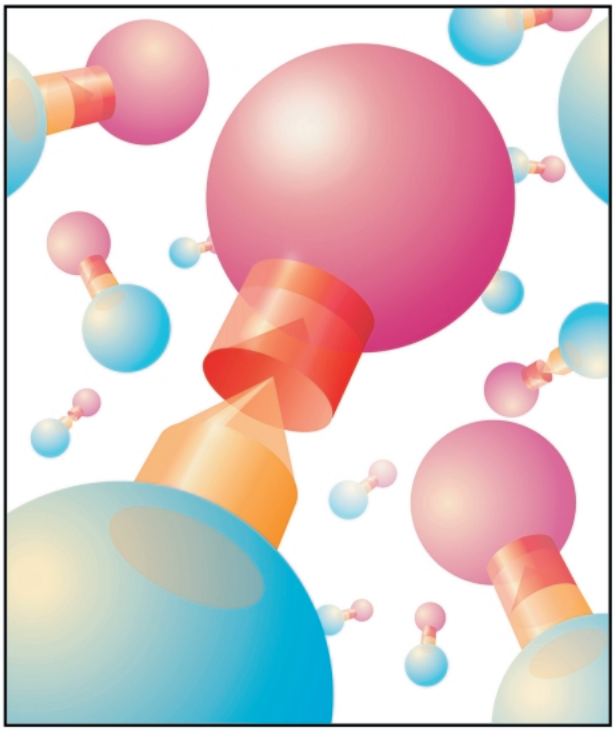
The Recombinant Protein Handbook

Protein Amplification and Simple Purification



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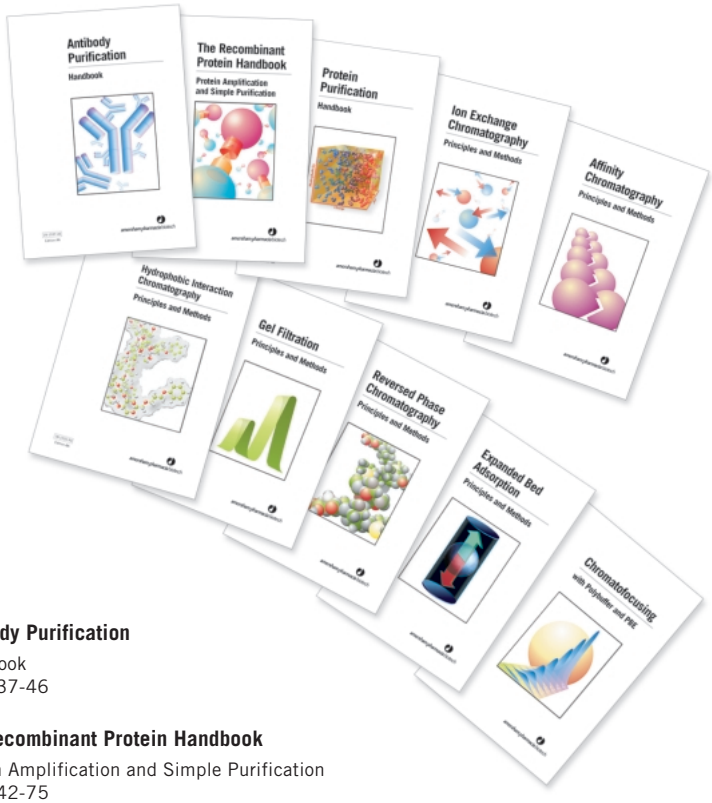

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The Recombinant Protein Handbook

Protein Amplification
and Simple Purification

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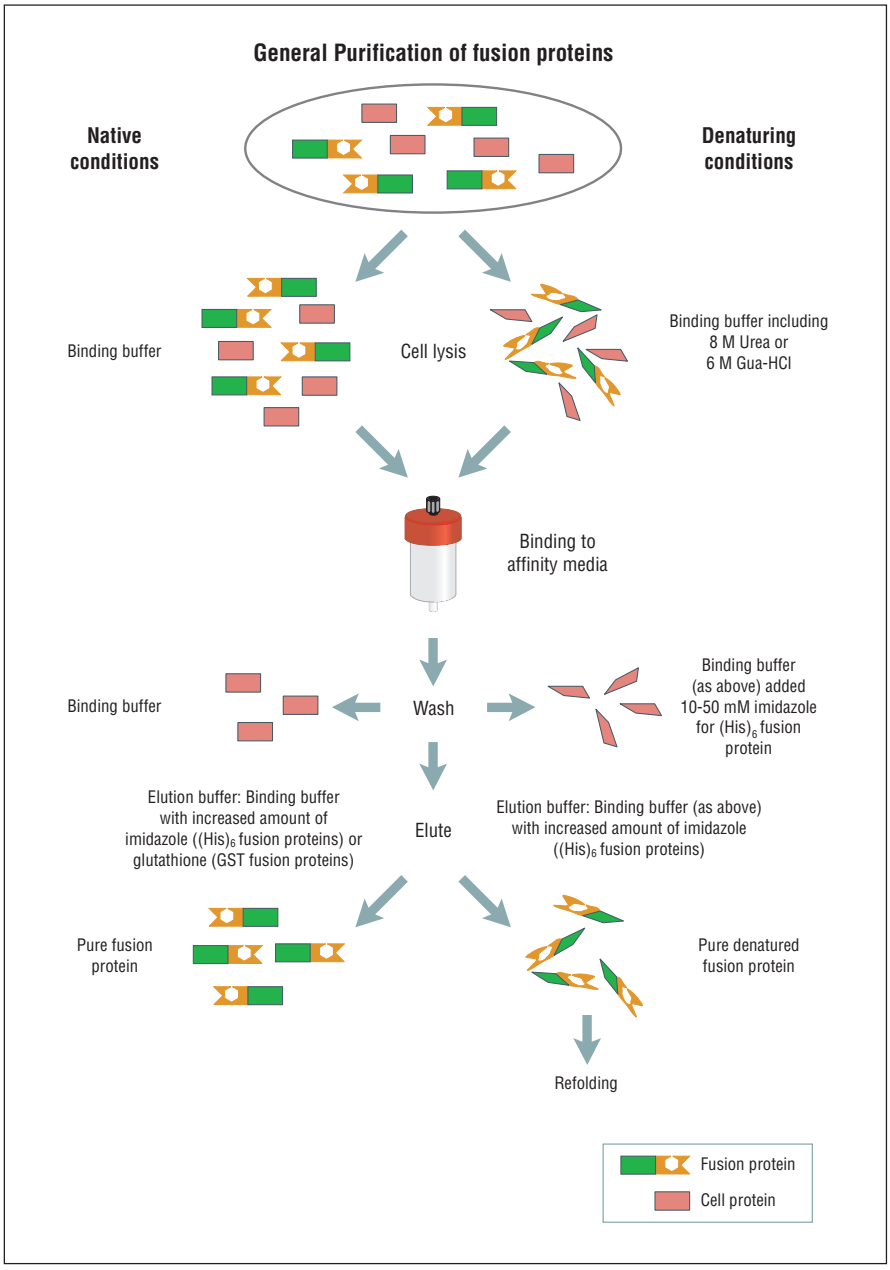

General Purification of fusion proteins
Native conditions
Denaturing conditions
Binding buffer
Cell lysis
Binding buffer including 8 M Urea or 6 M Gua-HCl
Binding to affinity media
Binding buffer
Wash
Binding buffer (as above) added 10-50 mM imidazole for (His)6 fusion protein
Elution buffer: Binding buffer with increased amount of imidazole ((His)6 fusion proteins) or glutathione (GST fusion proteins)
Elute
Elution buffer: Binding buffer (as above) with increased amount of imidazole ((His)6 fusion proteins)
Pure fusion protein
Pure denatured fusion protein
Refolding
Fusion protein
Cell protein

Introduction

This handbook is intended for the general reader interested in the amplification and purification of recombinant proteins and for everyday use at the laboratory bench.

The growth in the use of recombinant proteins has increased greatly in recent years, as has the wealth of techniques and products used for their amplification and purification. The advantages of using a fusion protein to facilitate purification and detection of the recombinant proteins are now widely recognised. This handbook introduces the reader to the initial considerations to be made when deciding upon host, vector and use of a fusion or non-fusion protein and covers general guidelines for successful protein amplification. General advice is also given on harvesting and extraction, handling of inclusion bodies, tag removal and removal of unvanted salts and small molecules.

The more that is known about the characteristics of a protein, the more easily it can be isolated and purified. Consequently, fusion proteins are simple and convenient to work with and, for many applications, a single purification step, using a commercially available affinity chromatography column, is sufficient. This is clearly demonstrated in the specific chapters on the amplification, purification and detection of the two most common fusion proteins (GST and $(His)_6$ tagged proteins) which include simple practical protocols for use in the laboratory. The handbook also gives suggestions for the successful purification of other fusion proteins by a single affinity chromatography step.

In situations where no fusion system is available, or when a higher degree of purity is required, a multi-step purification will be necessary. This can also become a straightforward task by following a Three Phase Purification Strategy reviewed in the final chapter.

Symbols

this symbol gives general advice which can improve procedures and provides recommendations for action under specific situations.

this symbol denotes advice which should be regarded as mandatory and gives a warning when special care should be taken in a procedure.

this symbol gives troubleshooting advice to help analyse and resolve any difficulties which may occur.

CHAPTER 1

Choice of host for protein amplification

Several host systems are available including phage, bacteria, yeast, plants, filamentous fungi, insect or mammalian cells grown in culture and transgenic animals. The final choice of host will depend upon the specific requirements and applications for the recombinant protein. Table 1 reviews commonly used host systems with their advantages and disadvantages.

The choice of host affects not only the amplification and isolation of the protein, but also the way in which the product can be subsequently purified. In order to decide which host is most suitable the amount and the degree of purity of the product as well as its biological integrity and potential toxicity should be considered. For example, bacterial expression systems are not suitable if post-translational modification is required to produce a fully functional recombinant product.

The location of product within the host will affect the choice of methods for isolation and purification of the product. For example, a bacterial host may secrete the protein into the growth media, transport it to the periplasmic space or store it as insoluble inclusion bodies within the cytoplasm.

Host	Advantages	Disadvantages
Bacteria *e.g.Escherichia coli*	Many references and much experience available	No post-translational modification
	Wide choice of cloning vectors	
	Gene expression easily controlled	Biological activity and immunogenicity may differ from natural protein
	Easy to grow with high yields (product can form up to 50% of total cell protein)	High endotoxin content in gram negative bacteria
	Product can be designed for secretion into the growth media	
Bacteria *e.g. Staphylococcus aureus*	Secretes fusion proteins into the growth media	Does not express such high levels as *E. coli*
		Pathogenic
Mammalian cells	Same biological activity as native proteins	Cells can be difficult and expensive to grow
	Mammalian expression vectors available	Cells grow slowly
	Can be grown in large scale cultures	Manipulated cells can be genetically unstable
		Low productivity as compared to micro-organisms
Yeasts	Lacks detectable endotoxins	Gene expression less easily controlled
	Generally Regarded As Safe (GRAS)	Glycosylation not identical to mammalian systems
	Fermentation relatively inexpensive	
	Facilitates glycosylation and formation of disulphide bonds	
	Only 0.5% native proteins are secreted so isolation of secreted product is simplified	
	Well established large scale production and downstream processing	
Cultured insect cells *Baculovirus vector*	Many processing mechanisms similar to eukaryotic cells	Lack of information on glycosylation mechanisms
	Safe, since few arthropods are adequate hosts for baculovirus	Product not always fully functional
	Baculovirus vector received FDA approval for a clinical trial	Few differences in functional and antigenic properties between product and native protein
	Virus stops host protein amplification. High level expression of product	

Table 1 (continued).

Host	Advantages	Disadvantages
Fungi *e.g.Aspergillus sp.*	Well established systems for fermentation of filamentous fungi	High level of expression not yet achieved
	Growth inexpensive	Genetics not well characterized
	A.niger is GRAS	No cloning vectors available
	Can secrete large quantities of product into growth media, source of many industrial enzymes	
Plants		Low transformation efficiency Long generation time

Table 1.

Choice of vectors

In order to clone the gene of interest all engineered vectors have a selection of unique restriction sites downstream of a transcription promotor sequence. The choice of vector family is governed by the host. Once the host has been selected, many different vectors are available for consideration, from simple expression vectors to those that secrete fusion proteins.

However, as for the selection of a suitable host system, the final choice of vector should take into consideration the specific requirements of the application and will, of course, be influenced by the behaviour of the target protein. One key factor that has led to the increased use of fusion protein vectors is that amplification of a fusion protein containing a tag of known size and biological function can greatly simplify subsequent isolation, purification and detection. In some cases the protein yield can also be increased. Table 2 reviews some of the features of fusion protein amplification that may influence the final choice of vector.

Maintenance and cloning protocols are highly specific for each vector and the instructions provided by the supplier should be followed carefully.

Advantages	Disadvantages
Fusion proteins	
Cell compartments can be targeted	Tag may interfere with protein structure and affect folding and biological activity
Provide a marker for expression	Cleavage site is not always 100% specific if tag needs to be removed
Simple purification using affinity chromatography under denaturing or non-denaturing conditions	
Easy detection	
Refolding achievable on a chromatography column	
Ideal for secreted proteins as the product is easily isolated from the growth media	
Non- fusion proteins	
No cleavage steps necessary	Purification and detection not as simple
	Problems with solubility may be difficult to overcome, reducing potential yield

Table 2.

Vectors for non-fusion proteins

Table 3 shows examples of non-fusion vectors.

Vector family	Comments
p*Trc* 99 A	Prokaryotic vector for expression of proteins encoded by inserts lacking a start codon, inducible by IPTG
pKK223-3	For over-expression of proteins under the control of the strong *tac* promotor in prokaryotes
pSVK 3	For *in vivo* expression in mammalian cell lines
PSVL SV40	For high level transient expression in eukaryotic cells
pMSG	For inducible expression in mammalian cells

Table 3.

Vectors for fusion proteins

Table 4 shows examples of vectors for fusion proteins together with the required purification product.

Vector family	Tag	Purification Products
pGEX	Glutathione S-transferase	GST MicroSpin™ Purification Module GSTrap™ Glutathione Sepharose™ Fast Flow
PQE	6 x Histidine	His MicroSpin Purification Module HisTrap™ HiTrap™ Chelating Chelating Sepharose Fast Flow
pET	6 x Histidine	His MicroSpin Purification Module HisTrap HiTrap Chelating Chelating Sepharose Fast Flow
pEZZ 18 (non-inducible expression)	IgG binding domain of protein A	IgG Sepharose 6 Fast Flow
pRIT2T(expression inducible by temperature change)	IgG binding domain of protein A	IgG Sepharose 6 Fast Flow

Table 4.

Please refer to Chapter 3 and 4 for further details of purification products for GST and $(His)_6$ fusion proteins.

Choice of fusion tag

The two most commonly used tags are glutathione S-transferase (GST tag) and 6 x histidine residues ($(His)_6$ tag. As for the selection of host and vectors, the decision to use either a GST or a $(His)_6$ tag must be made according to the needs of the specific application. Table 5 highlights some key features of these tags that should be considered.

GST tag	$(His)_6$ tag
Can be used in any expression system	Can be used in any expression system
Purification procedure gives high yields of pure product	Purification procedure gives high yields of pure product
Selection of purification products available for any scale	Selection of purification products available for any scale
pGEX6P PreScission™ protease vectors enable cleavage and purification in a single step	Small tag may not need to be removed e.g. tag is poorly immunogenic so fusion partner can be used directly as an antigen in antibody production
Site-specific proteases enable cleavage of tag if required	Site-specific proteases enable cleavage of tag if required. N.B. Enterokinase sites that enable tag cleavage without leaving behind extra amino acids are preferable
GST tag easily detected using an enzyme assay or an immunoassay	$(His)_6$ tag easily detected using an immunoassay
Simple purification. Very mild elution conditions minimize risk of damage to functionality and antigenicity of target protein	Simple purification, but elution conditions are not as mild as for GST fusion proteins. Purification can be performed under denaturing conditions if required. N.B. Neutral pH but imidazole may cause precipitation Desalting to remove imidazole may be necessary
GST tag can help stabilize folding of recombinant proteins	$(His)_6$ - dihydrofolate reductase tag stabilizes small peptides during expression
Fusion proteins form dimers	Small tag is less likely to interfere with structure and function of fusion partner
	Mass determination by mass spectrometry not always accurate for some $(His)_6$ fusion proteins*

Table 5.

**Geoghegan K.F. et al., Anal. Biochem. 267(1), 169-184, 1999.*

CHAPTER 2

Protein amplification

Cell culture conditions are dependent upon the host system. Follow the instructions of the supplier.

Monitor expression during growth and induction by one or more of the detection methods referred to in this handbook.

Retain small samples at key steps in all procedures for analysis of the purification method.

Before performing a large scale purification, check protein amplification in the culture or do a small pilot experiment to establish optimum conditions for expression.

Yield of fusion proteins is highly variable and is affected by the nature of the fusion protein, the host cell, and the culture conditions. Fusion protein yields can range from 0-10 mg/ml. Table 6 can be used to approximate culture volumes based on an average yield of 2.5 mg/ml.

Protein	12.5 µg	50 µg	1 mg	10 mg	50 mg
Culture Volume	5 ml	20 ml	400 ml	4 liters	20 liters
Volume of sonicate	0.5 ml	1 ml	20 ml	200 ml	1000 ml

Table 6.

Sample extraction

The various methods for sample extraction are reviewed in Chapter 7.

Troubleshooting protein amplification

(for specific details on GST or $(His)_6$ fusion proteins, see page 21 for detection of GST fusion proteins or page 50 for detection of $(His)_6$ fusion proteins).

High basal level of expression

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

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

No protein is detected in bacterial sonicate

Check DNA sequences. It is essential that protein-coding DNA sequences are cloned in the proper translation frame in the vectors. Cloning junctions should be sequenced to verify that inserts are in-frame.

Optimize culture conditions to improve yield. Investigate the effect of cell strain, medium composition, incubation temperature and induction conditions. Exact conditions will vary for each fusion protein expressed.

Analyse a small aliquot of an overnight culture by SDS-PAGE. Generally, a highly expressed protein will be visible by Coomassie™ blue staining when 5-10 µl of an induced culture whose A_{600} is ~1.0 is loaded on the gel. Non-transformed host *E. coli* cells and cells transformed with the parental vector should be run in parallel as negative and positive controls, respectively. The presence of the fusion protein in this total cell preparation and its absence from a clarified sonicate may indicate the presence of inclusion bodies.

Check for expression by immunoblotting. Some fusion proteins may be masked on an SDS-polyacrylamide gel by a bacterial protein of approximately the same molecular weight. Immunoblotting can be used to identify fusion proteins in these cases. Run an SDS-polyacrylamide gel of induced cells and transfer the proteins to a nitrocellulose or PVDF membrane (such as Hybond™-C or Hybond-P). Detect fusion protein using anti-GST or anti-His antibody.

Most of fusion protein is in the post-sonicate pellet

Check cell disruption procedure. Cell disruption is seen by partial clearing of the suspension or by microscopic examination. Addition of lysozyme (0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris™-HCl, pH 8.0) prior to sonication may improve results. Avoid frothing as this may denature the fusion protein.

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

Fusion protein may be produced as insoluble inclusion bodies. Try altering the growth conditions to slow the rate of translation.

- Lower the growth temperature (within the range of +20° to +30°C) to improve solubility.
- Decrease IPTG concentration to <0.1 mM to alter induction level.
- Alter time of induction.
- Induce for a shorter period of time.
- Induce at a higher cell density for a short period of time.
- Increase aeration. High oxygen transport can help prevent the formation of inclusion bodies.

