divided by the slurry concentration 0.75). Add additional 20% ethanol to achieve a 1:1 ratio of settled medium and overlayed 20% ethanol (= slurry concentration ~50%), and mix gently.
5. Pour the resuspended volume of the slurry into the column. Pouring down a glass rod held against the wall of the column will minimize the introduction of air bubbles.

When the slurry volume is greater than the total volume of the column, connect a second glass column to act as a reservoir (see Ordering information for details). This ensures that the slurry has a constant diameter during packing, minimizing turbulence and improving the column packing conditions.

6. Fill the column with 20% ethanol immediately.
7. Mount the column top piece and connect to a pump.
8. Open the column outlet and set the pump to the desired flow rate (Tables A6.2 to A6.4).

If the recommended flow rate is higher than the pump capacity, use the maximum flow rate that the pump can deliver. Do not exceed the maximum operating pressure of the medium or column.

9. Maintain the packing flow rate for at least 3 column volumes and after a constant bed height has been obtained. Mark the bed height on the column.
10. Stop the pump and close the column outlet. Remove the top piece (and the second glass column if that has been used) and carefully fill the rest of the column with 20% ethanol to form a convex surface at the top.
11. Insert the adapter into the column at an angle, ensuring that no air is trapped under the net.
12. Slide the adapter slowly down the column (the outlet of the adapter should be open) until it reaches to the mark. Lock the adapter in position.
13. Connect the column to the pump and set the pump to the desired flow rate (Tables A6.2 to A6.4). Reposition the adapter if necessary. The column can be stored in 20% ethanol until use.

Before the first chromatography run: Wash the column with 5 column volumes of water followed by 5 column volumes of binding buffer (see maximum flow rate, Table A6.5).

Recommended flow rates during column packing

Table A6.2. Recommended packing flow rates (ml/min) for GST Sepharose High Performance medium in 20% ethanol at room temperature. Ideally, GST Sepharose High Performance is packed in an XK, Tricorn, or HiScale column in a two-step procedure

	Packing flow rate (ml/min)	
	Step 1 (75 cm/h)	**Step 2 (200 cm/h)**
Tricorn 5/20[1]	0.25	0.6
Tricorn 5/50[1]	0.25	0.6
Tricorn 10/20[2]	1	2.6
Tricorn 10/50[2]	1	2.6
Tricorn 10/100[2]	1	2.6
XK 16/20[3] or HiScale 16/20[4]	2.5	6.7
XK 26/20[3] or HiScale 26/20[4]	6.5	17.7
XK50/20[5] or HiScale 50/20[4]	25	65

Max operating pressure:
1 *100 bar*
2 *50 bar*
3 *5 bar*
4 *20 bar*
5 *3 bar*

Table A6.3. Recommended packing flow rates (ml/min) for GST Sepharose 4 Fast Flow medium in 20% ethanol at room temperature

	Packing flow rate (ml/min)	
	Step 1 (60 cm/h)	**Step 2 (250 cm/h)**
Tricorn 5/20[1]	0.2	0.8
Tricorn 5/50[1]	0.2	0.8
Tricorn 10/20[2]	0.8	3.3
Tricorn 10/50[2]	0.8	3.3
Tricorn 10/100[2]	0.8	3.3
XK 16/20[3] or HiScale 16/20[4]	2	8.4
XK 26/20[3] or HiScale 26/20[4]	5.3	22
XK50/20[5] or HiScale 50/20[4]	20	82

Max operating pressure:
1 *100 bar*
2 *50 bar*
3 *5 bar*
4 *20 bar*
5 *3 bar*

Table A6.4. Recommended packing flow rates (ml/min) for GST Sepharose 4B medium in 20% ethanol at room temperature