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

Alter extraction conditions to improve solubilization of inclusion bodies (see Chapter 5).

Quantification of fusion proteins

Fusion proteins must be purified to homogeneity and quantified using a standard protein assay.

The relative yield of fusion protein can often be determined by measuring the absorbance at 280 nm (suitable for both GST and $(His)_6$ fusion proteins).

The yield of protein may also be determined by standard chromogenic methods (e.g. Lowry, BCA, Bradford, etc.).

Immunoassays can be used for quantification if a suitable standard curve can be produced. The immunoassay technique is also particularly suitable for screening large numbers of samples when a simple yes/no answer is required, as, for example, when testing fractions from a purification.

CHAPTER 3

GST fusion proteins

Amplification

Glutathione S-transferase (GST) Gene Fusion System is an integrated range of products for the amplification, purification and detection of GST fusion proteins in *E. coli*. The characteristics of GST are shown in Table 7 and Figure 1 shows the structure of Glutathione Sepharose used in the purification steps.

Glutathione S-transferase	Naturally occurring M_r 26 000 protein Can be expressed in *E. coli* with full enzymatic activity
Properties as 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 7.

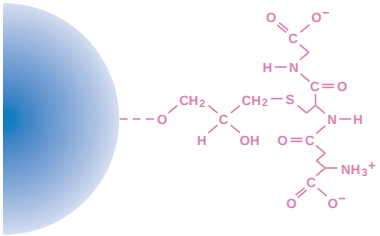


Fig. 1. Glutathione is attached to Sepharose by coupling to the oxirane group using epoxy-activation. The structure of glutathione is complementary to the binding site of the glutathione S-transferase binding site.

General considerations for the amplification of fusion proteins are discussed in Chapter 2.

In the GST gene fusion system expression is under control of the *tac* promoter, which is induced using the lactose analogue isopropyl β-D-thiogalactoside (IPTG). Induced cultures are allowed to express GST fusion proteins for several hours before cells are harvested.

The host

E. coli BL21 is a protease-deficient strain specifically selected to give high efficiency transformation and high level of expression of GST fusion proteins.

Genotype	F^-, *ompT*, *hsd*S (r_B^- , m_B^-), *gal* (52, 53)
Growth conditions	Resuspend lyophilized cultures in 1 ml of L-broth. Grow overnight before plating onto L-broth media plates
Long term storage	Mix equal volumes of stationary phase culture (grown in L-broth) and glycerol. Store at -70°C. Revive frozen glycerol stocks by streaking onto L-broth media plates

Table 8.

Use an alternative strain for cloning and maintenance of the vector (e.g. JM105).

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

The vectors

pGEX vectors (pGEX-T, pGEX-P, pGEX-X, pGEX-2TK) are available in all three reading frames with a range of cleavage recognition sites as shown in Table 9. The same multiple cloning sites in each vector ensure easy transfer of inserts. The vectors carry the *lacI*^{q} gene, so there are no specific host requirements for expression of fusion proteins. Vector control regions and the reading frame of the multiple cloning site for each pGEX vector are shown in Appendix 1.

pGEX-6P-1, pGEX-6P-2, pGEX-6P-3	PreScission Protease
pGEX-4T-1, pGEX-4T-2, pGEX-4T-3	Thrombin
pGEX-5X-1, pGEX-5X-2, pGEX-5X-3	Factor Xa
PGEX-2TK Allows detection of expressed proteins by direct labelling *in vitro*	Thrombin, c-AMP dependent protein kinase

Table 9.

pGEX6P PreScission Protease vectors offer the most efficient method for cleavage and purification of GST fusion proteins. Site specific cleavage is performed with simultaneous immobilization of the protease on the column. The protease has a high activity at a low temperature so that all steps can be performed in the cold room to protect protein integrity. Cleavage enzyme and GST tag are removed in a single step.

Purification

For simple, one step purification of GST fusion proteins, several products have been designed to meet specific purification needs, as shown in Table 10.

Column/loose**	Amount of GST fusion protein for a single purification	Comment
GST MicroSpin Purification Module	Up to 400 µg	Ready to use, pre-packed columns, buffers and chemicals High throughput when used with MicroPlex™ 24 Vacuum (up to 48 samples simultaneously)
GSTrap 1 ml	10-12 mg	Pre-packed column, ready to use
GSTrap 5 ml	50-60 mg	Pre-packed column, ready to use
Glutathione Sepharose 4B	8 mg per ml	For packing small columns and other formats
Glutathione Sepharose 4 Fast Flow	10-12 mg per ml	For packing high performance columns for use with purification systems and scaling up

Table 10. Summary of purification options for GST fusion proteins.

**Characteristics of GSTrap and Glutathione Sepharose are given in Appendix 5.

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

Batch preparation procedures are frequently mentioned in the literature. However the availability of pre-packed columns and easily packed high flow rate Glutathione Sepharose provide faster, more convenient alternatives.

Batch preparations are occasionally used if it appears that the tag is not fully accessible or when the protein in the lysate is at very low concentrations (both could appear to give a low yield from the first purification step). A more convenient alternative to improve yield is to decrease the flow rate or pass the sample through the column several times.

Monitor purification steps by using one or more of the detection methods referred to in this handbook. The choice of purification equipment should also be made according to the needs of the purification. Appendix 8 provides a guide to aid in the selection of the correct purification solution and key points to consider are highlighted here.

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

For purification of larger quantities of fusion proteins GSTrap columns provide the ideal solution and can be used with a syringe, a peristaltic pump or a chromatography system.

To increase capacity use several GSTrap columns (1 ml or 5 ml) in series or, for even larger capacity requirements, pack Glutathione Sepharose Fast Flow into a suitable column (details of column packing procedures are outlined in Appendix 6).

For simple and reproducible purification a chromatography system such as ÄKTA™*prime* is a significant advantage, recording the purification process and eliminating manual errors.

For laboratory environments in which all experimental data must be recorded and traceable, where method development, optimization and scale up are needed, a computer controlled ÄKTAdesign chromatography system is recommended.

Experiments such as protein refolding or method optimization require linear gradient elution steps that can only be performed by a chromatography system.

GST MicroSpin Purification Module

The GST MicroSpin Purification Module is useful for screening small or large numbers of lysates and for checking samples during the optimization of amplification or purification conditions. Each module contains reagents sufficient for 50 purifications.

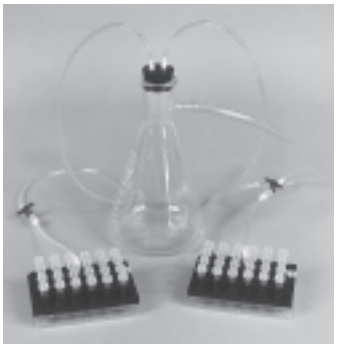
- 10X PBS: 1.4 M NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.3
- Reduced glutathione: 0.154 g
- Dilution buffer: 50 mM Tris-HCl, pH 8.0
- IPTG: 500 mg
- MicroSpin columns: 50 units

Reagents are prepared as follows:

1X PBS:	Dilute 10X PBS with sterile water. Store at +4°C.
Glutathione elution buffer:	Pour the entire contents of dilution buffer into the bottle containing the reduced glutathione. Shake until completely dissolved. Store as 1-20 ml aliquots at -20°C.
IPTG 100 mM:	Dissolve contents of the IPTG vial in 20 ml sterile water. Store as 1 ml aliquots at -20°C.

Alternative 1. High throughput purification using MicroPlex Vacuum

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



Also required:

- Vacuum source capable of providing 220 mm Hg (e.g. a house vacuum)
- Side arm flask, 500 ml or 1 litre
- Single hole rubber stop
- Vacuum tubing
- MicroPlex 24 Vacuum apparatus

1. Assemble the MicroPlex 24 Vacuum following the instructions supplied.
2. Resuspend the Glutathione Sepharose in each column by vortexing gently.
3. Remove the caps from the MicroSpin columns. Place the columns in the manifold, filling any unused holes with the plugs provided with MicroPlex 24 Vacuum.
4. Ensure the stopcock is in the closed position (i.e. perpendicular to the vacuum tubing) and that the manifold is placed squarely on the gasket.
5. Turn on vacuum supply at source. Open the stopcock (i.e. parallel to the vacuum tubing). After the column storage buffer has been drawn through all the columns into the collection tray, close the stopcock.
6. Allow 10-15 seconds for the vacuum pressure to dissipate. Remove the manifold and place it on a paper towel.
7. Apply up to 600 µl of lysate to the column and incubate at room temperature for 5-10 minutes.
8. Open the stopcock. After the lysates have been drawn through all the columns into the collection tray, close the stopcock.
9. Add 600 µl of 1X PBS wash buffer to each column. Open the stopcock. After buffer has been drawn through all the columns into the collection tray, close the stopcock.
10. Allow 10-15 seconds for the vacuum pressure to dissipate. Remove the manifold and reassemble the apparatus with a clean collection tray.
11. Add 200 µl of Glutathione elution buffer to each column. Incubate at room temperature for 5-10 minutes.
12. Open the stopcock. After elution buffer has been drawn through all the columns into the collection tray, close the stopcock.
13. Allow 10-15 seconds for the vacuum pressure to dissipate. Remove the manifold. Cover eluates with sealing tape until required for analysis.

Troubleshooting

See Purification and Detection Troubleshooting page 29.

Alternative 2. Purification of up to 12 samples using a microcentrifuge

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

1. Resuspend the Glutathione Sepharose in each column by vortexing gently.
2. Loosen the column caps one-fourth turn. Remove (and save) bottom closures.
3. Place each column into a clean 1.5 or 2 ml microcentrifuge tube. Spin for 1 minute at 735 x g.
4. Discard the buffer from each centrifuge tube and replace the bottom closures.
5. Apply up to 600 µl of lysate to the column.
6. Recap each column securely and mix by gentle, repeated inversion. Incubate at room temperature for 5-10 minutes.
7. Remove (and save) the top caps and bottom closures. Place each column into a clean, pre-labelled 1.5 or 2 ml microcentrifuge tube.
8. Spin for 1 minute at 735 x g to collect flow through.
9. Place each column into a clean, pre-labelled 1.5 or 2 ml microcentrifuge tube.
10. Apply 250-600 µl of 1X PBS wash buffer to each column. Repeat spin procedure.
11. Repeat step 10. Transfer and pool all washes into clean microcentrifuge tubes.
12. Add 100-200 µl of Glutathione elution buffer to each column. Replace top caps and bottom closures. Incubate at room temperature for 5-10 minutes.
13. Remove and discard top caps and bottom closures and place the column into a clean 1.5 or 2 ml microcentrifuge tube.
14. Spin all columns again to collect eluate. Save for analysis.

Troubleshooting

See Purification and Detection Troubleshooting page 29.

Alternative 3. Purification using MicroPlex

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

See Appendix 4 for recommended centrifugation systems.

1. Assemble the MicroPlex 24 unit following the instructions supplied. Two units can be processed simultaneously to handle 48 samples.
2. Resuspend the Glutathione Sepharose in each column by vortexing gently.
3. Remove the caps from the MicroSpin columns and snap off the bottom closures. Place the columns in the manifold.
4. Centrifuge the unit for 2 minutes following the instructions supplied.
5. Add up to 600 µl of lysate to each column. Incubate at room temperature for 5-10 minutes.
6. Centrifuge the unit for 2 minutes following the instructions supplied. Remove the manifold from each collection try and place on a clean paper towel. Reassemble each unit with a fresh collection tray.
7. Add 100-200 µl of glutathione elution buffer to each column. Incubate at room temperature for 5-10 minutes.
8. Centrifuge the unit for 2 minutes following the instructions supplied. Cover the eluted samples with sealing tape until required for analysis.

Troubleshooting

See Purification and Detection Troubleshooting page 29.

Purification using GSTrap 1 ml or 5 ml columns

GSTrap columns can be operated with a syringe, a peristaltic pump or a liquid chromatography system such as ÄKTA*prime*. Figure 2 shows a schematic of the simple steps needed for successful purification using a 1 ml GSTrap column.

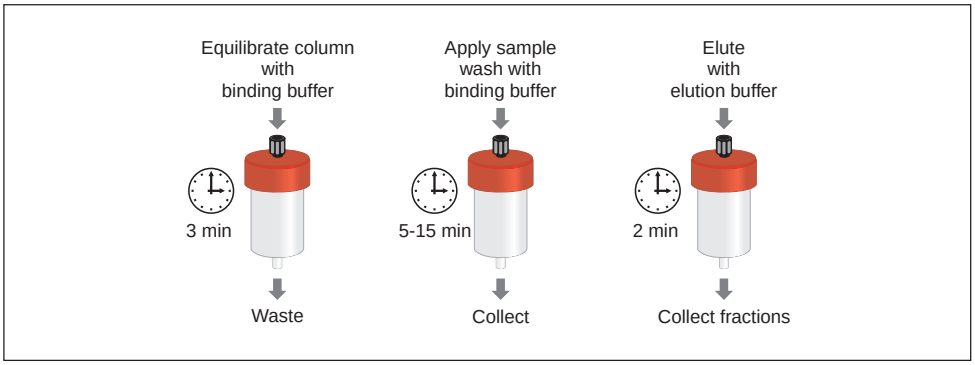


Fig. 2. Simple purification of GST fusion proteins using GSTrap.

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

GSTrap columns (1 ml or 5 ml) can be connected in series to increase binding capacity and hence scale of purification.

Characteristics, column regeneration and storage procedures for Glutathione Sepharose are given in Appendix 5.

Sample and buffer preparation

Use high quality water and chemicals. Filtration through 0.45 µm filters is recommended.

Samples should be centrifuged immediately before use and/or filtered through a 0.45 µm filter. If the sample is too viscous, dilute with binding buffer.

Sample binding properties can be improved by adjusting the sample to the composition of the binding buffer: dilute in binding buffer or perform a buffer exchange using a desalting column (see Chapter 7).

Alternative 1. Manual purification with a syringe

Binding buffer: 1X PBS, pH 7.3 (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.

Fig. 3. Using GSTrap with a syringe. **A** Prepare buffers and sample. Remove the column's top cap and twist off the end. **B** Load the sample and begin collecting fractions. **C** Elute and continue collecting fractions.

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

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

Alternative 2. Simple purification with ÄKTA*prime*

ÄKTA*prime* contains a pre-programmed template for purification of GST fusion proteins using a single GSTrap column.

Binding buffer: 20 mM sodium phosphate, 0.15 M NaCl, pH 7.3 (or the buffer used in Alternative 1).
Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0.

Prepare at least 500 ml of each eluent.

1. Follow instructions supplied on the ÄKTA*prime* cue card to connect the column and load the system with binding buffer.
2. Select the Application Template.
3. Start the method.
4. Enter the sample volume and press OK to start.

Connecting the column.

Preparing the fraction collector.

Fig. 4. Typical procedures when using ÄKTA*prime*.

Figure 5 shows a typical purification of GST fusion protein on GSTrap 1 ml, using a system, and an SDS-PAGE analysis of the purified protein.

Column regeneration, storage procedures and the characteristics of Glutathione Sepharose 4 Fast Flow (packed in the GSTrap column) are described in Appendix 5.

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

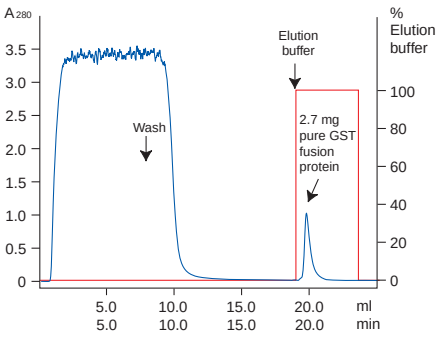


Fig. 5a. Purification of GST fusion protein on GSTrap 1 ml.

Lane 1: Low Molecular Weight (LMW) Calibration kit, reduced, Amersham Pharmacia Biotech
Lane 2: Cytoplasmic extract of *E. coli* expressing GST fusion protein, 1 g cell paste/10 ml
Lane 3: GST fusion protein eluted from GSTrap 1 ml

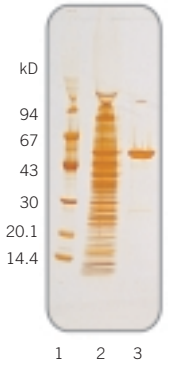


Fig. 5b. SDS-PAGE on ExelGel™ SDS Gradient 8-18% using Multiphor™ II (Amersham Pharmacia Biotech) followed by silver staining.

Troubleshooting

See Purification and Detection Troubleshooting page 29.

Optimization of GST fusion protein purification

Following the instructions supplied for each pre-packed GSTrap column will generally provide very good results.

Dimer formation is inevitable with GST fusion proteins since GST itself is a homodimer when folded. Use gel filtration chromatography to remove the dimers. A column pre-packed with Superdex™ will give the highest possible resolution between two molecules of such similar molecular weight.

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

Volumes and times used for elution may vary among fusion proteins.

Further elution with higher concentrations of glutathione (20-50 mM) may improve yield. At concentrations above 15 mM glutathione the buffer concentration should also be increased to maintain the pH within the range 8-9.

Detection of GST fusion proteins

Table 11 reviews the methods available for detection of GST fusion proteins. These methods can be selected according to the experimental situation, for example, SDS-PAGE analysis, performed frequently during amplification and purification to monitor results, may not be the method of choice for routine monitoring of samples from high throughput screening. Functional assays are useful, but need to be developed for each specific protein.

Detection method	Comments
GST 96 Well Detection module for ELISA assay	Ideal for screening expression systems and chromatographic fractions. Useful when amount of expressed protein is unknown or when increased sensitivity is required.
GST Detection Module for enzymatic assay	Rapid assay, ideal for screening.
Western blot analysis using anti-GST antibody and ECL™ detection systems	Highly specific, detects only GST fusion protein. Little or no background detectable when used with optimized concentrations of secondary HRP conjugated antibody. ECL detection systems enhance detection in Western blot. ECL provides adequate sensitivity for most recombinant expression applications. For higher sensitivity use ECL Plus.
SDS-PAGE with Coomassie or silver staining	Provides information on size and % purity. Detects fusion protein and contaminants.
Functional assays	Useful to assess activity of the purified GST fusion protein, but may require development and optimization.

Table 11. Detection methods for GST fusion proteins.

Alternative 1. SDS-PAGE Analysis

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

1. Add 2 µl of 6X SDS loading buffer to 5-10 µl of supernatant from crude extracts, cell lysates or purified fractions as appropriate.
2. Vortex briefly and heat for 5 minutes at +90° to +100°C.
3. Load the samples onto an SDS-polyacrylamide gel.
4. Run the gel for the appropriate length of time and stain with Coomassie Blue or silver stain.

The percentage of acrylamide in the SDS-gel should be selected according to the expected molecular weight of the protein of interest (see Table 12).

% Acrylamide in resolving gel	Separation size range (M_r x 10^{-3})
Single percentage:	
5%	36-200
7.5%	24-200
10%	14-200
12.5%	14-100
15%	14-60[1]
Gradient:	
5-15%	14-200
5-20%	10-200
10-20%	10-150

[1]The larger proteins fail to move significantly into the gel.

Table 12.

If using horizontal SDS pre-cast gels, refer to the Gel Media Guide from Amersham Pharmacia Biotech.

Troubleshooting

If the fusion protein is absent, it may be insoluble or expressed at very low levels: refer to Troubleshooting protein amplification (page 9).

If no fusion protein is detected by Coomassie Blue, try silver staining or Western blotting to enhance sensitivity.

Transformants expressing the fusion protein will be identified by the absence from total cellular proteins of the parental M_r 29 000 GST and by the presence of a novel, larger fusion protein. In some cases both the M_r 29 000 GST and fusion protein may be present. This can be caused by intracellular cleavage of the fusion protein, or by translational pausing at the junction between GST and the fusion partner.

Interpretation is sometimes complicated when fusion proteins break down and release the M_r 26 000 GST moiety. Such cases are usually recognized by the reduced level of the M_r 26 000 species, and by the series of larger, partial proteolytic fragments above it.

Alternative 2. GST Detection Module

The GST Detection Module is designed for the rapid enzymatic detection of GST fusion proteins produced using the pGEX vectors using the GST substrate 1-chloro-2,4-dinitrobenzene (CDNB). The GST-mediated reaction of CDNB with glutathione produces a conjugate that is measured by absorbance at 340 nm using a UV/vis spectrophotometer, such as an Ultrospec™ 1000, or a plate reader. The CDNB assay is performed in less than 10 minutes on crude bacterial sonicates, column eluates, or purified GST fusion protein. Figure 6 shows typical results from a CDNB assay. Each detection module contains reagents sufficient for 50 detections.

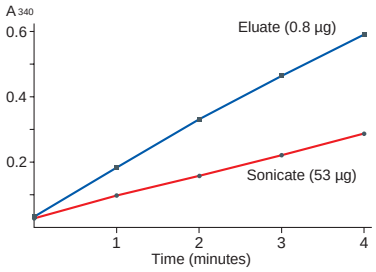


Fig. 6. Typical results of a CDNB assay for GST fusion proteins. 53 µg of total protein from an *E. coli* TG1/pGEX-4T-Luc sonicate and 0.8 µg of total protein eluted from Glutathione Sepharose were assayed according to the instructions for the GST Detection Module.

- 10X Reaction buffer: 1 M potassium phosphate buffer, pH 6.5.
- CDNB: 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol.
- Reduced glutathione powder for glutathione solution. Dissolve 100 mM reduced glutathione in sterile distilled water. Aliquot into microcentrifuge tubes.
  Store at -20°C. Avoid more than five freeze/thaw cycles.
- Goat/anti-GST antiserum for use in Western blots.

Measurement of GST activity by CDNB assay

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 before performing a CDNB assay.

1. In a microcentrifuge tube, combine the following:
   - Distilled water 880 µl
   - 10X Reaction buffer 100 µl
   - CDNB 10 µl
   - Glutathione solution 10 µl
   - Total Volume 1000 µl
2. Cap and mix by inverting the tube several times.

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

3. Transfer 500 µl volumes of the above solution into two UV-transparent cuvettes. Add sample (5-50 µl) to the "sample cuvette". To the "blank cuvette", add a volume of 1X Reaction buffer equal to the sample volume in the sample cuvette.
4. Cover each cuvette with wax film and invert to mix.
5. Place the blank cuvette in 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 one-minute intervals for 5 minutes 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

   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 minutes

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

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

Adapt the assay to give absolute fusion protein concentrations by constructing a standard curve of ΔA_{340}/min versus fusion protein amount.

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

Troubleshooting

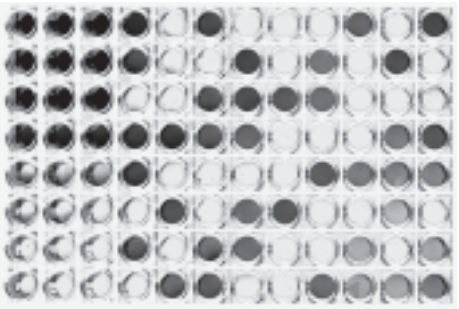
The reaction rate is linear provided that an A_{340} of approximately 0.8 is not exceeded during the five-minute time course. Plot initial results to verify that the reaction rate is linear over the time course. Adjust the amount of sample containing the GST fusion protein to maintain a linear reaction rate.

If a low absorbance is obtained using the CDNB assay, a Western blot using the Anti-GST Antibody may reveal high levels of protein expression.

Under standard assay conditions at +22°C and in the absence of GST, glutathione and CDNB react spontaneously to form a chemical moiety that produces a baseline drift at ΔA_{340}/min of approximately 0.003 (or 0.015 in 5 minutes). Correct for baseline drift by blanking the spectrophotometer with the blank cuvette before each reading of the sample cuvette.

Alternative 3: GST 96 Well Detection Module

The GST 96 Well Detection Module provides a highly sensitive ELISA assay for testing clarified lysates and intermediate purification fractions. Each detection module contains reagents sufficient for 96 detections:



- GST 96 Well detection plates in which each well is coated with anti-GST antibody, blocked and dried.
- Horse radish peroxidase conjugated to goat polyclonal anti-GST antibody.
- Purified recombinant glutathione S-transferase test protein.

Additional reagents to be prepared:

PBS:	140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.
Wash buffer:	0.05% Tween™ 20 in PBS (500 ml/96 well plate). Store at room temperature until needed.
Blocking buffer:	1 x conc. 3% non-fat dry milk in PBS with 0.05% Tween 20 (10 ml/96 well plate). 2 x conc. 6% non-fat dry milk in PBS with 0.1% Tween 20 (5 ml/96 well plate).

Prepare fresh buffers daily.

Run recombinant GST (rGST) protein as a standard control in every assay.

As each fusion protein is captured uniquely, prepare standards of rGST protein and the target fusion protein using a dilution series from 100 ng/100 µl to 10 pg/µl in 1X Blocking buffer if quantification is required.

Screening of GST expression clones or chromatographic fractions

1. Bring each test sample to a final volume of 50 µl with 1X PBS.
2. Mix with 50 µl of 2X Blocking buffer.
3. For screening: dilute rGST protein standard to 1 ng/100 µl in 1X Blocking buffer.
4. For quantification: use dilution series from 100 ng/100 µl to 10 pg/µl in 1X Blocking buffer for rGST protein and for the target fusion protein.
5. Remove one 96-well plate from the foil pouch. If using less than 96 wells, carefully remove the well strips from the holder by pushing up on the wells from below. Store unused swell strips in the pouch with the dessicant provided.
6. Pipette 100 µl of sample into each well.
7. Incubate for 1 hour at room temperature in a humidified container or incubator.
8. Empty contents of the well by flicking the inverted plate.
   (Biohazardous material should be pipetted or aspirated into a suitable container.)
9. Blot the inverted well or strips on to a paper towel to remove excess liquid.
10. Wash each well 5 times with wash buffer (inverting and flicking out the contents each time).
11. Blot the inverted well or strips on to a paper towel to remove excess wash buffer.
12. Dilute HRP/anti-GST conjugate 1:10 000 (1 µl:10 ml) in 1X Blocking buffer. One 96 well plate will require 10 ml of the diluted solution.
13. Add 100 µl of diluted HRP/anti-GST conjugate to each well and incubate for 1 hour at room temperature in a humidified container or incubator.
14. Empty well contents and wash twice with wash buffer as previously described.
15. Add soluble horseradish peroxidase substrate* to each well and incubate according to supplier's instructions.

*3,3',5,5'-tetramethyl benzidine (A_{450}) or 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.

Troubleshooting

See also Purification and Detection Troubleshooting page 29.

Low absorbance detected in samples

Check that samples were sufficiently induced and lysed (see Troubleshooting protein amplification page 9).

If clarified lysate is being tested, mix initial GST sample with 2X Blocking buffer to give a final concentration of 1X Blocking buffer.

Poor day to day reproducibility between identical samples

Ensure that all incubation times are consistent. Reduction in GST capture incubation time can be reduced to >30 minutes with slightly reduced signal, but HRP/anti-GST conjugate incubation time can significantly reduce signal with every 15 minute decrease.

Alternative 4. Western blot analysis

Amplification and purification can also be monitored by Western blot analysis, using ECL or ECL Plus detection systems to enhance sensitivity. Figure 7 shows an example of a typical Western blot analysis.

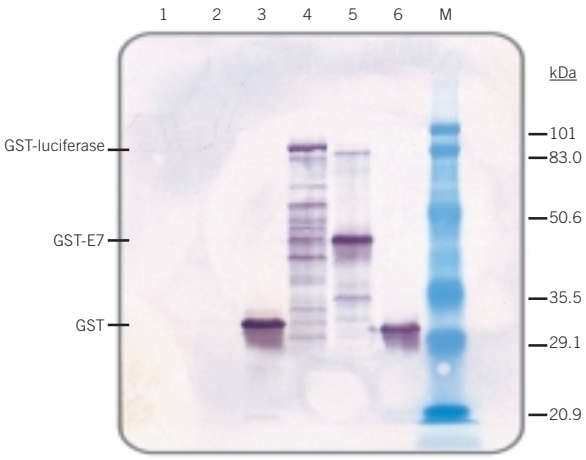


Fig. 7. Western blot *E. coli* cell lysates containing GST fusion proteins using anti-GST Antibody and anti-goat IgG alkaline phosphatase conjugate.

Anti-GST Antibody

Blocking/Incubation buffer: 5% (w/v) non-fat 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)

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

1. Separate the protein samples by SDS-PAGE.

Although anti-GST antibody from Amersham Pharmacia Biotech has been cross-adsorbed with *E. coli* proteins, low levels of cross-reacting antibodies may remain. It is recommended to always run a sample of an *E. coli* sonicate that does not contain a recombinant pGEX plasmid as a control.

2. Transfer the separated proteins from the electrophoresis gel to an appropriate membrane, such as Hybond ECL (for subsequent ECL detection) or Hybond P (for subsequent ECL Plus detection).

Electrophoresis and protein transfer may be accomplished using a variety of equipment and reagents. For further details, refer to the Protein Electrophoresis Technical Manual (from Amersham Pharmacia Biotech) and Hybond ECL instruction manual.

Blocking of membrane

1. Transfer the membrane onto which the proteins have been blotted to a container such as a Petri dish.
2. Add 50-200 ml of blocking/incubation buffer.
3. Incubate for 1-16 hours at ambient temperature with gentle shaking.

Longer incubation times (up to 16 hours) with blocking buffer may reduce background signal.

4. Decant and discard the buffer.

Anti-GST antibody

1. Prepare an appropriate dilution of anti-GST antibody with blocking/incubation buffer e.g. 5-10 µl of antibody to 50 ml of buffer. Refer to Amersham Pharmacia Biotech Application Note 18-1139-13 for further information on optimization.
2. Pour the antibody-buffer mixture into the container with the membrane.
3. Incubate for 1 hour at ambient temperature with gentle shaking.
4. Decant and discard the antibody-buffer.
5. Rinse twice with 20-30 ml of blocking or wash buffer to remove most of the unbound antibody.
6. Decant and discard the rinses.
7. Wash the membrane with 20-30 ml of blocking or wash buffer for 10-60 minutes at ambient temperature with gentle shaking.
8. Discard the wash and repeat.

Secondary antibody

1. Dilute an appropriate anti-goat secondary antibody with blocking/incubation buffer according to the manufacturer's recommendation. Refer to Amersham Pharmacia Biotech Application Note 18-1139-13 for further information on optimization.
2. Pour the antibody-buffer mixture into the container with the membrane.
3. Incubate for 1 hour at ambient temperature with gentle shaking.
4. Decant and discard the antibody-buffer.
5. Rinse twice with 20-30 ml of blocking or wash buffer to remove most of the unbound antibody.
6. Decant and discard the rinses.
7. Wash the membrane with 20-30 ml of blocking or wash buffer for 10-60 minutes at ambient temperature with gentle shaking.
8. Discard the wash and repeat.
9. Develop the blot with the appropriate substrate for the conjugated secondary antibody.

Troubleshooting

See also Purification and Detection Troubleshooting page 29.

Multiple bands seen on Western blot analysis

Anti-GST antibody from Amersham Pharmacia Biotech has been cross-absorbed against *E. coli* proteins and tested for its lack of non-specific background binding in a Western blot. Some sources of the anti-GST antibody may contain antibodies that react with various *E. coli* proteins present in the fusion protein sample. Cross-adsorb the antibody with an *E. coli* sonicate to remove anti-*E. coli* antibodies.

Purification and detection troubleshooting

Column has clogged

Cell debris in the sample may clog the column. Clean the column according to Appendix 5 and ensure that samples have been filtered or centrifuged.

Fusion protein does not bind to purification column

Over-sonication may have denatured the fusion protein. Check by using a microscope to monitor cell breakage. Use mild sonication conditions during cell lysis.

Add 5 mM DTT prior to cell lysis. This can significantly increase binding of some GST fusion proteins to Glutathione Sepharose.

Check that the column has been equilibrated with a buffer 6.5 < pH < 8.0 (e.g. PBS) before application of the fusion protein. The correct pH range is critical for efficient binding.

Decrease the flow rate to improve binding.

If re-using a pre-packed column, check that the column has been regenerated correctly (see Appendix 5). Replace with fresh Glutathione Sepharose or a new column if binding capacity does not return after regeneration.

Check the binding of a cell sonicate prepared from the parental pGEX plasmid. If GST produced from the parental plasmid binds with high affinity, then the fusion partner may have altered the conformation of GST, thereby reducing its affinity. Try reducing the binding temperature to +4°C and limit the number of washes.

Column capacity may have been exceeded. If using GSTrap columns (1 ml or 5 ml) link 2 or 3 columns in series to increase capacity or pack a larger column.

Fusion protein is poorly eluted

Increase concentration of glutathione in the elution buffer. Above 15 mM glutathione the buffer concentration should be increased to maintain pH.

Increase pH of the elution buffer. Values up to pH 9 may improve elution without requiring an increase in the concentration of glutathione.

Increase ionic strength of the elution buffer by addition of 0.1-0.2 M NaCl. Note that very hydrophobic proteins may precipitate under high salt conditions. If this is the case, addition of a non-ionic detergent may improve results (see below).

Decrease the flow rate to improve elution.

Add a non-ionic detergent (0.1% Triton™ X-100 or 2% N-octyl glucoside) to the elution buffer to reduce non-specific hydrophobic interactions that may prevent solubilization and elution of fusion proteins

Try over-night elution at room temperature or +4°C.

Multiple bands seen on SDS-PAGE or Western blot analysis

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

Check that a protease-deficient host such as *E. coli* B21 has been used.

Add protease inhibitors such as 1 mM PMSF to the lysis solution. A non-toxic, water soluble alternative to PMSF is 4-(2-amino-ethyl)- benzenesulfonyl fluoride hydrochloride (AEBSF), commercially available as Pefabloc™ SC from Boehringer Mannheim.

Serine protease inhibitors must be removed prior to cleavage by PreScission Protease, thrombin or Factor Xa.

Use pre-packed GSTrap columns or Glutathione Sepharose 4 Fast Flow. These can be used at higher flow rates to process samples more quickly and so avoid degradation.

Decrease sonication. Addition of lysozyme (0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris-HCl pH 8.0) prior to sonication may improve results. Avoid frothing as this may denature the fusion protein.

Include an additional purification step. A variety of proteins known as chaperonins that are involved in the correct folding of nascent proteins in *E. coli* may co-purify with GST fusion proteins, including a M_r 70 000 protein (see below).

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

Pre-incubate the protein solution with 2 mM ATP, 10 mM $MgSO_4$, 50 mM Tris-HCl (pH 7.4) for 10 minutes at +37°C prior to purification in order to dissociate the complex. This M_r 70 000 protein is probably a protein product of the *E. coli* gene *dna*K and involved in the degradation of "abnormal" proteins in *E. coli*. Reports suggest that this protein can be removed by ion exchange chromatography (Analects and Separations p24, 1996, Amersham Pharmacia Biotech and *(http://bionet.hgmp.mrc.ac.uk/hypermail/methods/methods.199406/0813.html)*) or by passage of the sample over ATP agarose (Myers, M., BIOSCI posting, 7 July 1993).
Thain, A., *et al. Trends Genet.* 12, 209-210 (1996) and Sherman, M. and Goldberg, A. L., *J. Biol. Chem*, 269, 31479-31483, (1994) suggest washing the column with ATP or GroES rather than using a subsequent IEX step.

Tag removal by enzymatic cleavage

In most cases, functional tests can be performed using the intact fusion with GST. If removal of the GST tag is necessary, it is highly recommended to produce the fusion proteins with a PreScission Protease cleavage site. The GST tag then can be removed and the protein purified in a single step on the column (see Figure 8). This protease also has the useful property of being maximally active at +4°C thus allowing cleavage to be performed at low temperatures and so improving the stability of the target protein.

Thrombin or Factor Xa recognition sites may be cleaved either while bound on the column or in solution after elution from the column (see Figure 9). However, the cleavage enzyme must be removed in a second step, usually requiring the development of a simple ion exchange chromatographic separation (see Appendix 9).

On-column cleavage is generally recommended as the method of choice since many potential contaminants can be washed through the column and the target protein eluted with a higher level of purity.

The amount of enzyme, temperature and length of incubation required for complete digestion varies according to the specific GST fusion protein produced. Determine optimal conditions in pilot experiments.

Remove samples at various time points and analyse by SDS-PAGE to estimate the yield, purity and extent of digestion.

Approximate molecular weights for SDS-PAGE analysis.

PreScission Protease	M_r 46 000
Bovine thrombin	M_r 37 000
Bovine Factor Xa	M_r 48 000

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

Cleavage of fusion proteins is most commonly performed on milligram quantities of fusion protein suitable for purification on GSTrap. The following protocols describe a manual cleavage and purification using a syringe and a 1 ml GSTrap column. The protocols can be adapted for use with GST MicroSpin columns to work at smaller scales or scaled up onto larger columns to run on ÄKTAdesign systems.

PreScission Protease cleavage and purification

PreScission Protease is a fusion protein of GST and human rhinovirus 3C protease. The protease specifically recognizes the amino acid sequence Leu-Glu-Val-Leu-Phe-Gln↓Gly-Pro cleaving between the Gln and Gly residues. Since the protease is fused to GST, it is easily removed from cleavage reactions using GSTrap or Glutathione Sepharose. This protease also has the useful property of being maximally active at +4°C thus allowing cleavage to be performed at low temperatures and so improving the stability of the target protein.

Enzymatic cleavage

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

Cleavage should be complete following a 4 hour treatment at +5°C with ≤10 cleavage units/mg of fusion protein.

Incubation times may be reduced by adding a greater amount of PreScission Protease.
(continued on page 34)

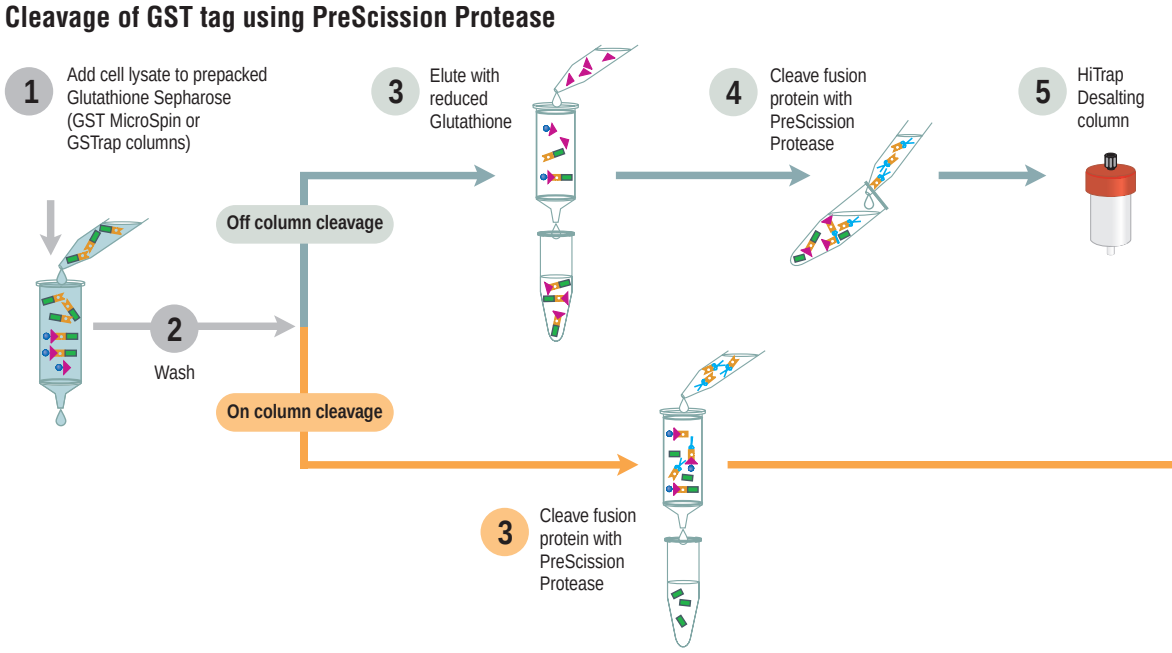


Fig. 8. Flow chart of the affinity purification procedure and PreScission Protease cleavage of glutathione S-transferase fusion proteins.