	Packing flow rate (ml/min)	
	Step 1 (30 cm/h)	**Step 2 (60 cm/h)**
Tricorn 5/20[1]	0.1	0.2
Tricorn 5/50[1]	0.1	0.2
Tricorn 10/20[2]	0.4	0.8
Tricorn 10/50[2]	0.4	0.8
Tricorn 10/100[2]	0.4	0.8
XK 16/20[3] or HiScale 16/20[4]	1	2
XK 26/20[3] or HiScale 26/20[4]	2.7	5.3
XK50/20[5] or HiScale 50/20[4]	10	20

Max operating pressure:
1 *100 bar*
2 *50 bar*
3 *5 bar*
4 *20 bar*
5 *3 bar*

Table A6.5. Maximum running flow rates (ml/min) for aqueous buffers and solutions at room temperature

	Maximum flow rate (ml/min)		
	Sepharose High Performance	**Sepharose 4 Fast Flow**	**Sepharose 4B**
	(150 cm/h)	**(200 cm/h)**	**(75 cm/h)**
Tricorn 5/20[1]	0.5	0.65	0.25
Tricorn 5/50[1]	0.5	0.65	0.25
Tricorn 10/20[2]	2	2.6	1
Tricorn 10/50[2]	2	2.6	1
Tricorn 10/100[2]	2	2.6	1
XK 16/20[3] or HiScale 16/20[4]	5	6.7	2.5
XK 26/20[3] or HiScale 26/20[4]	13	18	6.5
XK50/20[5] or HiScale 50/20[4]	50	65	25

Max operating pressure:

1 *100 bar*
2 *50 bar*
3 *5 bar*
4 *20 bar*
5 *3 bar*

Appendix 7
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 tagged protein sample. Use the following protocols to prepare an immobilized *E. coli* lysate that can be used to cross-adsorb anti-*E. coli* antibodies. The antibody available from GE Healthcare 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 lysate

Reagents required

2× YT medium:	Dissolve 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl in 900 ml of distilled water. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 l with distilled water. Sterilize by autoclaving for 20 min. To prepare as a solid medium, add 1.2% to 1.5% agar.
Coupling buffer:	Dissolve 29 g of NaCl and 8.4 g of $NaHCO_3$ in 800 ml of distilled water. Adjust the pH to 8.3 with HCl. Bring final volume to 1 l with distilled water. Store at room temperature for no longer than 1 wk.

Procedure

1. Use a single colony of nontransformed *E. coli* cells to inoculate 30 ml of 2× YT medium.
2. Incubate for 12 to 15 h at 37°C with vigorous shaking.
3. Transfer 25 ml of the culture into 2 l of prewarmed 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 to 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 lysate at -70°C until needed.

Preparation of immobilized lysate

Reagents required

CNBr-activated Sepharose 4B

Coupling buffer:	Dissolve 29 g of NaCl and 8.4 g of $NaHCO_3$ in 800 ml of distilled water. Adjust the pH to 8.3 with HCl. Bring the final volume to 1 l with distilled water. Store at room temperature for no longer than 1 wk.
Acetate buffer:	Dissolve 8.2 g of sodium acetate and 29 g of NaCl in 800 ml of distilled water. Adjust the pH to 4.0 with acetic acid. Bring the final volume to 1 l with distilled water. Store at room temperature.
Tris buffer:	Dissolve 12.1 g of Tris-base and 29 g of NaCl in 800 ml of distilled water. Adjust the pH to 8.0 with HCl. Bring the final volume to 1 l with distilled water. Store at room temperature.
PBS:	140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3

Procedure

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* lysate (thawed if necessary). Close the container securely and incubate at 4°C for 12 to 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 PBS to the medium. Stir to suspend.
9. Split the suspension equally into four 100 ml centrifuge bottles. Add 50 ml of 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 lysate

1. Add 60 ml of crude anti-GST antiserum to each of the four bottles containing the immobilized *E. coli* lysate.
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 8
Converting from flow velocity (cm/h) to volumetric flow rates (ml/min) and *vice versa*

It is convenient when comparing results for columns of different sizes to express flow as flow velocity (cm/h). However, flow is usually measured in volumetric flow rate (ml/min). To convert between flow velocity and volumetric flow rate use one of the formulas below:

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

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

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

where

Y = flow velocity 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 flow velocity is 150 cm/h?