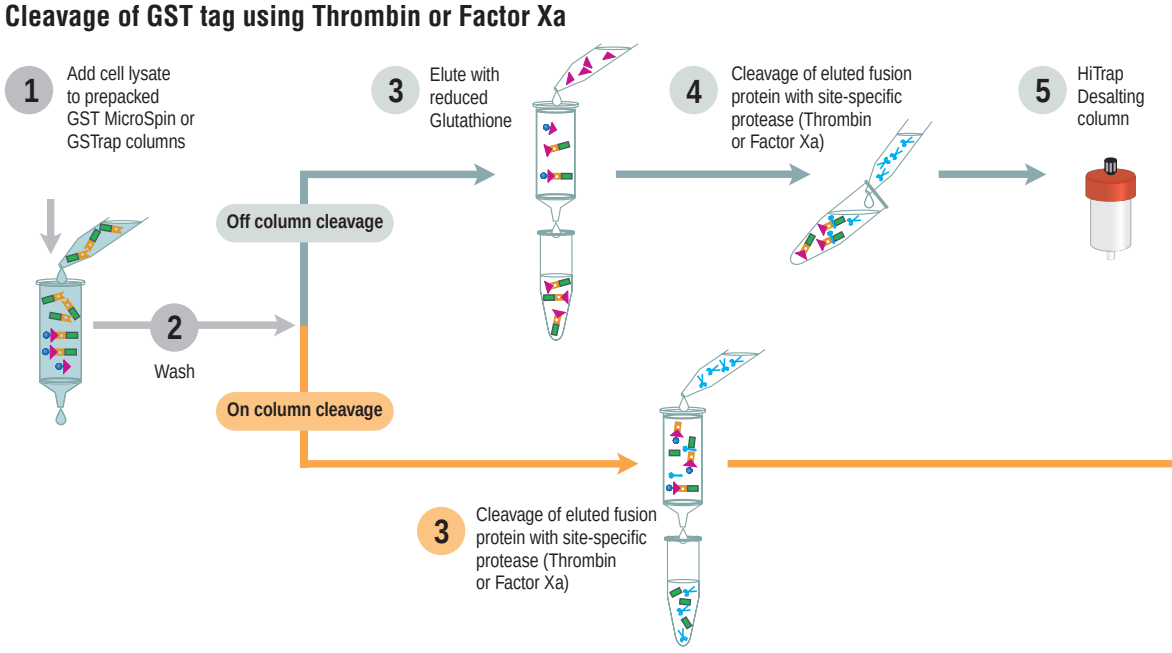
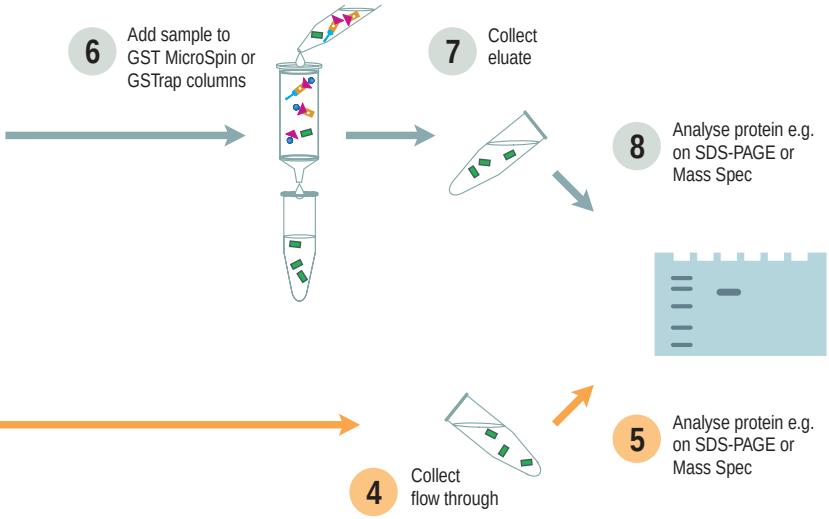
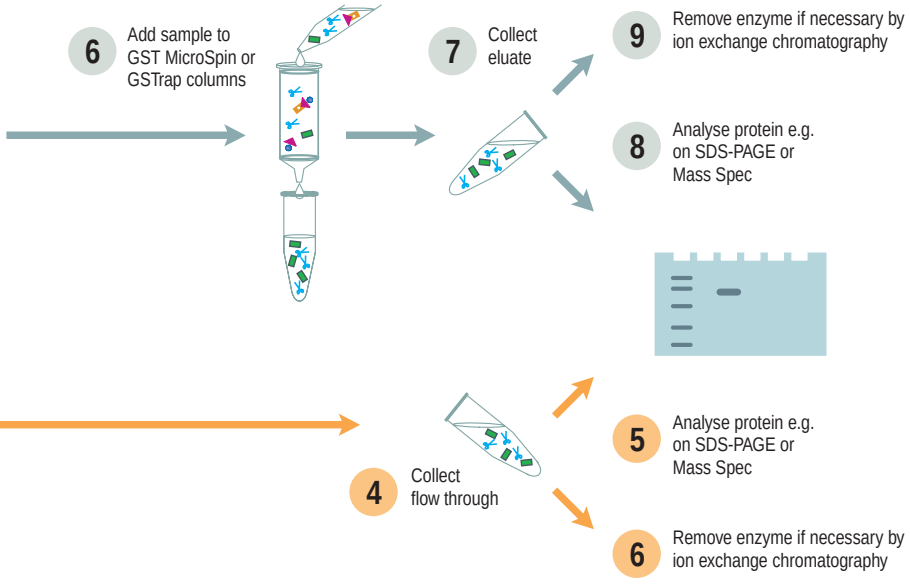
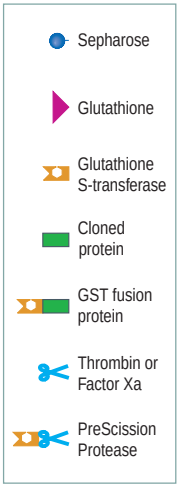


Fig. 9. Flow chart of the affinity purification procedure and Thrombin or Factor Xa cleavage of glutathione S-transferase fusion proteins.


6
Add sample to GST MicroSpin or GSTrap columns
7
Collect eluate
8
Analyse protein e.g. on SDS-PAGE or Mass Spec
5
Analyse protein e.g. on SDS-PAGE or Mass Spec
4
Collect flow through


6
Add sample to GST MicroSpin or GSTrap columns
7
Collect eluate
9
Remove enzyme if necessary by ion exchange chromatography
8
Analyse protein e.g. on SDS-PAGE or Mass Spec
5
Analyse protein e.g. on SDS-PAGE or Mass Spec
4
Collect flow through
6
Remove enzyme if necessary by ion exchange chromatography


Sepharose
Glutathione
Glutathione S-transferase
Cloned protein
GST fusion protein
Thrombin or Factor Xa
PreScission Protease

Alternative 1. On-column cleavage and purification

Assumption: 8 mg GST fusion protein bound/ml gel

1. Fill the syringe with binding buffer.
2. Connect the column to the syringe using the adaptor supplied ("drop to drop" to avoid introducing air into the column).
3. Remove the twist-off end.
4. Equilibrate the column with 5 column volumes of binding buffer.
5. Apply the sample using the syringe. For best results, maintain a flow rate of 0.2-1 ml/min as the sample is applied.
6. Wash with 5-10 column volumes of binding buffer. Maintain flow rates of 1-2 ml/min during the wash.
7. Wash the column with 10 column volumes of PreScission cleavage buffer.
8. Prepare the PreScission Protease mix: mix 80 µl (160 units) of PreScission Protease with 920 µl of PreScission cleavage buffer at +5°C.
9. Load the PreScission Protease mix onto the column using a syringe and the adaptor supplied. Seal the column with the top cap and the domed nut supplied.
10. Incubate the column at +5°C for 4 hours.
11. Fill a syringe with 3 ml of PreScission cleavage buffer. Remove the top cap and domed nut. Avoid introducing air into the column. Begin elution and collect the eluate (0.5 ml-1 ml/tube).

N.B. The eluate will contain the protein of interest, while the GST moiety of the fusion protein and the PreScission Protease will remain bound to GSTrap.

Alternative 2. Off-column cleavage and purification

Assumption: 8 mg GST fusion protein bound/ml gel

1. Follow steps 1-5 above.
2. Elute with 5-10 column volumes of elution buffer. Maintain flow rates of 1-2 ml/min during elution.
3. Remove the reduced glutathione from the eluate using a quick buffer exchange on HiTrap Desalting or HiPrep™ 26/10 Desalting depending on the sample volume.
4. Add 1 µl (2 U) of PreScission Protease for each 100 µg of fusion protein in the eluate. If the amount of fusion protein in the eluate has not been determined, add 80 µl (160 units) of PreScission Protease.
5. Incubate at +5°C for 4 hours.
6. Once digestion is complete, apply the sample to an equilibrated GSTrap column to remove the GST moiety of the fusion protein and the PreScission Protease. The protein of interest will be found in the flow-through, while the GST moiety of the fusion protein and the PreScission Protease will remain bound to the column.

Troubleshooting

Incomplete PreScission Protease cleavage

Check that the PreScission Protease to fusion protein ratio is correct (although saturation of the purification column is rarely a problem).

Increase the incubation time to 20 hours or longer at +5°C and increase the amount of PreScission Protease used in the reaction.

Verify presence of the PreScission Protease cleavage site. Compare the DNA sequence of the construct with the known PreScission Protease cleavage sequence. Verify that the optimal PreScission Protease recognition site, Leu-Glu-Val-Leu-Phe-Gln↓Gly-Pro, has not been altered.

Remove possible PreScission Protease inhibitors by extensive washing of the purification column before cleaving with PreScission Protease. The presence of Zn^{2+} as well as Pefabloc SC or chymostatin may interfere with PreScission Protease activity.

Multiple bands seen on SDS Gel after cleavage

Determine when the bands appear. Additional bands seen prior to PreScission Protease cleavage may be the result of proteolysis in the host bacteria. *E. coli* BL21 is a recommended protease-deficient strain.

Check the sequence of the fusion partner for the presence of additional PreScission Protease recognition sites. PreScission Protease optimally recognizes the sequence Leu-Glu-Val-Leu-Phe-Gln↓Gly-Pro and cleaves between the Gln and Gly residues but similar secondary sites may exhibit some propensity for cleavage. Adjusting reaction conditions (e.g. time, temperature, salt concentration) may result in selective cleavage at the desired site. If adjustment of the conditions does not correct the problem, reclone the insert into a pGEX T (thrombin) or pGEX X (Factor Xa) expression vector.

Fusion partner is contaminated with PreScission Protease after purification

Pass the sample over fresh Glutathione Sepharose to remove residual PreScission Protease (the Glutathione Sepharose may have been saturated with GST fusion protein in the first purification). Alternatively, a conventional ion exchange chromatography separation can be developed to remove the PreScission Protease and other minor contaminants (see Appendix 9).

Thrombin cleavage and purification

Enzymatic cleavage

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 cleavage units in 0.5 ml of PBS pre-chilled to +4°C. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.

With a specific activity >7500 units/mg protein one cleavage unit of thrombin will digest ≥ 90% of 100 µg of a test fusion protein in 16 hours at +22°C in elution buffer. A cleavage unit is approximately equal to 0.2 NIH units.

Cleavage should be complete following overnight treatment with ≤10 cleavage units/mg of fusion protein.

For some applications, thrombin should be subsequently removed from the sample by chromatography using Benzamidine Sepharose (specific instructions are supplied with the product). Alternatively, a conventional ion exchange chromatography separation can be developed to remove the protease and other minor contaminants (see Appendix 9).

Alternative 1. On-column cleavage and purification

Assumption: 8 mg GST fusion protein bound/ml gel

1. Follow steps 1-6 under Prescission Protease cleavage.
2. Prepare the thrombin mix: mix 80 µl thrombin solution (1 U/µl) with 920 µl PBS.
3. Load the thrombin solution onto the column using a syringe and the adaptor supplied. Seal the column with the top cap and the domed nut supplied.
4. Incubate the column at room temperature (+22° to +25°C) for 2-16 hours.
5. Fill a syringe with 3 ml PBS. Remove the top cap and domed nut from the column. Avoid introducing air into the column. Begin elution and collect the eluate (0.5 ml-1 ml/tube).

N.B. The eluate will contain the protein of interest and thrombin, while the GST moiety of the fusion protein will remain bound to GSTrap.

Alternative 2. Off-column cleavage and purification

Assumption: 8 mg GST fusion protein bound/ml gel

1. Follow steps 1-7 under Prescission Protease cleavage.
2. Add 10 µl (10 units) of thrombin solution for each mg of fusion protein in the eluate. If the amount of fusion protein in the eluate has not been determined, add 80 µl (80 U) thrombin solution.
3. Incubate at room temperatue (+22° to +25°C) for 2-16 hours.
4. Once digestion is complete, GST can be removed by first removing glutathione using a quick buffer exchange on HiTrap Desalting or HiPrep 26/10 Desalting depending on the sample volume.
5. Apply the sample to an equilibrated GSTrap column. The purified protein of interest and thrombin will be found in the flow-through.

Troubleshooting

Incomplete Thrombin cleavage

Check that the thrombin to fusion protein ratio is correct.

Increase the reaction time to 20 hours at +5°C and increase the amount of thrombin used in the reaction.

Check the sequence of the fusion partner for the thrombin recognition sequence. In most cases, thrombin cleaves following Arg/Lys in -Pro-Arg↓/Lys.
Ref: Chang, J-Y., Eur. J. Biochem. 151, p217 (1985)

Check DNA sequence of construct to verify presence of thrombin site. Compare with known sequence, and verify that the thrombin recognition site has not been altered.

Multiple bands SDS-PAGE after cleavage

Determine when the bands appear. Additional bands seen prior to cleavage may be the result of proteolysis in the host bacteria. *E. coli* BL21 is a protease-deficient strain that is recommended.

Check the sequence of the fusion partner for the presence of thrombin recognition sites. The optimum cleavage site for thrombin can have the following two structures: P4-P3-Pro-Arg/Lys↓P1´-P2´ where P3 and P4 are hydrophobic amino acids and P1´ and P2´ are non-acidic amino acids. The Arg/Lys↓P1´ bond is cleaved.
Ref: Chang, J-Y., Eur. J. Biochem. 151, p217 (1985)

Examples:

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

In A, the Arg↓Gly bond is cleaved very fast by thrombin. In B, the Lys↓Leu bond is cleaved. C is the recognition sequence found on the thrombin series of pGEX plasmids and the Arg↓Gly bond is cleaved.

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

Examples:

	P2	R/K↓P1´
A)	Ala	Arg↓Gly
B)	Gly	Lys↓Ala

In A, the Arg↓Gly bond is cleaved efficiently. In B, the Lys↓Ala bond is cleaved. Adjusting time and temperature of digestion can result in selective scission at the desired thrombin site. If adjustment of conditions does not correct the problem, reclone the insert into a pGEX-6P (PreScission) or pGEX X expression vector.

Factor Xa cleavage and purification

Enzymatic cleavage

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

Factor Xa solution: Dissolve 400 cleavage units of Factor Xa in +4°C water to give a final solution of 1 U/µl. Swirl gently.

Store solution in small aliquots at -80°C to preserve activity.

With a specific activity of >800 units/mg protein one cleavage unit of Factor Xa will digest ≥ 90% of 100 µg of a test fusion protein in 16 hours at +22°C in Factor Xa cleavage buffer.

Cleavage should be complete following overnight treatment at +22°C with a Factor Xa to substrate ratio of 1% (w/w).

For some applications, Factor Xa should be removed from the sample using a HiTrap Heparin column. Alternatively, a conventional ion exchange chromatography separation can be developed to remove the Factor Xa and other minor contaminants (see Appendix 9).

Factor Xa consists of two subunits linked by disulphide bridges. Since glutathione can disrupt disulphide bridges, it should be removed from the sample prior to the cleavage reaction. Glutathione can be easily and rapidly removed from the fusion protein using a desalting column (Chapter 7) with Factor Xa cleavage buffer as eluent.

Alternative 1. On-column cleavage and purification

Assumption: 8 mg GST fusion protein bound/ml gel

1. Follow steps 1-6 under Prescission Protease cleavage.
2. Wash GSTrap with 10 column volumes of Factor Xa cleavage buffer.
3. Prepare the Factor Xa mix: Mix 80 µl Factor Xa solution with 920 µl Factor Xa cleavage buffer.
4. Load the mix onto the column using a syringe and the adaptor supplied. Seal the column with the top cap and the domed nut supplied.
5. Incubate the column at room temperature (+22° to +25°C) for 2-16 hours.
6. Fill a syringe with 3 ml (1 ml column) or 15 ml (5 ml column) Factor Xa cleavage buffer. Remove the top cap and domed nut from the column. Avoid introducing air into the column. Begin elution and collect the eluate (0.5 ml-1 ml/tube).

N.B. The eluate will contain the protein of interest and Factor Xa, while the GST moiety of the fusion protein will remain bound to GSTrap.

Alternative 2. Off-column cleavage and purification

Assumption: 8 mg GST fusion protein bound/ml gel

1. Follow steps 1-7 under Prescission Protease cleavage.
2. Remove the reduced glutathione from the eluate using a quick buffer exchange on HiTrap Desalting or HiPrep 26/10 Desalting depending on sample volume.
3. Add 10 units of Factor Xa solution for each mg fusion protein in the eluate. If the amount of fusion protein in the eluate has not been determined, add 80 µl (80 units) of Factor Xa solution.
4. Incubate the column at room temperature (+22° to +25°C) for 2-16 hours.
5. Once digestion is complete, apply the sample to an equilibrated GSTrap column to remove the GST moiety of the fusion.

N.B. The protein of interest will be found in the flow-through together with Factor Xa.

Troubleshooting

Incomplete Factor Xa cleavage

Functional Factor Xa requires activation of Factor X with Russell's viper venom. Factor Xa from Amersham Pharmacia Biotech has been preactivated, but other sources may not be activated. Activation conditions: Incubate 1% (w/w) of Russell's viper venom to Factor X in 8 mM Tris-HCl (pH 8.0), 70 mM NaCl, 8 mM $CaCl_2$ at +37°C for 5 minutes.

Check the sequence of the fusion partner to see if the first three nucleotides after the Factor Xa recognition sequence code for Arg or Pro. The recognition sequence for Factor Xa is Ile-Glu-Gly-Arg↓X, where X can be any amino acid except Arg or Pro.

Check that the Factor Xa to fusion protein ratio is correct.

Check that glutathione has been removed as recommended.

In some cases increase substrate concentration up to 1 mg/ml may improve results.

Add <0.5% w/v SDS to the reaction buffer. This can significantly improve Factor Xa cleavage with some fusion proteins. Various concentrations of SDS should be tested to find the optimum concentration.

Increase incubation time to 20 hours or longer at +22°C and the amount of Factor Xa (for some fusion proteins, Factor Xa can be increased up to 5%).

Verify presence of Factor Xa site: Check DNA sequence of construct. Compare with known sequence, and verify that the Factor Xa recognition site has not been altered.

Multiple bands on SDS-PAGE after cleavage

Determine when the bands appear. Additional bands seen prior to cleavage may be the result of proteolysis in the host bacteria. *E. coli* BL21 is a protease-deficient strain that is recommended.

Check the sequence of the fusion partner for the presence of Factor Xa recognition sites. Factor Xa is highly specific for the recognition sequence Ile-Glu-Gly-Arg↓. The bond following the Arg residue is cleaved Adjusting time and temperature of digestion can result in selective scission at the desired Factor Xa site. If adjustment of conditions does not correct the problem, reclone the insert into a pGEX-6P (PreScission) or pGEX T (Thrombin) expression vector.

CHAPTER 4

$(His)_6$ fusion proteins

Amplification

General considerations for the amplification of fusion proteins are discussed in Chapter 2.

The vectors and hosts

There is a wide variety of hosts and vectors for the amplification of $(His)_6$ fusion proteins. The factors that should be considered when selecting the host and vector are discussed in Chapter 1.

Purification

Figure 10 gives an overview of a typical purification flow scheme for $(His)_6$ fusion proteins, including purification under denaturing conditions. On-column purification and refolding of $(His)_6$ fusion proteins is described in Chapter 5.

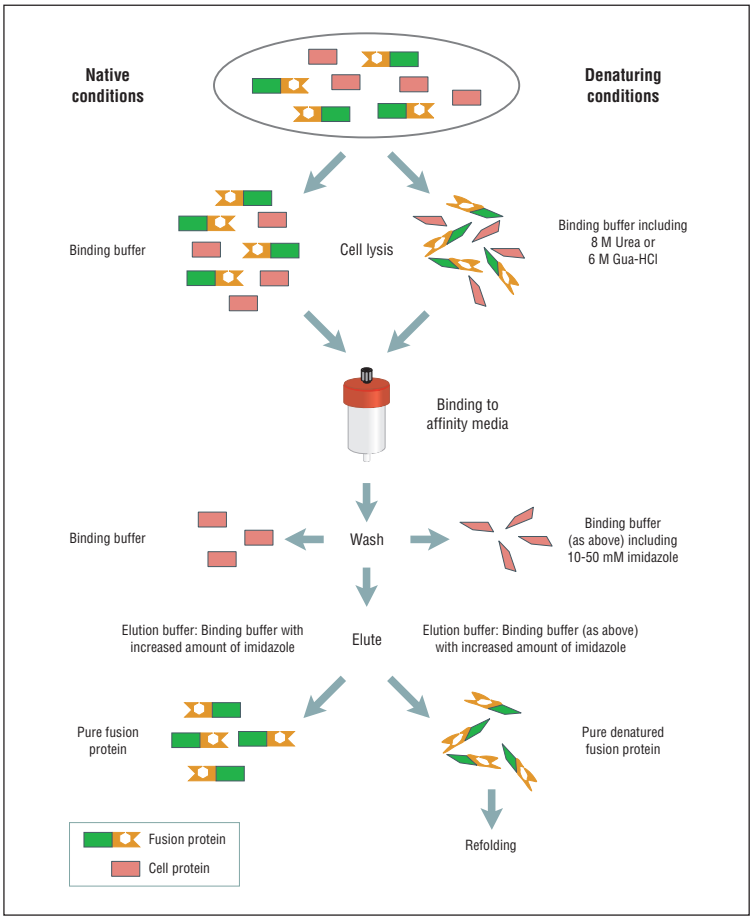


Fig. 10.

For simple, one step purification of $(His)_6$ fusion proteins there is a range of products designed to meet specific purification needs, as shown in Table 13.

Chelating Sepharose, when charged with Ni^{2+} ions, selectively retains proteins if complex-forming amino acid residues, in particular histidine, are exposed on the protein surface. $(His)_6$ fusion proteins can be easily desorbed with buffers containing imidazole.

If the $(His)_6$ fusion proteins are expressed as inclusion bodies, see Chapter 5 for solubilization, refolding and purification information.

Column/loose**	Amount of protein for a single purification	Comment
His MicroSpin Purification Module	Up to 100 µg	Ready to use, pre-packed columns, buffers and chemicals
		High throughput when used with MicroPlex 24
		Vacuum (up to 48 samples simultaneously)
HiTrap Chelating 1 ml	Up to 12 mg*	Pre-packed column, ready to use
HisTrap Kit	Up to 12 mg/column*	As above, but includes buffers for up to 12 purifications using a syringe
HiTrap Chelating 5 ml	Up to 60 mg*	Pre-packed column, ready to use
Chelating Sepharose Fast Flow	12 mg $(His)_6$ fusion protein* per ml	Supplied loose for packing columns and scale up
	*estimate for a protein of M_r 27 600, binding capacity varies according to specific protein	

Table 13. Summary of purification options for $(His)_6$ fusion proteins.

**Characteristics of HiTrap Chelating and Chelating Sepharose Fast Flow are given in Appendix 5.

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

Batch preparation procedures are frequently mentioned in the literature. However the availability of pre-packed columns and easily packed high flow rate Chelating Sepharose provide faster, more convenient alternatives.

Batch preparations are occasionally used if it appears that the tag is not fully accessible or when the protein in the lysate is at very low concentrations (both could appear to give a low yield from the first purification step). A more convenient alternative to improve yield is to decrease the flow rate or pass the sample through the column several times.

Monitor purification steps by one or more of the detection methods referred to in this book.

The choice of purification equipment should also be made according to the needs of the purification. Appendix 8 provides a guide to aid in the selection of the correct purification solution and key points to consider are highlighted below.

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

For purification of larger quantities of fusion proteins HisTrap Kit or HiTrap Chelating columns are ready to use with a syringe, a peristaltic pump or a chromatography system.

To increase capacity use several HiTrap Chelating columns (1 ml or 5 ml) in series or, for even larger capacity requirements, pack Chelating Sepharose Fast Flow into a suitable column (details of column packing procedures are outlined in Appendix 6).

For simple and reproducible purification a chromatography system such as ÄKTA*prime* is a significant advantage, recording the purification process and eliminating manual errors.

For laboratory environments in which all experimental data must be recorded and traceable or where method development, optimization and scale up are required, a computer controlled ÄKTAdesign chromatography system is recommended.

Experiments such as protein refolding or method optimization require linear gradient elution steps that can only be performed by a chromatography system.

His MicroSpin Purification Module

The His MicroSpin Purification Module is useful for screening small or large numbers of lysates and for checking samples during the optimization of amplification or purification conditions. Each module contains reagents sufficient for 50 purifications.

- 10X PBS: 1.4 M NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.3
- 8X Phosphate/NaCl buffer: 160 mM phosphate, 4 M NaCl, pH 7.4
- 4 M Imidazole elution buffer (avoid contact with skin or eyes)
- IPTG: 500 mg
- His MicroSpin columns: 50 units

Reagents are prepared as follows:

1X PBS:	Dilute 10X PBS with sterile water. Store at +4°C.
PNI_{20} wash buffer:	Mix 6.25 ml of 8X Phosphate/NaCl buffer with 0.25 ml imidazole elution buffer. Add distilled water to a final volume of 50 ml.
PNI_{400} elution buffer:	Mix 6.25 ml of 8X Phosphate/NaCl buffer with 5 ml imidazole elution buffer. Add distilled water to a final volume of 50 ml.
IPTG 100 mM:	Dissolve contents of the IPTG vial in 20 ml sterile water. Store as 1 ml aliquots at -20°C.

Alternative 1. High throughput purification using MicroPlex Vacuum

Do not apply more than 400 µl at a time to a His MicroSpin column. This procedure will accommodate lysates from 2 to 8 ml of culture.

Also required:

- Vacuum source capable of providing 220 mm Hg (e.g. a house vacuum)
- Side arm flask, 500 ml or 1 litre
- Single hole rubber stop
- Vacuum tubing
- MicroPlex 24 Vacuum apparatus

1. Assemble the MicroPlex 24 Vacuum following the instructions supplied.
2. Remove the caps from the MicroSpin columns. Place the columns in the manifold, filling any unused holes with the plugs provided with MicroPlex 24 Vacuum.
3. Apply 400 µl of lysate to the column.

If using < 400 µl adjust volume to 400 µl with 1X PBS beforehand.

4. Recap each column securely and mix by gentle, repeated inversion. Incubate at room temperature for 5-10 minutes.
5. Remove columns from the manifold and remove top caps and bottom closures. Return columns to the manifold.
6. Ensure the stopcock is in the closed position (i.e. perpendicular to the vacuum tubing) and that the manifold is placed squarely on the gasket.
7. Turn on vacuum supply at source. Open the stopcock (i.e. parallel to the vacuum tubing). After lysates have been drawn through all the columns into the collection tray, close the stopcock.
8. Allow 10-15 seconds for the vacuum pressure to dissipate. Remove the manifold and place it on a paper towel.
9. Discard eluate and re-use collection plate or save eluate in the collection plate for later analysis and use a new collection plate for the next step.
10. Add 600 µl of PNI_{20} wash buffer to each column. Open the stopcock. After buffer has been drawn through all the columns into the collection tray, close the stopcock.
11. Allow 10-15 seconds for the vacuum pressure to dissipate. Remove the manifold and reassemble the apparatus with a clean collection tray.
12. Add 200 µl of PNI_{400} elution buffer to each column. Incubate at room temperature for 5-10 minutes.
13. Open the stopcock. After elution buffer has been drawn through all the columns into the collection tray, close the stopcock.
14. Allow 10-15 seconds for the vacuum pressure to dissipate. Remove the manifold. Cover eluates with sealing tape until required for analysis.

Troubleshooting

See Purification and Detection Troubleshooting page 53.

Alternative 2. Purification of up to 12 samples using a microcentrifuge

Do not apply more than 400 µl at a time to a His MicroSpin column. This procedure will accommodate lysates from 2 to 8 ml of culture.

1. Remove (and save) each column cap.
2. Apply 400 µl of lysate to the column.

If using < 400 µl adjust volume to 400 µl with 1X PBS beforehand.

3. Recap each column securely and mix by gentle, repeated inversion. Incubate at room temperature for 5-10 minutes.
4. Remove (and save) the top cap and bottom closure from each column. Place column into a clean 1.5 or 2 ml microcentrifuge tube. Spin for 1 minute at 735 x g.
5. Remove the MicroSpin columns and place into a clean 1.5 or 2 ml microcentrifuge tube.
6. Apply 600 µl of PNI_{20} wash buffer to each column. Repeat spin procedure.
7. Replace the bottom closure on each column. Add 100-200 µl of PNI_{400} elution buffer to each column. Replace top cap and incubate at room temperature for 5-10 minutes.
8. Remove and discard top cap and bottom closure from each column and place the column into a clean 1.5 or 2 ml microcentrifuge tube.
9. Spin all columns again to collect eluate. Save for analysis.

Troubleshooting

See Purification and Detection Troubleshooting page 53.

Alternative 3. Purification using MicroPlex

Do not apply more than 400 µl at a time to a His MicroSpin column. This procedure will accommodate lysates from 2 to 8 ml of culture.
See Appendix 4 for recommended centrifugation systems.

1. Assemble the MicroPlex 24 unit following the instructions supplied. Two units can be processed simultaneously to handle 48 samples.
2. Remove the caps from the MicroSpin columns and place the columns in the manifold.
3. Apply 400 µl of lysate to each column.

If using < 400 µl adjust volume to 400 µl with 1X PBS beforehand.

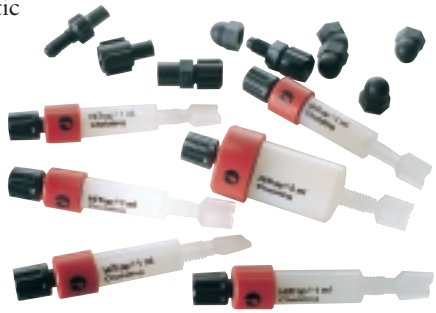
4. Recap each column securely and mix by gentle, repeated inversion. Incubate at room temperature for 5-10 minutes.
5. Remove columns from the manifold and remove top caps and bottom closures. Return columns to the manifold.
6. Centrifuge the unit for 2 minutes following the instructions supplied.
7. Add 600 µl of PNI_{20} wash buffer to each column.
8. Centrifuge the unit for 2 minutes following the instructions supplied. Remove the manifold from each collection try and place on a clean paper towel. Reassemble each unit with a fresh collection tray.
9. Add 100-200 µl of PNI_{400} elution buffer to each column. Incubate at room temperature for 5-10 minutes.
10. Centrifuge the unit for 2 minutes following the instructions supplied. Cover the eluted samples with sealing tape until required for analysis.

Troubleshooting

See Purification and Detection Troubleshooting page 53.

Purification using HiTrap Chelating 1 ml or 5 ml columns

HiTrap Chelating columns are pre-packed and ready for use. They can be operated with a syringe, a peristaltic pump or a liquid chromatography system such as ÄKTA*prime*. Figure 10 illustrates the simple steps for purification of a $(His)_6$ fusion protein.



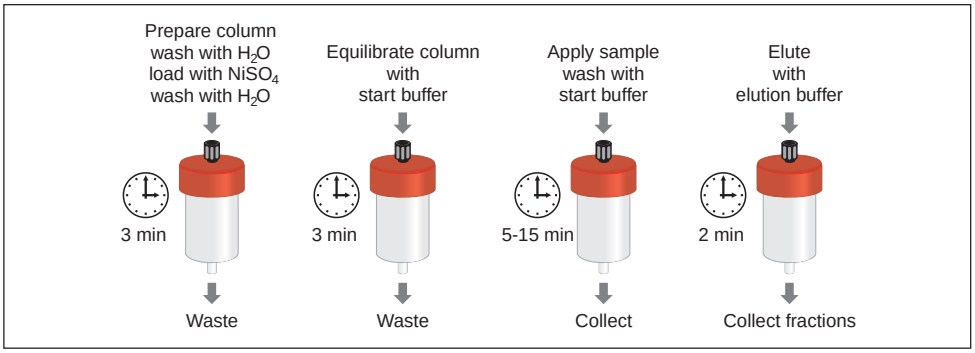


Fig. 10. HiTrap Chelating (1 ml) and a schematic overview of $(His)_6$ fusion protein purification.

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

HiTrap Chelating columns (1 ml and 5 ml) can be connected in series to increase the binding capacity. At larger scale columns can be packed with Chelating Sepharose Fast Flow (for column packing see Appendix 6).

Sample and buffer preparation

Use high quality water and chemicals. Filtration through 0.45 µm filters is recommended.

Samples should be centrifuged immediately before use and/or filtered through a 0.45 µm filter. If the sample is too viscous, dilute with binding buffer.

Sample binding properties can be improved by adjusting the sample to the composition of the binding buffer: dilute in binding buffer or perform a buffer exchange using a desalting column (see Chapter 7).

If the fusion protein is expressed as an inclusion body, add 6 M guanidinium hydrochloride or 8 M urea to all buffers.

During purification there is a balance between the amount of imidazole needed to prevent non-specific binding of contaminants and the amount of imidazole needed to elute the $(His)_6$ fusion protein, conditions may need to be optimized.

Chelating Sepharose characteristics, column regeneration and storage procedures are described in Appendix 5.

Alternative 1. Purification using HisTrap Kit

HisTrap kit includes everything needed for 12 preparations using a syringe. Three ready-to-use HiTrap Chelating 1 ml columns and ready-made buffer concentrates are supplied with easy-to-follow instructions.

• HiTrap Chelating columns	3 x 1 ml
• Phosphate buffer, 8X Stock solution, pH 7.4	2 x 50 ml
• 2 M imidazole, pH 7.4	50 ml
• 0.1 M $NiSO_4$	10 ml
• Syringe	1 x 5 ml
• All necessary connectors	

Purification protocols

Column preparation

1. Fill syringe with distilled water. Remove stopper and connect the column to the syringe with the adaptor provided ("drop to drop" to avoid introducing air into the column).
2. Remove the twist-off end.
3. Wash the column with 5 ml distilled water, using the syringe.

Do not use buffer to wash away the 20% ethanol solution as nickel salt precipitation can occur in the next step. If air is trapped in the column, wash the column with distilled water until the air disappears.

4. Disconnect syringe and adaptor from the column, fill syringe with 0.5 ml of the 0.1 M nickel solution supplied and load onto the column.
5. Wash column with 5 ml distilled water.

A. Basic purification protocol for high yield

In all steps use the syringe supplied.

1. Prepare 24 ml start buffer. Mix 3 ml phosphate buffer 8X Stock solution with 0.12 ml 2 M imidazole and add water up to 24 ml. Check pH and adjust to pH 7.4-7.6 if necessary. This buffer will contain 20 mM phosphate, 0.5 M NaCl and 10 mM imidazole.
2. Prepare 8 ml elution buffer. Mix 1 ml phosphate buffer 8X Stock solution with 2 ml 2 M imidazole and add distilled water up to 8 ml. Check pH and adjust to pH 7.4-7.6 if necessary. This buffer will contain 20 mM phosphate (1x), 0.5 M NaCl and 500 mM imidazole.
3. Equilibrate the column with 10 ml start buffer.
4. Apply sample at a flow rate 1-4 ml/min. Collect the flow-through fraction. A pump is more suitable for application of sample volumes greater than 15 ml.
5. Wash with 10 ml start buffer. Collect wash fraction.
6. Elute with 5 ml elution buffer.

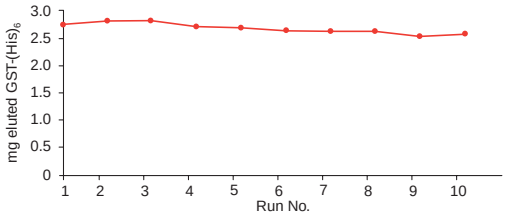
Avoid dilution of the eluate by collecting the eluate in 1 ml fractions.

7. Check the purification by analysing aliquots of starting material, flow through and eluent, for example, by SDS-PAGE. The purified protein is most likely to be found in the 2nd + 3rd ml of the elution step.

Imidazole absorbs at 280 nm. Use elution buffer as blank when monitoring absorbance. If imidazole needs to be removed use a desalting column (see Chapter 7).

8. After the protein has been eluted, regenerate the column by washing with 10 ml of start buffer. The column is now ready for a new purification and there is rarely a need to reload with metal if the same $(His)_6$ protein is to be purified (see Figure 11).

Samples: 2.5 ml GST-$(His)_6$ cell extract
Binding buffer: 1X Binding buffer, 20 mM imidazole, pH 7.4
Elution buffer: 1X Binding buffer, 500 mM imidazole, pH 7.4
Flow rate: 2 ml/min, 312 cm/h
Note: No Ni^{2+} re-loading of the column between the runs



Result:

Run No.	Eluted GST-$(His)_6$, total mg
1	2.76
2	2.82
3	2.83
4	2.72
5	2.71
6	2.65
7	2.64
8	2.63
9	2.54
10	2.59

Fig. 11. 10 repetitive purifications of GST-$(His)_6$ without reloading the column with Ni^{2+} between the runs.

Troubleshooting

See Purification and Detection Troubleshooting page 53.

B. Optimization purification protocol for high purity

After optimization the same protein can be purified as in the Basic Protocol.

In all steps use the syringe supplied.