Y = flow velocity = 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 flow velocity (cm/hour)

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

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

where

Z = volumetric flow velocity in ml/min
d = column inner diameter in cm

Example:

What is the flow velocity in a Tricorn 5/50 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

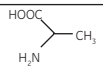
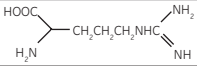
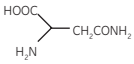
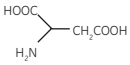
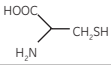
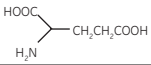
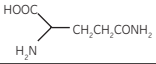
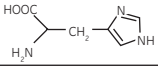
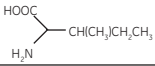
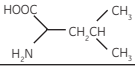
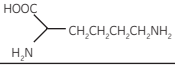
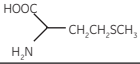
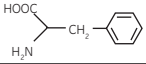
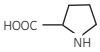
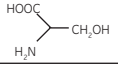
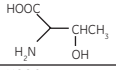
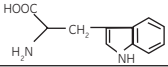
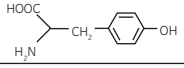
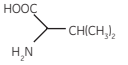
$$\text{Flow velocity} = 1 \times 60 \times \frac{4}{\pi \times 0.5 \times 0.5} \text{ cm/h}$$

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

From ml/min to drops/min 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 9
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 ($-H_20$) Formula	M_r	Charge at pH 6.0–7.0	Hydrophobic (nonpolar)	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 10
Protein conversion data

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

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

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.

Related literature

	Code number
Data files, Application note, and Selection guide for protein preparation and detection of GST-tagged proteins	
Data file: Glutathione S-transferase (GST) Gene Fusion System	28-9622-84
Data file: Glutathione Sepharose and prepacked columns	28-9941-47
Data file: GST MultiTrap FF, GST MultiTrap 4B	28-4081-57
Application note: Efficient, rapid protein purification and on-column cleavage using GSTrap FF columns	18-1146-70
Selection guide: Solutions for protein preparation and detection of GST-tagged proteins	28-9168-33

Handbooks

2-D Electrophoresis using immobilized pH gradients: Principles and Methods	80-6429-60
Affinity Chromatography: Principles and Methods	18-1022-29
ÄKTA Laboratory-scale Chromatography Systems: Instrument Management	29-0108-31
Antibody Purification	18-1037-46
Biacore Assay	29-0194-00
Biacore Sensor Surface	BR-1005-71
Cell Separation Media: Methodology and Applications	18-1115-69
Gel Filtration: Principles and Methods	18-1022-18
GST Gene Fusion System	18-1157-58
High-throughput Process Development with PreDictor Plates: Principles and Methods	28-9403-58
Hydrophobic Interaction and Reversed Phase Chromatography: Principles and Methods	11-0012-69
Imaging: Principles and Methods	29-0203-01
Ion Exchange Chromatography and Chromatofocusing: Principles and Methods	11-0004-21
Isolation of mononuclear cells: Methodology and Applications	18-1152-69
MicroCal Calorimetry—Achieving High Quality Data	29-0033-51
Microcarrier Cell Culture: Principles and Methods	18-1140-62
Nucleic Acid Sample Preparation for Downstream Analyses: Principles and Methods	28-9624-00
Protein Sample Preparation	28-9887-41
Purifying Challenging Proteins: Principles and Methods	28-9095-31
Recombinant Protein Purification[1]: Principles and Methods	18-1142-75
Spectrophotometry	29-0331-82
Strategies for Protein Purification	28-9833-31
Western Blotting: Principles and Methods	28-9998-97

[1] *The Recombinant Protein Purification Handbook includes information on recombinant protein expression and purification, including GST-tagged 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 histidine-tagged protein purification.*

	Code number
Column packing	
Column Packing - The Movie CD-ROM	18-1165-33

Additional reading

Data File	
HiTrap Benzamidine FF (high sub) and Benzamidine Sepharose 4 Fast Flow	18-1139-38
Brochures	
Pure simplicity for tagged proteins	28-9353-68
Years of experience in every column	28-9090-92
MultiTrap 96-well plates —Applications and guidelines	28-9511-27
ÄKTA protein purification by design	28-4026-97
Selection guides	
Solutions for protein preparation and detection of GST-tagged proteins	28-9168-33
Protein sample preparation	29-0009-71
Protein and Nucleic Acid Sample Preparation	28-9337-00
Protein and Peptide Purification, Technique Selection Guide	18-1128-63
Affinity chromatography columns and media	18-1121-86
Fast Desalting and Buffer Exchange of Proteins and Peptides	18-1128-62
Ion exchange columns and media	18-1127-31
Gel filtration columns and media	18-1124-19
Prepacked Chromatography Columns for ÄKTA design systems	28-9317-78
Amersham ECL Gel System Compatibility Guide	29-0251-27
Book	
Protein Purification, Principles, High Resolution Methods and Applications, J.C. Janson and L. Ryden, 1998, 2nd ed. Wiley VCH	18-1128-68

Product index[1]

[1] *Bold page numbers indicate main entry for product(s).*

Ordering information

Product	Quantity	Code number
pGEX vectors		
pGEX-4T-1	25 µg	28-9545-49
pGEX-4T-2	25 µg	28-9545-50
pGEX-4T-3	25 µg	28-9545-52
pGEX-5X-1	25 µg	28-9545-53
pGEX-5X-2	25 µg	28-9545-54
pGEX-5X-3	25 µg	28-9545-55
pGEX-2TK	25 µg	28-9546-46
pGEX-6P-1	25 µg	28-9546-48
pGEX-6P-2	25 µg	28-9546-50
pGEX-6P-3	25 µg	28-9546-51
pGEX-2T	25 µg	28-9546-53
pGEX-3X	25 µg	28-9546-54
pGEX-1λT EcoRI/BAP	5 µg	28-9546-56
GST vector primers for sequencing		
pGEX 5' Sequencing Primer 5'-d[GGGCTGGCAAGCCACGTTTGGTG]-3'	0.05 A_{260} unit	27-1410-01
pGEX 3' Sequencing Primer 5'-d[CCGGGAGCTGCATGTGTCAGAGG]-3'	0.05 A_{260} unit	27-1411-01
Small-scale purification products		
GST SpinTrap	50 columns	28-9523-59
GST Bulk Kit	1 kit	27-4570-01
GST GraviTrap	10 columns	28-9523-60
GST MultiTrap FF	4 × 96-well filter plates	28-4055-01
GST MultiTrap 4B	4 × 96-well filter plates	28-4055-00
GST Buffer Kit	1 kit	28-9523-61
Detection		
Anti-GST Antibody 0.5 ml	50 detections	27-4577-01
GST Detection Module	50 detections	27-4590-01
GST 96-Well Detection Module	5 plates	27-4592-01
Anti-GST HRP Conjugate	75 µl	RPN1236
ECL GST Western Blotting Detection Kit	1 kit	RPN1237
Cleavage		
Thrombin	500 units	27-0846-01
Factor Xa	400 units	27-0849-01
PreScission Protease	500 units	27-0843-01
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