1. Prepare buffers according to Table 14. Use 1X Phosphate buffer including 10 mM imidazole as start buffer and 6 steps ranging up to 500 mM imidazole as elution buffers. Check pH after mixing and adjust to pH 7.4-7.6 if necessary.

Imidazole concentration in buffer	Phosphate buffer 8X Stock solution pH 7.4	2 M Imidazole pH 7.4	Distilled water
10 mM	3.0 ml	0.12 ml	to 24 ml
20 mM	1.0 ml	0.08 ml	to 8 ml
40 mM	1.0 ml	0.16 ml	to 8 ml
60 mM	1.0 ml	0.24 ml	to 8 ml
100 mM	1.0 ml	0.40 ml	to 8 ml
300 mM	1.0 ml	1.20 ml	to 8 ml
500 mM	1.0 ml	1.20 ml	to 8 ml

Table 14. General mixing table for one purification using HisTrap buffers.

2. Load the column with nickel ions according to "Column preparation" (page 45).
3. Equilibrate the column with 10 ml start buffer (1X Phosphate buffer, 10 mM imidazole pH 7.4).
4. Apply the sample. Collect the flow-through fraction.
5. Wash with 10 ml start buffer. Collect the wash fraction.
6. Begin elution with 5 ml 1X Phosphate buffer containing 20 mM imidazole. Avoid dilution by collecting the eluate in 1 ml fractions.
7. Proceed with the next imidazole concentration, i.e. elute with 5 ml 1X Phosphate buffer containing 40 mM imidazole. Collect the eluate in 1 ml fractions as above.
8. Proceed with the buffers of increasing imidazole concentration, as described in steps 6 and 7. The purified protein is most likely to be found in the 2nd + 3rd ml of one of the elution steps.
9. Check the collected fractions, for example, by SDS-PAGE.
10. Regenerate column by washing with 10 ml start buffer.

From these results select the elution buffer that eluted the $(His)_6$ fusion protein and the start buffer with a lower concentration of imidazole. Using the highest possible concentration of imidazole in the start buffer will give the highest purity. Use these buffers for the next purification of an identical protein.

Imidazole absorbs at 280 nm. Use elution buffer as blank when monitoring absorbance. If imidazole needs to be removed use a desalting column (see Chapter 7).

Perform a blank run to elute non-specifically bound metal ions. Add 5 column volumes start buffer followed by 5 column volumes elution buffer. Re-equilibrate the column with 5-10 column volumes of start buffer before sample application.

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

Troubleshooting

See Purification and Detection Troubleshooting page 53.

Alternative 2. Simple purification with ÄKTA*prime*

ÄKTA*prime* contains a pre-programmed template for purification of $(His)_6$ fusion proteins on a single HiTrap Chelating column.

Start buffer:	20 mM sodium phosphate, 0.5 M NaCl, pH 7.4
Elution buffer:	20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4
Eluent:	Distilled water
Metal loading solution:	0.1 M $NiSO_4$ x $6H_2O$ in distilled water

Prepare at least 500 ml of each buffer.

1. Follow the instructions supplied on ÄKTA*prime* cue card to connect the column and load the system with binding buffer.
2. Select the Application Template.
3. Start the method.
4. Enter the sample volume and press OK.

Connecting the column.

Preparing the fraction collector.

Fig. 12. Typical procedures when using ÄKTA*prime*.

Sample:	5 ml cytoplasmic extract containing (His)-tagged Glutathione S-transferase, GST-$(His)_6$ The clone was a kind gift from Dr. J. Lidholm, Pharmacia & Upjohn Diagnostics, Sweden
Column:	HiTrap Chelating 1 ml, Ni^{2+}-loaded according to the instructions
Start buffer:	1X Phosphate buffer, 20 mM imidazole, pH 7.4
Elution buffer:	1X Phosphate buffer, 500 mM imidazole, pH 7.4
Flow rate:	2 ml/min, 312 cm/h
Result:	Eluted GST-$(His)_6$, 4 ml, A_{280}: 1.65 Total amount: 4.46 mg

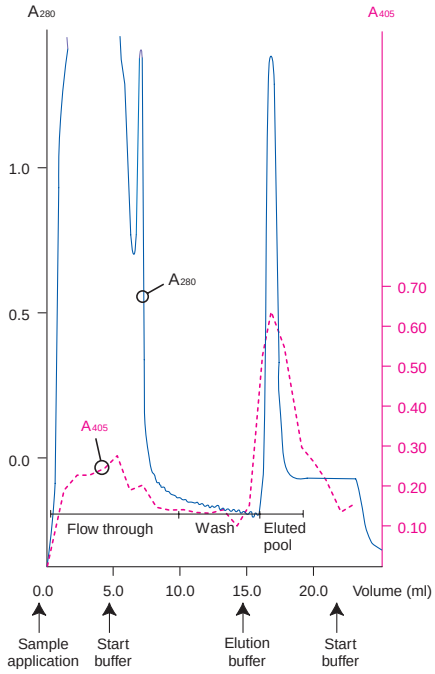


Fig. 13a. Purification of histidine-tagged Glutathione S-transferase from a cytoplasmic extract.

Lane 1: Low Molecular Weight Calibration Kit (LMW)
Lane 2: Starting material, cytoplasmic extract, dil. 1:20
Lane 3: Flow-through, dil. 1:10
Lane 4: Wash
Lane 5: Eluted GST-$(His)_6$, dil 1:20
Lane 6: Eluted GST-$(His)_6$, dil 1:10
Lane 7: GST standard, 0.5 mg/ml
Lane 8: LMW

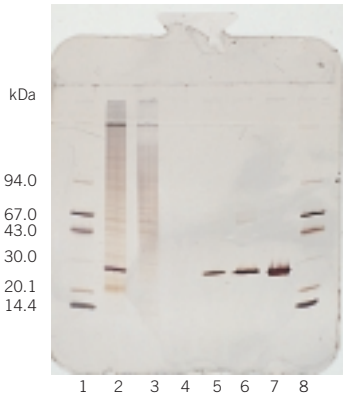


Fig. 13b. SDS electrophoresis on PhastSystem™ using PhastGel™ 10–15, silver staining.

Fig. 13.

Figure 13 shows an example of the purification of a $(His)_6$ fusion protein using a chromatography system.

Column regeneration, storage procedures and the characteristics of Chelating Sepharose High Performance (contained in the HiTrap Chelating and HisTrap columns) are described in Appendix 5.

Troubleshooting

See Purification and Detection Troubleshooting page 53.

An example of the purification and refolding of an insoluble $(His)_6$ fusion protein is shown on page 59 and clearly demonstrates the advantage of using a chromatography system for this type of work.

Detection of $(His)_6$ fusion proteins

Table 15 reviews the methods available for detection of $(His)_6$ fusion proteins. These methods can be selected according to the experimental situation, for example, SDS-PAGE analysis, performed frequently during amplification and purification to monitor results, may not be the method of choice for routine monitoring of samples from high throughput screening. Functional assays specific for the protein of interest are useful but not often available.

Detection method	Comments
ELISA assay using conjugated IgG	Highly specific, detects only $(His)_6$ fusion protein
Western blot analysis using anti-His antibody and ECL detection systems	Highly specific, detects only $(His)_6$ fusion protein Little or no background when used at optimized concentrations with secondary HRP conjugated antibody ECL detection systems enhance detection in Western blot ECL provides adequate sensitivity for most recombinant expression applications For higher sensitivity use ECL Plus
SDS-PAGE with Coomassie or silver staining	Provides information on size and % purity Detects fusion protein and contaminants
Functional assays	Useful to assess if the purified $(His)_6$ fusion protein is active Not always available May require development and optimization

Table 15. Detection methods for $(His)_6$ fusion proteins.

Alternative 1: SDS-PAGE analysis

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

1. Add 2 µl of 6X SDS loading buffer to 5-10 µl of supernatant from crude extracts, cell lysates or purified fractions as appropriate.
2. Vortex briefly and heat for 5 minutes at +90° to +100°C.
3. Load the samples onto an SDS-polyacrylamide gel.
4. Run the gel for the appropriate length of time and stain with Coomassie Blue or silver stain.

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

% Acrylamide in resolving gel	Separation size range (M_r x 10^{-3})
Single percentage:	
5%	36-200
7.5%	24-200
10%	14-200
12.5%	14-100
15%	14-60[1]
Gradient:	
5-15%	14-200
5-20%	10-200
10-20%	10-150

[1]The larger proteins fail to move significantly into the gel.

Table 16.

If using horizontal SDS pre-cast gels, refer to the Gel Media Guide from Amersham Pharmacia Biotech.

Troubleshooting

If the fusion protein is absent, it may be insoluble or expressed at very low levels; refer to protein amplification troubleshooting (page 9).

If no fusion protein is detected by Coomassie Blue, try silver staining or Western blotting to enhance sensitivity.

Try silver staining or Western blot analysis to increase the sensitivity of the detection technique

If there appears to be aggregation of protein in the gel, use at least 7.5 mM 2-mercaptoethanol in the SDS-PAGE sample buffer. This may be caused by aggregation between N terminal histidine helices.

Alternative 2. Western blot analysis

Amplification and purification can be monitored by Western blot analysis using ECL or ECL Plus detection systems to enhance sensitivity, if required.

Anti-His Antibody

Blocking/Incubation buffer: 5% (w/v) non-fat 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)

Secondary Antibody to detect the anti-His antibody (such as HRP-linked anti-mouse Ig, NA931).

1. Separate the protein samples by SDS-PAGE.

Anti-His antibody from Amersham Pharmacia Biotech is a monoclonal preparation avoiding the presence of low levels of cross-reacting antibodies. However, it is recommended to always run a sample of an *E. coli* sonicate that does not contain a recombinant $(His)_6$ plasmid as a control.

2. Transfer the separated proteins from the electrophoresis gel to an appropriate membrane, such as Hybond ECL (for subsequent ECL detection) or Hybond P (for subsequent ECL Plus detection).

Electrophoresis and protein transfer may be accomplished using a variety of equipment and reagents. For further details, refer to the Protein Electrophoresis Technical Manual and Hybond ECL instruction manual from Amersham Pharmacia Biotech.

Blocking of membrane

1. Transfer the membrane onto which the proteins have been blotted to a container such as a Petri dish.
2. Add 50-200 ml of blocking/incubation buffer.
3. Incubate for 1-16 hours at ambient temperature with gentle shaking.

Longer incubation times (up to 16 hours) with blocking buffer may reduce background signal.

4. Decant and discard the buffer.

Anti-His antibody

1. Prepare an appropriate dilution of anti-His antibody with blocking/incubation buffer e.g. 5-10 µl of antibody to 50 ml of buffer. Refer to Amersham Pharmacia Biotech Application Note 18-1139-13 for further information on optimization.
2. Pour the antibody-buffer mixture into the container with the membrane.
3. Incubate for 1 hour at ambient temperature with gentle shaking.
4. Decant and discard the antibody-buffer.
5. Rinse twice with 20-30 ml of blocking or wash buffer to remove most of the unbound antibody.
6. Decant and discard the rinses.
7. Wash the membrane with 20-30 ml of blocking or wash buffer for 10-60 minutes at ambient temperature with gentle shaking.
8. Discard the wash and repeat.

Secondary antibody

1. Dilute an appropriate anti-mouse secondary antibody with blocking/incubation buffer according to the manufacturer's recommendation. Refer to Amersham Pharmacia Biotech Application Note 18-1139-13 for further information on optimization.
2. Pour the antibody-buffer mixture into the container with the membrane.
3. Incubate for 1 hour at ambient temperature with gentle shaking.
4. Decant and discard the antibody-buffer.
5. Rinse twice with 20-30 ml of blocking or wash buffer to remove most of the unbound antibody.
6. Decant and discard the rinses.
7. Wash the membrane with 20-30 ml of blocking or wash buffer for 10-60 minutes at ambient temperature with gentle shaking.
8. Discard the wash and repeat.
9. Develop the blot with the appropriate substrate for the conjugated secondary antibody.

Troubleshooting

See also Purification and Detection Troubleshooting page 53.

Multiple bands seen on Western blot analysis

Anti-His antibody from Amersham Pharmacia Biotech is a monoclonal preparation and has been tested for its lack of non-specific background binding in a Western blot. Some sources of the anti-His antibody may contain antibodies that react with various *E. coli* proteins present in the fusion protein sample. Cross-adsorb the antibody with an *E. coli* sonicate to remove anti-*E. coli* antibodies.

Purification and detection troubleshooting

Column has clogged

Cell debris in the sample may clog the column. Clean the column according to Appendix 5 and ensure that samples have been filtered or centrifuged.

Protein is precipitating in solution or on the column

Add a non-ionic detergent (0.1% Triton X-100 or Tween 20) to the elution buffer to reduce non-specific hydrophobic interactions that may prevent solubilization and elution of fusion proteins.

Triton X-100 has a high absorbance at A_{280} and cannot be removed by buffer exchange procedures.

Alternative solubilizing agents include: 2 M NaCl, 50 mM CHAPS, 50% glycerol, (8 M urea, 6 M guanidinium hydrochloride or 20 mM 2-mercaptoethanol).
Mix gently for 30 minutes.

Fusion protein does not bind or elutes in the start buffer

Over-sonication may have denatured the fusion protein. Check by using a microscope to monitor cell breakage. Use mild sonication conditions during cell lysis.

Sonication may be insufficient: Check using a microscope or monitor by measuring the release of nucleic acids at A_{260}. Addition of lysozyme (0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris-HCl, pH 8.0) prior to sonication may improve results.

Check that the correct buffers and pH have been used. Ensure that no chelating or reducing agents are present.

If re-using a pre-packed column check that it has been regenerated correctly (see Appendix 5). Replace with fresh Chelating Sepharose or a new column if the binding capacity does not return after regeneration.

Decrease the concentration of imidazole in the binding buffer (MicroSpin columns are pre-packed with 20 mM imidazole, wash the column once with phosphate/NaCl buffer and re-equilibrate with buffer containing the desired concentration of imidazole).

Tag may be inaccessible. Purify under denaturing conditions or move tag to opposite end of the protein. For denaturing conditions use 20 mM sodium phosphate, 8 M urea or 6 M guanidinium hydrochloride and imidazole concentrations in the range 10-500 mM pH 7.4.

Tag may be degraded. Check that tag is not associated with part of the protein that is processed.

Column capacity is exceeded. Join 2 or 3 HiTrap Chelating 1 ml or 5 ml columns in series or use an even larger column.

Fusion protein is poorly eluted

The elution conditions are too mild: increase concentration of imidazole in the elution buffer. Concentrations >400 mM may be more effective. Elute with an increasing imidazole gradient or decreasing pH to determine the optimal elution conditions.

Fusion protein may be precipitating (see Protein is precipitating).

Stabilizing cofactors such as Mg^{2+} may be needed to prevent precipitation.

Try over-night elution at room temperature or +4°C.

Increase ionic strength of the elution buffer by addition of 0.1-0.2 M NaCl. Note that very hydrophobic proteins may precipitate under high salt conditions. If this is the case, addition of a non-ionic detergent may improve results.

Purification needs to be optimized

Following the instructions supplied with HiTrap Chelating columns will generally give good results. Further optimization for a specific fusion protein may be possible by adjusting the imidazole concentration in the start and elution buffers.

Optimize the imidazole concentration in the wash buffer: Follow instructions on page 47 (HisTrap Kit protocols). A more stringent wash buffer will remove more impurities from the column resulting in a higher purity of fusion protein in the elution buffer.

Increasing the purity by increasing the imidazole concentration may decrease the yield of protein as some of the $(His)_6$ fusion proteins may be washed away. Always determine the optimum combination of purity and yield for the specific application.

Yields of $(His)_6$ fusion proteins may sometimes be increased by repeating the elution step two or three times and pooling the eluents or by reducing the flow rate of elution.

Contaminants are co-eluted with fusion protein (multiple bands on SDS PAGE)

Over-sonication can lead to co-purification of host proteins with the fusion protein. Check conditions as described earlier. Avoid frothing.

Follow optimization instructions on page 47 (HisTrap Kit protocols).

Increase detergent levels (e.g. up to 2% Triton X-100 or 2% Tween 20) or add glycerol (up to 50%) to the start buffer to disrupt non-specific interactions.

Add 2-mercaptoethanol (up to 20 mM) to disrupt disulphide bonds. N.B. this can affect binding properties.

If contaminants are truncated forms of fusion protein, check for premature termination sites (N-terminal tag) or internal translation starts (C-terminal tag). Work at +4°C or add protease inhibitors to prevent proteolysis.

If contaminants have a high affinity for nickel ions add imidazole to the sample at the same concentration as in the start buffer.

Use further chromatographic purification steps (see Chapter 9).

Tag removal by enzymatic cleavage

In most cases, functional tests can be performed using the intact fusion with $(His)_6$ tag. If removal of the tag is necessary, for example, in cases where the tag forms a large part of the sequence, then procedures similar to GST tag removal can be followed i.e. specific recognition sites are incorporated to allow subsequent enzymatic cleavage. The precise protocols required for cleavage and purification will depend upon the original vectors and the properties of the specific enzymes used for cleavage.

There is no PreScission Protease recognition site available for use with $(His)_6$ fusion proteins.

rTEV protease (Life Technologies Cat. No. 10127-017) has a $(His)_6$ tag and recognizes the amino acid sequence Glu-Asn-Leu-Tyr-Phe-Gln*Gly. Glu, Tyr, Gln and Gly are needed for cleavage between the Gln and Gly residues(*). N-terminal $(His)_6$ tags can be removed. The advantage of this enzymatic cleavage is that the protein of interest can be repurified using the same Chelating Sepharose. The $(His)_6$ tag and the $(His)_6$ tag rTEV protease will both bind to the column and the protein of interest can be collected in the flow through.

The amount of enzyme, temperature and length of incubation required for complete digestion varies according to the specific fusion protein produced. Determine optimal conditions in preliminary experiments.

Remove samples at various time points and analyse by SDS-PAGE to estimate the yield, purity and extent of digestion.

Approximate molecular weights for SDS-PAGE analysis.

rTEV protease*	M_r 29 000
Carboxypeptidase A**	M_r 94 000

*** for the removal of C-terminal $(His)_6$ tags.*

All cleavage procedures will require a second purification step to be performed to remove the cleavage enzyme or other contaminants. Conventional chromatographic separation techniques such as ion exchange or hydrophobic intercaction chromatography will need to be developed (see Appendix 9).

CHAPTER 5

Handling inclusion bodies

Amplification can often be controlled so that recombinant protein accumulates in the intracellular space or is secreted into the periplasmic space. While secretion is advantageous in terms of protein folding, solubility and cysteine oxidation, the yield is generally much higher when using intracellular expression.

However, recombinant protein accumulated intracellularly is frequently laid down in the form of inclusion bodies, insoluble aggregates of mis-folded protein lacking biological activity. So, whilst the presence of inclusion bodies can make preliminary isolation steps very simple, the isolation of proteins from inclusion bodies often leads to difficulties with re-folding of the protein, correct re-formation of disulphide bonds and thus full recovery of biological activity. Table 17 summarizes the advantages and disadvantages of working with recombinant products expressed as inclusion bodies.

Advantages	Disadvantages
High expression levels can reduce fermentation costs	Re-folding shifts difficulties and costs downstream
Easily monitored by SDS-PAGE or immunoblotting	Amplification cannot be monitored directly by functional assays
Cytoplasmic proteins are easily washed away	Minor contaminants are often hydrophobic, poorly soluble membrane proteins and cell wall fragments
Major contaminants are oligomers and misfolded or proteolyzed forms of the protein	Can be difficult to separate multiple forms of the same protein
pL promoter with T induction often yields protein where other systems fail	If the protein does not fold well, another expression system will be needed

Table 17.