Product	Quantity	Code number
Scale-up purification products		
GSTrap HP columns	5 × 1 ml*	17-5281-01
	1 × 5 ml	17-5282-01
	5 × 5 ml*	17-5282-02
Glutathione Sepharose High Performance	25 ml	17-5279-01
	100 ml	17-5279-02
GSTrap FF columns	2 × 1 ml	17-5130-02
	5 × 1 ml*	17-5130-01
	1 × 5 ml	17-5131-01
	5 × 5 ml*	17-5131-02
GSTPrep FF 16/10 column	1 × 20 ml	28-9365-50
Glutathione Sepharose 4 Fast Flow	25 ml	17-5132-01
	100 ml	17-5132-02
	500 ml	17-5132-03
GSTrap 4B columns	5 × 1 ml*	28-4017-45
	1 × 5 ml	28-4017-47
	5 × 5 ml*	28-4017-48
Glutathione Sepharose 4B	10 ml	17-0756-01
	100 ml	17-0756-05
	300 ml	17-0756-04
Glutathione Sepharose 4B (prepacked disposable column)	2 × 2 ml	17-0757-01

Companion products

Reagents		
E. coli BL21	1 vial	27-1542-01
illustra GFX PCR DNA and Gel Band Purification Kit	100 purifications	28-9034-70
	250 purifications	28-9034-71
Ready-To-Go T4 DNA Ligase	50 reactions	27-0361-01
Adenosine 5'-Triphosphate, 100 mM Solution (ATP)	25 µmol	27-2056-01
illustra PuReTaq Ready-To-Go PCR Beads (multiwell plate)	96 reactions	27-9557-01
	5 × 96 reactions	27-9557-02
illustra PuReTaq Ready-To-Go PCR Beads (0.5 ml tubes)	100 reactions	27-9558-01
Taq DNA Polymerase (cloned)	250 units†	27-0798-04
	4 × 250 units†	27-0798-05
	10 × 250 units†	27-0798-06
	1000 units	28-9373-45
	5000 units	28-9373-46
	25 000 units	28-9373-48
dNTP Set, 100 mM Solutions	4 × 25 µmol	28-4065-51
(dATP, dCTP, dGTP, dTTP)	4 × 100 µmol	28-4065-52
	4 × 500 µmol	28-4065-53
Illustra plasmidPrep Midi Flow Kit	25 purifications	28-9042-67
	100 purifications	28-9042-68
illustra plasmidPrep Mini Spin Kit	50	28-9042-69
	250	28-9042-70

* *100-pack size available by special order.*

† *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) Taq DNA Polymerase (T. aquaticus) also provided.*

Product	Quantity	Code number
Columns, media, and equipment		
Disposable PD-10 Desalting Columns	30 columns	17-0851-01
PD SpinTrap G-25	50 columns	28-9180-04
PD MultiTrap G-25	4 × 96-well plates	28-9180-06
PD MiniTrap™ G-25	50 columns	28-9180-07
PD MidiTrap™ G-25	50 columns	28-9180-08
PD MiniTrap G-10	50 columns	28-9180-10
PD MidiTrap G-10	50 columns	28-9180-11
HiTrap Desalting	5 × 5 ml	17-1408-01*
HiPrep 26/10 Desalting	1 × 53 ml	17-5087-01
HiPrep 26/10 Desalting	4 × 53 ml	17-5087-02
CNBr-activated Sepharose 4B	15 g	17-0430-01
Superdex Peptide 10/300 GL	1 × 24 ml	17-5176-01
Superdex 75 10/300 GL	1 × 24 ml	17-5174-01
Superdex 200 10/300 GL	1 × 24 ml	17-5175-01
Superdex 75 5/150	1 × 3 ml	28-9205-04
Superdex 200 5/150	1 × 3 ml	28-9065-61
HiLoad 16/600 Superdex 30 prep grade	1 × 120 ml	28-9893-31
HiLoad 16/600 Superdex 75 prep grade	1 × 120 ml	28-9893-33
HiLoad 16/600 Superdex 200 prep grade	1 × 120 ml	28-9893-35
HiLoad 26/600 Superdex 30 prep grade	1 × 320 ml	28-9893-32
HiLoad 26/600 Superdex 75 prep grade	1 × 320 ml	28-9893-34
HiLoad 26/600 Superdex 200 prep grade	1 × 320 ml	28-9893-36
Empty Disposable PD-10 Columns	50	17-0435-01
Collection plate, 500 µl V-bottom (for use with multiwell plates)	5 × 96-well plates	28-4039-43
LabMate PD-10 Buffer Reservoir	10	18-3216-03
Column Packing—The Movie	1 CD	18-1165-33
Concentration devices		
Vivaspin 500 MWCO 3000	25	28-9322-18
Vivaspin 500 MWCO 5000	25	28-9322-23
Vivaspin 500 MWCO 10 000	25	28-9322-25
Vivaspin 500 MWCO 30 000	25	28-9322-35
Vivaspin 500 MWCO 50 000	25	28-9322-36
Vivaspin 500 MWCO 100 000	25	28-9322-37
Vivaspin 2 MWCO 3000	25	28-9322-40
Vivaspin 2 MWCO 5000	25	28-9322-45
Vivaspin 2 MWCO 10 000	25	28-9322-47
Vivaspin 2 MWCO 30 000	25	28-9322-48
Vivaspin 2 MWCO 50 000	25	28-9322-57
Vivaspin 2 MWCO 100 000	25	28-9322-58
Vivaspin 6 MWCO 3000	25	28-9322-93
Vivaspin 6 MWCO 5000	25	28-9322-94
Vivaspin 6 MWCO 10 000	25	28-9322-96
Vivaspin 6 MWCO 30 000	25	28-9323-17
Vivaspin 6 MWCO 50 000	25	28-9323-18
Vivaspin 6 MWCO 100 000	25	28-9323-19