Solubilization of inclusion bodies

The solubility of a recombinant protein can be made more favourable by modification of culture conditions (see Chapter 1 and 2).

If culture modifications do not significantly improve the yield of soluble fusion proteins, then common denaturants such as 4-6 M guanidinium hydrochloride, 4-8 M urea, detergents, alkaline pH (> 9), organic solvents or N-lauroyl-sarcosine can be used to solubilize inclusion bodies.

For each denaturant the success of solubilization will be affected by time, temperature, ionic strength, the ratio of denaturant to protein and the presence of thiol reagents.

Solubilized proteins can often be purified at this stage by using a suitable purification technique that will also remove the denaturant and allow refolding of the protein (see below).

Success of affinity purification in the presence of denaturing agents will depend on the nature of the fusion protein. For example: GST fusion proteins from inclusion bodies have been solubilized and purified in the presence of 2-3 M guanidinium hydrochloride or urea. Other denaturants (up to 2% Tween 20; 1% CTAB, 10 mM DTT; or 0.03% SDS) have also been used.

Refolding of solubilized recombinant proteins

Following solubilization, proteins must be properly refolded to regain function. Denaturing agents must always be removed to allow refolding of the protein and formation of the correct intramolecular associations. Critical parameters during refolding include pH, presence of thiol reagents, the speed of denaturant removal, and the relative concentrations of host proteins and recombinant protein. Table 17 compares conventional methods for refolding of insoluble recombinant proteins with on-column affinity purification and refolding.

Refolding techniques	Advantages/Disadvantages
Step dialysis	Takes several days Uses large volumes of buffer
Dilution into near neutral pH	Dilutes the protein of interest
Gel filtration	Slow Requires a second column to be run Only small volumes can be processed per column
On-column refolding	Fast and simple No sample volume limitations

Table 17. Comparison of methods for protein refolding.

On-column refolding

Using a $(His)_6$ fusion protein enables the use of a simple, but efficient, purification and on-column refolding procedure that produces soluble protein exhibiting the desired biological activity. The protocol shown in Figure 14 has been used successfully for several different $(His)_6$ fusion proteins.

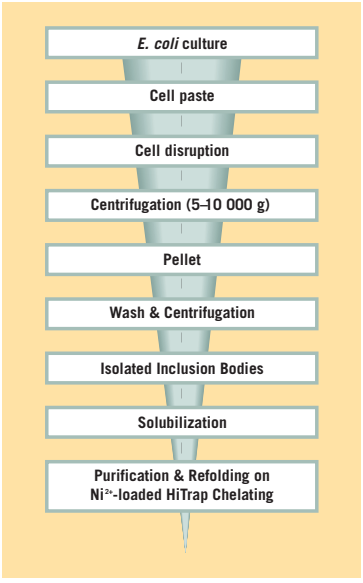


Fig. 14. General scheme for the extraction, solubilization and refolding of $(His)_6$ fusion proteins produced as inclusion bodies in *E. coli* cells.

High concentrations of chaotropic agents (such as urea or guanidinium hydrochloride) enhance the binding of the histidine tract to immobilized divalent metal ions. Consequently, $(His)_6$ fusion proteins can be solubilized by chaotropic extraction and bound to Chelating Sepharose. Removal of contaminating proteins and refolding by exchange to non-denaturing buffer conditions can be performed before elution of the protein from the column (*Colangeli et al., J. Chrom. B, 714, 223-235, 1998*).

Once refolded, protein may be purified further by other techniques (see Chapter 9) if a higher degree of purity is required.

Purification and on-column refolding of an insoluble $(His)_6$ fusion protein from a 100 ml *E. coli* culture

Resuspension buffer:	20 mM Tris-HCl, pH 8.0
Isolation buffer:	2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton X-100, pH 8.0
Solubilization buffer:	6 M guanidinium hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0
Refolding buffer:	6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0
Wash buffer:	20 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0
Elution buffer:	20 mM Tris-HCl, 0.5 M NaCl, 0.5 M imidazole, 1 mM 2-mercaptoethanol, pH 8.0
For column preparation:	Distilled water 100 mM $NiSO_4 \times 6H_2O$ in distilled H_2O

Phosphate buffers can also be used instead of Tris-HCl (see earlier purification protocols for $(His)_6$ fusion proteins).

Use high purity water and chemicals. Filter buffers through a 0.45 µm filter before use. Prepare at least 500 ml of each eluent when using ÄKTA*prime*.

This protocol uses 10 mM imidazole in the elution buffer. After preliminary investigations it may be possible to increase the concentration of imidazole and narrow the elution gradient. The optimum concentration and gradient elution will be dependent on the fusion protein.

Disruption, wash and isolation of inclusion bodies

1. Resuspend the cell paste from 100 ml culture in 4 ml 20 mM Tris-HCl, pH 8.0.
2. Disrupt cells with sonication on ice (e.g. 4 × 10 sec.).
3. Centrifuge at high speed for 10 min. at +4°C.
4. Resuspend pellet in 3 ml cold isolation buffer and sonicate as above.
5. Centrifuge at high speed for 10 min. at +4°C.
6. Repeat steps 4 and 5.

At this stage the pellet material can be washed once in buffer lacking urea and stored frozen for later processing.

Solubilization and sample preparation

1. Resuspend pellet in 5 ml solubilization buffer.
2. Stir for 30-60 min. in room temperature.
3. Centrifuge for 15 min. at high speed, +4°C.
4. Remove particles by passing sample through a 0.45 µm filter.

Optimal concentration of reducing 2-mercaptoethanol (0-5 mM) must be determined experimentally for each individual protein.

Refolding and purification

The requirement for linear gradient formation for refolding and elution makes the use of a chromatography system essential.

When using ÄKTA*prime*, select the Application Template and enter sample volume. The following steps will be performed automatically.

Preparation of the column

1. Wash HiTrap Chelating 1 ml column with 5 ml distilled water.
2. Load 0.5 ml 0.1 M $NiSO_4$ solution and continue to wash with 5 ml distilled water. Equilibrate column with 5-10 ml solubilization buffer.

Loading and washing

1. Load sample and wash column with 10 ml solubilization buffer.
2. Wash with 10 ml refolding buffer.

Refolding

1. Refolding of the bound protein is performed by the use of a linear 6-0 M urea gradient, starting with the refolding buffer above and finishing with the wash buffer. A gradient volume of 30 ml or higher and a flow rate of 0.1-1 ml/min can be used. The optimal refolding rate should be determined experimentally for each protein.
2. Continue to wash with 5 ml of wash buffer after the gradient has come to its endpoint.

Elution and Purification

1. Elute refolded recombinant protein using a 10-20 ml linear gradient starting with wash buffer and ending with the elution buffer.
2. Check collected fractions with, for example, SDS-PAGE and pool as suitable. Figure 15 illustrates an example of this on-column refolding and purification procedure.

Column:	Ni^{2+}-loaded HiTrap Chelating 1 ml
Sample:	N-terminal $(His)_6$ recombinant protein produced in *E. coli*
Flow rates:	0.1–1 ml/min, sample loading and refolding 1 ml/min, wash and elution
Binding buffer:	20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidinium hydrochloride, 1 mM 2-mercaptoethanol, pH 8.0
Washing buffer:	20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 6 M urea, 1 mM 2-mercaptoethanol, pH 8.0
Refolding buffer:	20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0
Refolding gradient:	30 ml
Elution buffer:	20 mM Tris-HCl, 0.5 M NaCl, 500 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0
Elution gradient:	10 ml
Fraction volumes:	3 ml sample loading, wash and refolding 1 ml elution

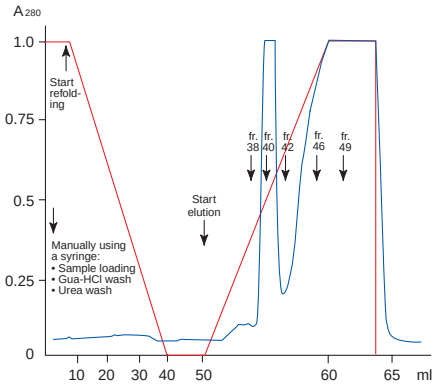


Fig. 15.

Imidazole is easily removed using a desalting column (see Chapter 7).

CHAPTER 6

Harvesting and extraction of recombinant proteins

This section reviews the most common harvesting and extraction procedures for recombinant proteins. Samples should be clear and free from particles before beginning any isolation or purification. Extraction procedures should be selected according to the source of the protein, such as bacterial, plant or mammalian, intracellular or extracellular. Selection of an extraction technique is dependent as much upon the equipment available and scale of operation as on the type of sample. Examples of common extraction processes are shown in Table 18.

Use procedures which are as gentle as possible since disruption of cells or tissues leads to the release of proteolytic enzymes and general acidification.

Extraction should be performed quickly, at sub-ambient temperatures, in the presence of a suitable buffer to maintain pH and ionic strength and stabilize the sample.

Extraction process	Typical conditions	Protein source	Comment
Gentle Cell lysis (osmotic shock)	2 volumes water to 1 volume packed pre-washed cells	*E. coli* periplasm: intracellular proteins	lower product yield but reduced protease release
Enzymatic digestion	lysozyme 0.2 mg/ml, +37°C, 15 mins.	bacteria: intracellular proteins	lab scale only, often combined with mechanical disruption
Moderate Grinding with abrasive e.g. sand	follow equipment instructions	bacteria, plant tissues	
Vigorous Ultrasonication or bead milling	"	cell suspensions: intracellular proteins in cytoplasm, periplasm, inclusion bodies	small scale, release of nucleic acids may cause viscosity problems, inclusion bodies must be resolubilized
Manton-Gaulin homogeniser	"	cell suspensions	large scale only
French press	"	bacteria, plant cells	
Fractional precipitation	see section on fractional precipitation	extracellular: secreted recombinant proteins, cell lysates	precipitates must be resolubilized

Table 18. Common sample extraction processes.

If lysates are too viscous to handle (caused by the presence of a high concentration of host nucleic acid) continue to sonicate for a longer period or follow one of the following procedures:

1. Add DNase I to a final concentration of 10 µg/ml.
2. Add RNase A to a final concentration of 10 µg/ml and DNase I to 5 µg/ml, and incubate on ice for 10-15 min.
3. To avoid use of enzymes, draw the lysate through a syringe needle several times.

For optimal conditions for growth, induction and cell lysis conditions of your recombinant fusion protein, refer to recommended protocols. Below is a general protocol for sample preparation.

Start buffer: PBS, pH 7.4 or other recommended buffer.

Additives such as 8 M urea or 6 M guanidinium hydrochloride can be included if solubilization of the protein is needed (e.g. if the protein is expressed as an inclusion body).

To prevent non-specific binding of host cell proteins, 5-50 mM imidazole can be included in the start buffer when working with $(His)_6$ fusion proteins.

1. Harvest cells by centrifugation (e.g. at 7 000-8 000 x g for 10 minutes or 1 000-1 500 x g for 30 minutes at +4°C).
2. Discard supernatant. Place bacterial pellet on ice.
3. Using a pipette, resuspend the cell pellet by adding 50 µl of ice-cold start buffer pH 7-8.5 per ml of cell culture.
4. Disrupt suspended cells e.g. sonicate on ice in short 10 seconds bursts. Save an aliquot of the sonicate for analysis by SDS-PAGE.

Sonicate for the minimum time necessary to disrupt the cells. Prolonged sonication may destroy protein functionality. Avoid frothing as this may denature the fusion protein and can lead to co-purification of host proteins with the fusion protein.

5. Sediment cell debris by centrifugation (e.g. at 12 000 x g for 10 minutes at +4°C).
6. Carefully transfer the supernatant, without disturbing the pellet, to a fresh container. Save aliquots of the supernatant and the cell debris pellet for analysis by SDS-PAGE.

Samples containing 8 M urea can be analysed directly, but, if 6 M guanidinium hydrochloride is present, this must be exchanged for 8 M urea by using a pre-packed desalting column (see Chapter 7) before loading onto an electrophoresis gel.

7. The sample should be fully dissolved prior to loading the column and adjusted to pH 7-8 by dilution or buffer exchange on a desalting column.
8. Store sample at -20°C when not in use.

Fractional precipitation

Fractional precipitation is often used for extraction and clarification at laboratory scale. The sample can be partially purified and may also be concentrated.

Fractional precipitation should be used before a first chromatographic step.

Most precipitation techniques are not suitable for large scale preparations.

Precipitation techniques are affected by temperature, pH and sample concentration. These parameters must be controlled to ensure reproducible results. Precipitation can be used in three different ways, as shown in Figure 16.

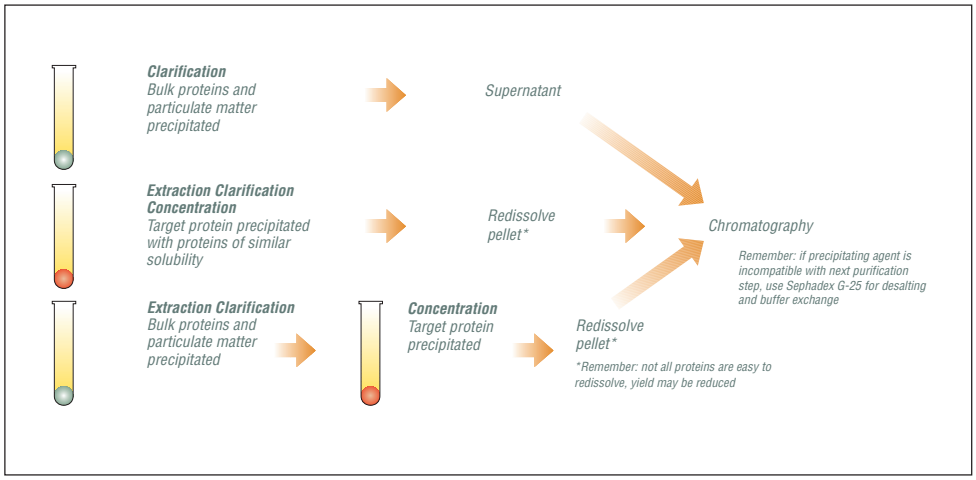


Fig. 16. Three ways to use precipitation.

Precipitation techniques are reviewed in Table 19 and the most common method is described in more detail.

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulphate	as described	>1mg/ml proteins especially immuno-globulins	stabilizes proteins, no denaturation, supernatant can go directly to HIC
Dextran sulphate	as described	samples with high levels of lipoprotein e.g ascites	precipitates lipoprotein
Polyvinylpyrrolidine	Add 3% (w/v), stir 4 hours, centrifuge, discard pellet	"	alternative to dextran sulphate
Polyethylene glycol (PEG, M_r >4000)	up to 20% w/vol	plasma proteins	no denaturation, supernatant goes direct to IEX or AC, complete removal may be difficult
Acetone	up to 80% vol/vol at $\pm$0°C	useful for peptide precipitation or concentration of sample for electrophoresis	may denature protein irreversibly
Polyethyleneimine	0.1% w/v		precipitates aggregated nucleoproteins
Protamine sulphate	1%		"
Streptomycin sulphate	1%		precipitation of nucleic acids

Table 19. Examples of precipitation techniques.

Details taken from:
Protein Purification, Principles and Practice, R.K. Scopes. 1994, Springer.
Protein Purification, Principles, High Resolution Methods and Applications, J.C. Janson and L. Rydén, 1998, 2nd ed. Wiley Inc. and other sources.

Ammonium sulphate precipitation

Saturated ammonium sulphate solution

Add 100 g ammonium sulphate to 100 ml distilled water, stir to dissolve.

1 M Tris-HCl, pH 8.0.

Buffer for first purification step.

1. Filter (0.45 µm) or centrifuge (refrigerated, 10000g) sample.
2. Add 1 part 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently. Add ammonium sulphate solution, drop by drop (solution becomes milky at about 20% saturation). Add up to 50% saturation*. Stir for 1 hour.
4. Centrifuge 20 minutes at 10000g.
5. Discard supernatant. Wash pellet twice by resuspension in an equal volume of ammonium sulphate solution of the same concentration (i.e. a solution that will not redissolve the precipitated protein or cause further precipitation). Centrifuge again.
6. Dissolve pellet in a small volume of the purification buffer.
7. Ammonium sulphate is removed during clarification/buffer exchange steps with Sephadex G-25 or during hydrophobic interaction separations.

*The % saturation can be adjusted either to precipitate a target molecule or to precipitate contaminants.

The quantity of ammonium sulphate required to reach a given degree of saturation varies according to temperature. Table 20 shows the quantities required at +20°C.

	Final percent saturation to be obtained																
	20	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**	**65**	**70**	**75**	**80**	**85**	**90**	**95**	**100**
Starting percent saturation	**Amount of ammonium sulphate to add (grams) per litre of solution at +20°C**																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

Table 20. Quantities of ammonium sulphate required to reach given degrees of saturation at +20°C.

CHAPTER 7

Buffer exchange and desalting of recombinant proteins

Dialysis is frequently mentioned in the literature as a technique used to remove salt or other small molecules and exchange buffer composition of a sample. However dialysis is generally a slow technique, requiring large volumes of buffer and with a risk of losing material during handling or because of proteolytic breakdown or non-specific binding of samples to the dialysis membranes.

A simpler and much faster technique is to use desalting columns that perform a group separation between high and low molecular weight substances. The columns are packed with Sephadex™ G-25, a gel filtration product that separates molecules on the basis of size.

In a single step, the sample is desalted, exchanged into a new buffer, and low molecular weight materials, such as unwanted salts, are removed.

Desalting columns are used not only for the removal of low molecular weight contaminants such as salt, but also for buffer exchange prior to or after different chromatographic steps or for the rapid removal of reagents to terminate a reaction.

Sample volumes up to 30% of the total volume of the desalting column can be processed. The high speed and high capacity of the separation allows even large sample volumes to be processed rapidly and efficiently. Figure 17 shows a typical desalting and buffer exchange separation.

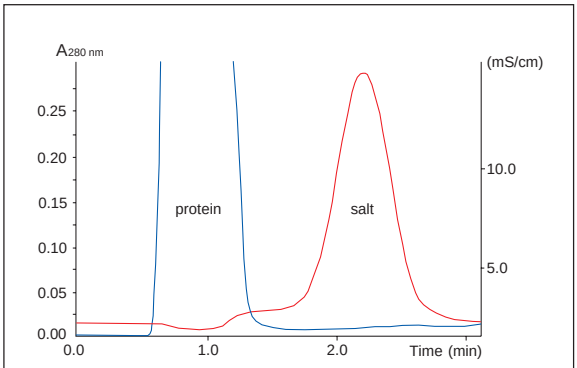


Fig. 17. Desalting and buffer exchange.

Table 21 shows a selection guide for pre-packed, ready to use desalting and buffer exchange columns.

Column	Sample volume	Volume after elution
MicroSpin G-25	0.1-0.15 ml	0.1-0.15 ml
HiTrap Desalting	0.25-1.5 ml	1.0-2.0 ml
HiPrep 26/10 Desalting	2.5-15 ml	7.5-20 ml

Table 21.

To desalt larger sample volumes

-connect up to 5 HiTrap Desalting columns in series to increase the sample volume capacity e.g. 2 columns: sample volume 3 ml, 5 columns: sample volume 7.5 ml.

-connect up to 4 HiPrep 26/10 Desalting columns in series to increase the sample volume capacity, e.g. 2 columns: sample volume 30 ml, 4 columns: sample volume 60 ml. Even with 4 columns in series the sample can be processed in 20 to 30 minutes.

Instructions are supplied with each column. Desalting and buffer exchange will take less than 5 minutes per sample with greater than 95% recovery for most proteins.

A salt concentration of at least 25 mM NaCl in the chosen buffer is recommended to prevent possible ionic interactions.