* *100-pack size available by special order.*

Product	Quantity	Code number
Vivaspin 20 MWCO 3000	12	28-9323-58
Vivaspin 20 MWCO 5000	12	28-9323-59
Vivaspin 20 MWCO 10 000	12	28-9323-60
Vivaspin 20 MWCO 30 000	12	28-9323-61
Vivaspin 20 MWCO 50 000	12	28-9323-62
Vivaspin 20 MWCO 100 000	12	28-9323-63
Lysis/protein extraction		
Yeast Protein Extraction Buffer Kit	1 kit	28-9440-45
Mammalian Protein Extraction Buffer	1 × 500 ml	28-9412-79
Western blotting		
Hybond-P	10 sheets	RPN2020F
Hybond ECL	10 sheets	RPN2020D
Amersham ECL Western Blotting Detection Reagents	125 ml/each reagent	RPN2209
Amersham ECL Prime Western Blotting Detection Reagent	125 ml/each reagent	RPN2232
Amersham ECL Select Western Blotting Detection Reagent	125 ml/each reagent	RPN2235
Amersham ECL Prime Blocking Reagent	40 g	RPN418
10 x Amersham ECL Gel, 10%, 10 wells	pack of 10	28-9898-04
10 x Amersham ECL Gel 4-20%, 10 wells	pack of 10	28-9901-54
Amersham ECL Gel Running Buffer	250 ml	28-9902-52
Amersham ECL Gel Box	1	28-9906-08
Amersham ECL DualVue Western Markers	250 µl	RPN810
Amersham Low-Range Rainbow Molecular Weight Markers	250 µl	RPN755E

* *100-pack size available by special order.*

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

GE Healthcare Europe, GmbH
Munzinger Strasse 5
D-79111 Freiburg
Germany

GE Healthcare Bio-Sciences Corp.
800 Centennial Avenue, P.O. Box 1327
Piscataway, NJ 08855-1327
USA

GE Healthcare Japan Corp.
Sanken Bldg., 3-25-1, Hyakunincho
Shinjuku-ku, Tokyo 169-0073
Japan

For local office contact information,
please visit www.gelifesciences.com/contact

www.gelifesciences.com/protein-purification
www.gelifesciences.com/sampleprep

GE Healthcare UK Limited
Amersham Place
Little Chalfont
Buckinghamshire, HP7 9NA
UK

imagination at work

18-1157-58 AD 11/2014