Sample concentration does not influence the separation as long as the concentration of proteins does not exceed 70 mg/ml when using normal aqueous buffers.

The sample should be fully dissolved. Centrifuge or filter (0.45 µm filter) to remove particulate material if necessary.

The protocols below describe desalting and buffer exchange using HiTrap Desalting. These procedures can be adapted to process smaller sample volumes (on MicroSpin G-25 columns) or larger sample volumes (on HiPrep 26/10 Desalting columns using a chromatography system).

Alternative 1. Desalting with HiTrap Desalting using a syringe or pipette

1. Fill the syringe with buffer. Remove the stopper. To avoid introducing air into the column, connect the column "drop to drop" to the syringe (via the adaptor).
2. Remove the twist-off end.
3. Wash the column with 25 ml buffer at 5 ml/min to completely remove the 20% ethanol (supplied as storage buffer). If air is trapped in the column, wash with degassed buffer until the air disappears. Air introduced onto the column by accident during sample application does not influence the separation.
4. Connect the syringe to the column with the luer adaptor supplied.

To deliver more precise volumes, a multi-dispensing pipette (Eppendorf model 4780 Multipipette™) can also be used for sample application and elution. Use the M6 threaded stopper from the HiTrap column as an adaptor by piercing a hole through the bottomend of the stopper. Connect the modified "stopper" to the top of the column and, by using gentle force, drive the pipette tip (Combitip with a pipette tip mounted) into the stopper.

When dispensing liquid with the Multipipette, do not exceed the maximum flow rate for the column.

Take care that all liquid is dispensed for each stroke before a new stroke is delivered.

5. Apply the sample using a flow rate between 1-10 ml/min. Discard the liquid eluted from the column.

The maximum recommended sample volume is 1.5 ml. See Table 22 for the effect of varying the sample volume applied to the column using a syringe.

If the sample volume is less than 1.5 ml, add buffer until a total of 1.5 ml buffer is eluted. Discard the eluted liquid.

6. Change to the elution buffer and elute the target protein with the volumes listed in Table 22. Collect the desalted protein in the volume indicated.

Sample load ml	Add buffer ml	Elute and collect ml	Yield %	Remaining salt %	Dilution factor
0.25	1.25	1.0	>95	0.0	4.0
0.50	1.0	1.5	>95	<0.1	3.0
1.00	0.5	2.0	>95	<0.2	2.0
1.50	0	2.0	>95	<0.2	1.3

Table 22. Recommended sample and elution volumes using a syringe or Multipipette.

A simple peristaltic pump can also be used to apply sample and buffers.

Alternative 2. Simple desalting with ÄKTA*prime*

ÄKTA*prime* contains pre-programmed templates for individual HiTrap Desalting and HiPrep 26/10 Desalting columns.

Prepare at least 500 ml of the required buffer.

1. Follow the instructions supplied on ÄKTA*prime* cue card to connect the column and load the system with buffer.
2. Select the Application Template.
3. Start the method.
4. Enter the sample volume (up to 1.5 ml) and press OK.

Figure 18 shows a typical desalting procedure using ÄKTA*prime*. The UV and conductivity traces enable the appropriate desalted fractions to be pooled.

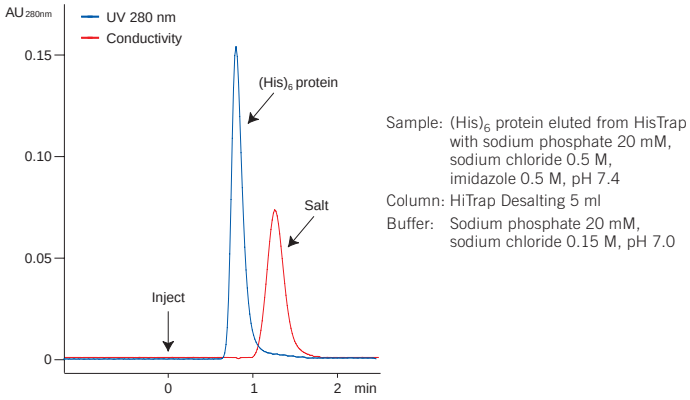


Fig. 18. Desalting of a $(His)_6$ fusion protein on ÄKTA*prime*.

CHAPTER 8

Simple purification of other recombinant proteins

GST MicroSpin and His MicroSpin Purification Modules, GSTrap, HiTrap Chelating and HisTrap are products that use affinity chromatography to isolate and purify a specific fusion protein. However, many other fusion and non-fusion proteins can also be isolated to a satisfactory degree of purity by a single step purification using affinity chromatography.

Affinity chromatography isolates a specific protein or a group of proteins with similar characteristics. The technique separates proteins on the basis of a reversible interaction between the protein(s) and a specific ligand attached to a chromatographic matrix. Whenever a suitable ligand is available for the protein(s) of interest, a single affinity purification step offers high selectivity, hence high resolution, and usually high capacity for the target protein(s). The basic principles of affinity chromatography are outlined in Appendix 9.

Ready to use affinity purification columns

Table 23 shows the applications for which affinity purification with HiTrap columns are already available. All columns are supplied with a detailed protocol that outlines the buffers and steps required for optimum results. For larger scale work HiTrap columns can often be linked together to increase the capacity of a single purification step. Loose media are also available for packing larger columns.

Application	Column	Capacity (mg/ml affinity medium)
Isolation of immunoglobulins IgG classes, fragments and subclasses from all sources	HiTrap rProtein A	Human IgG 50 mg/ml
IgG classes, fragments and subclasses from all sources	HiTrap Protein A	Human IgG 20 mg/ml
IgG classes, fragments and subclasses including human IgG_3. Strong affinity for monoclonal mouse IgG_1 and rat IgG	HiTrap Protein G	Human IgG 25 mg/ml
Monoclonal and polyclonal IgG from ascites fluid, serum and cell culture supernatant	MAbTrap™ GII (HiTrap Protein G column (1 ml), accessories, pre-made buffers)	Human IgG 25 mg/ml
IgY from egg yolk	HiTrap IgY Purification	IgY 20 mg/ml
Monoclonal and human IgM	HiTrap IgM Purification	Mouse IgM 5 mg/ml
Group Specific Media Glycoproteins or polysaccharides Specificity: branched mannoses, carbohydrates with terminal mannose or glucose(α Man> α Glc> GlcNAc)	HiTrap Con A	Transferrin 4 mg/ml
Specificity: branched mannoses with fucose linked α(1,6) to the N-acetyl-glucosamine, (α Man>α Glc> GlcNAc) N-acetyl glucosamine binding lectins	HiTrap Lentil Lectin	Thyroglobulin 4 mg/ml
Specificity: Terminal β-galactose, (Gal β 1,3 GalNAc > α and β Gal)	HiTrap Peanut Lectin	Asialofetuin 3 mg/ml
Specificity: chitobiose core of N-linked oligosaccharides, [GlcNAc (β 1,4GlcNAc) 1-2 > β GlcNAc]	HiTrap Wheat Germ Lectin	Ovomucoid 4 mg/ml
Specificity: as listed for Con A, Lentil Lectin, Peanut Lectin, Wheat Germ Lectin	HiTrap Lectin Test Kit	as listed above
Group Specific Media Albumin, various nucleotide-requiring enzymes, coagulation factors, DNA binding proteins, α_2-macroglobulin	HiTrap Blue	HSA 20 mg/ml
Proteins and peptides with exposed amino acids: His (Cys, Trp) e.g. α_2-macroglobulin and interferon	HiTrap Chelating	$(His)_6$ fusion protein (M_r 27 600) 12 mg/ml
Biotin and biotinylated substances	HiTrap Streptavidin	Biotinylated BSA 6 mg/ml
Coagulation factors, lipoprotein lipases, steroid receptors, hormones, DNA binding proteins, interferon, protein synthesis factors	HiTrap Heparin	Antithrombin III (bovine) 3 mg/ml
Matrix for preparation of affinity columns Coupling of primary amines	HiTrap NHS-activated	ligand specific

Table 23. Ready to use HiTrap columns for affinity purification.

Making a specific purification column

In cases when a ready made affinity medium is unavailable, it may be considered worthwhile to develop a "home-made" affinity purification column, for example, when a specific recombinant protein needs to be prepared efficiently on a regular basis.

The ligand must be prepared (following procedures specific for the type of ligand e.g. by raising antibodies), tested for affinity to the target protein and purified before linking to a chromatographic matrix. For further details on general purification strategies for proteins see Chapter 9 and the Protein Purification Handbook from Amersham Pharmacia Biotech. A detailed account of the principles of affinity chromatography can be found in the Affinity Chromatography, Principles and Methods Handbook also available from Amersham Pharmacia Biotech.

Preparation of NHS-activated HiTrap to create a simple affinity purification column

NHS-activated Sepharose High Performance is a chromatographic matrix specifically designed for the covalent coupling of ligands containing primary amino groups (the most common form of attachment). The matrix is based on highly cross-linked agarose beads with 6-atom spacer arms attached to the matrix by epichlorohydrin and activated by N-hydroxysuccinimide. The substitution level is ~10 µmol NHS-groups/ml gel. Non-specific adsorption of proteins (which can reduce binding capacity of the target protein) is negligible due to the excellent hydrophilic properties of the base matrix.

The protocol below describes the preparation of a pre-packed NHS-activated HiTrap column and is generally applicable to all NHS-activated Sepharose products.

Optimum binding and elution conditions for purification of the target protein must be determined separately for each ligand.

This procedure can be performed using a HiTrap column with a syringe, a peristaltic pump or a liquid chromatography system such as ÄKTA*prime*.

The activated product is supplied in 100% isopropanol to preserve the stability prior to coupling. Do not replace the isopropanol until it is time to couple the ligand.

Buffer preparation

Acidification solution:	1 mM HCl (kept on ice)
Coupling buffer:	0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3

Use high quality water and chemicals. Filtration through 0.45 µm filters is recommended.

Coupling within pH range 6.5-9, maximum yield is achieved at pH ~8.

Column preparation

1. Dissolve desired ligand in the coupling buffer to a concentration of 0.5-10 mg/ml (for protein ligands) or perform a buffer exchange using a desalting column (see Chapter 7). The optimum concentration depends on the ligand. Optimum sample volume is equivalent to one column volume.
2. Remove top-cap and apply a drop of ice cold 1 mM HCl to the top of the column to avoid air bubbles.
3. Connect the top of the column to the syringe or system.
4. Remove the twist-off end.

Ligand coupling

1. Wash out the isopropanol with 3 x 2 column volumes of ice-cold 1 mM HCl.

Do not use excessive flow rates. The column contents can be irreversibly compressed.

2. Inject one column volume of ligand solution onto the column.
3. Seal the column. Leave to stand for 15-30 minutes at +25°C (or 4 hours at +4°C).

If larger volumes of ligand solution are used, re-circulate the solution. For example, when using a syringe connect a second syringe to the outlet of the column and gently pump the solution back and forth for 15-30 minutes or, if using a peristaltic pump, simply re-circulate the sample through the column.

If required the coupling efficiency can be measured at this stage. These procedures are supplied with each HiTrap NHS-activated column.

Washing and deactivation

This procedure deactivates any excess active groups that have not coupled to the ligand and washes out non-specifically bound ligands.

Buffer A: 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3
Buffer B: 0.1 M acetate, 0.5 M NaCl, pH 4

1. Inject 3 x 2 column volumes of buffer A.
2. Inject 3 x 2 column volumes of buffer B.
3. Inject 3 x 2 column volumes of buffer A.
4. Let the column stand for 15-30 min.
5. Inject 3 x 2 column volumes of buffer B.
6. Inject 3 x 2 column volumes of buffer A.
7. Inject 3 x 2 column volumes of buffer B.
8. Inject 2-5 column volumes of a buffer with neutral pH.

The column is now ready for use.

Store the column in storage solution, e.g. 0.05 M Na_2HPO_4, 0.1% NaN_3, pH 7.

Purification

Optimum binding and elution conditions for purification of the target protein must be determined separately for each ligand.

Literature references and textbooks may give good guidelines. Below is a general protocol that can be used initially.

Perform a blank run to ensure that loosely bound ligand is removed.

Use high quality water and chemicals. Filtration through 0.45 µm filters is recommended.

Samples should be centrifuged immediately before use and/or filtered through a 0.45 µm filter. If the sample is too viscous, dilute with binding buffer.

Sample binding properties can be improved by adjusting the sample to the composition of the binding buffer. Dilute in binding buffer or perform a buffer exchange using a desalting column (see Chapter 7).

Prepare the column

1. Wash with 2 column volumes of start buffer.
2. Wash with 3 column volumes of elution buffer.
3. Equilibrate with 5-10 column volumes of start buffer.

Purification

1. Apply sample. Optimal flow rate is dependent on the binding constant of the ligand, but a recommended flow rate range is, for example, 0.5-1 ml/min on a HiTrap 1 ml column.
2. Wash with 5-10 column volumes of start buffer, or until no material appears in the eluent.

Avoid excessive washing if the interaction between the protein of interest and the ligand is weak, since this may decrease the yield.

3. Elute with 2-5 column volumes of elution buffer (larger volumes may be necessary).
4. If required purified fractions can be desalted and exchanged into the buffer of choice using pre-packed desalting columns (see Chapter 7).

Re-equilibrate the column

Re-equilibrate the column by washing with 5-10 column volumes of start buffer.

CHAPTER 9

Multi-step purification of recombinant proteins (fusion and non-fusion)

Fusion systems are simple and convenient and, for many applications, a single purification step using affinity chromatography is sufficient to achieve the desired level of purity. However, if there is no suitable fusion system so that affinity chromatography cannot be used, or if a higher degree of purity is required, a multi-step purification will be necessary.

A significant advantage when working with recombinant products is that there is often considerable information available about the product and contaminants. With this information, detection assays and sample preparation and extraction procedures in place, the Three Phase Purification Strategy (Capture, Intermediate Purification, Polishing) can be applied (Figure 19). This strategy is used in both the pharmaceutical industry and in the research laboratory to ensure faster method development, a shorter time to pure product and good economy.

This section gives a brief overview of the approach recommended for any multi-step protein purification. The Protein Purification Handbook (from Amersham Pharmacia Biotech) is highly recommended as a guide to planning efficient and effective protein purification strategies.

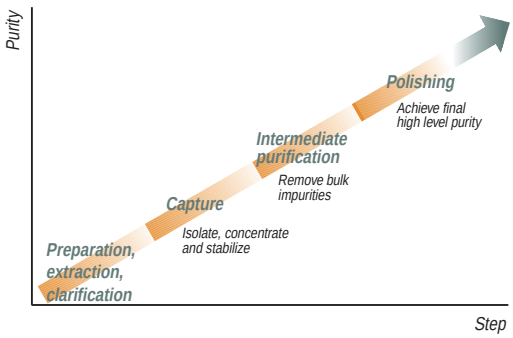


Fig. 19. Preparation and the Three Phase Purification Strategy.

The Three Phase Purification Strategy is applied as follows:

Imagine the purification has three phases Capture, Intermediate Purification and Polishing.

Assign a specific objective to each step within the purification process.

The purification problem associated with a particular step will depend greatly upon the properties of the starting material. Thus, the objective of a purification step will vary according to its position in the process i.e. at the beginning for *isolation* of product from crude sample, in the middle for *further purification* of partially purified sample, or at the end for final clean up of an almost pure product.

In the *capture phase* the objectives are to *isolate, concentrate and stabilize* the target product. The product should be concentrated and transferred to an environment that will conserve potency/activity.

During the *intermediate purification phase* the objectives are to *remove most of the bulk impurities*, such as other proteins and nucleic acids, endotoxins and viruses.

In the *polishing phase* most impurities have already been removed except for trace amounts or closely related substances. The objective is to *achieve final purity* by removing any remaining trace impurities or closely related substances.

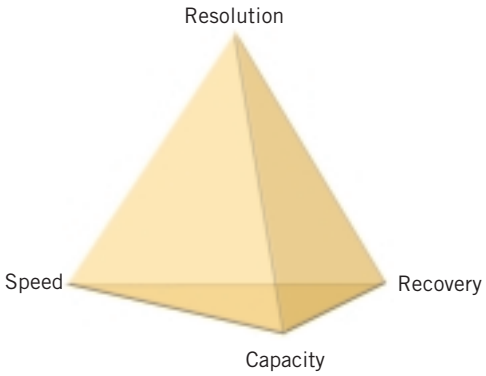
The optimum selection and combination of purification techniques for Capture, *Intermediate Purification and Polishing* is crucial for an efficient purification.

Selection and combination of purification techniques

Proteins are purified using chromatographic purification techniques that separate according to differences in specific properties, as shown in Table 24.

Protein property	Technique
Charge	Ion exchange (IEX)
Size	Gel filtration (GF)
Hydrophobicity	Hydrophobic interaction (HIC), Reversed phase (RPC)
Biorecognition (ligand specificity)	Affinity (AC)
Charge, ligand specificity or hydrophobicity	Expanded bed adsorption (EBA) follows the principles of AC, IEX or HIC

Table 24. Protein properties used during purification.



Every technique offers a balance between resolution, capacity, speed and recovery.

Capacity, in the simple model shown, refers to the amount of target protein loaded during purification. In some cases the amount of sample which can be loaded may be limited by volume (as in gel filtration) or by large amounts of contaminants rather than by the amount of the target protein.

Speed is of the highest importance at the beginning of purification where contaminants such as proteases must be removed as quickly as possible.

Recovery becomes increasingly important as the purification proceeds because of the increased value of the purified product. Recovery is influenced by destructive processes in the sample and unfavourable conditions on the column.

Resolution is achieved by the selectivity of the technique and the ability of the chromatographic matrix to produce narrow peaks. In general, resolution is most difficult to achieve in the final stages of purification when impurities and target protein are likely to have very similar properties.

Select a technique to meet the objectives for the purification step.

Choose logical combinations of purification techniques based on the main benefits of the technique and the condition of the sample at the beginning or end of each step.

A guide to the suitability of each purification technique for the stages in the Three Phase Purification Strategy is shown in Table 25.

Technique	Main features	Capture	Intermediate	Polish	Sample Start condition	Sample End condition
IEX	high resolution high capacity high speed	★★★	★★★	★★★	low ionic strength sample volume not limiting	high ionic strength or pH change **concentrated sample**
HIC	good resolution good capacity high speed	★★	★★★	★	high ionic strength sample volume not limiting	low ionic strength **concentrated sample**
AC	high resolution high capacity high speed	★★★	★★★	★★	specific binding conditions sample volume not limiting	specific elution conditions **concentrated sample**
GF	high resolution using Superdex media		★	★★★	limited sample volume (<5% total column volume) and flow rate range	buffer exchanged (if required) diluted sample
RPC	high resolution		★	★★★	requires organic solvents	in organic solvent, risk loss of biological activity

Table 25. Suitability of purification techniques for the Three Phase Purification Strategy.

Minimize sample handling between purification steps by combining techniques to avoid the need for sample conditioning. The product should be eluted from the first column in conditions suitable for the start conditions of the next column (see Table 25).

Ammonium sulphate precipitation is a common sample clarification and concentration step and so HIC (which requires high salt to enhance binding to the media) is ideal as the capture step. The salt concentration and the total sample volume will be significantly reduced after elution from the HIC column. Dilution of the fractionated sample or rapid buffer exchange using a desalting column will prepare it for the next IEX or AC step.

GF is well suited for use after any of the concentrating techniques (IEX, HIC, AC, EBA) since the target protein will be eluted in a reduced volume and the components from the elution buffer will not affect the gel filtration separation. Gel filtration is a non-binding technique with limited volume capacity and is unaffected by buffer conditions.

Selection of the final strategy will always depend upon specific sample properties and the required level of purification. Logical combinations of techniques are shown in Figure 19.

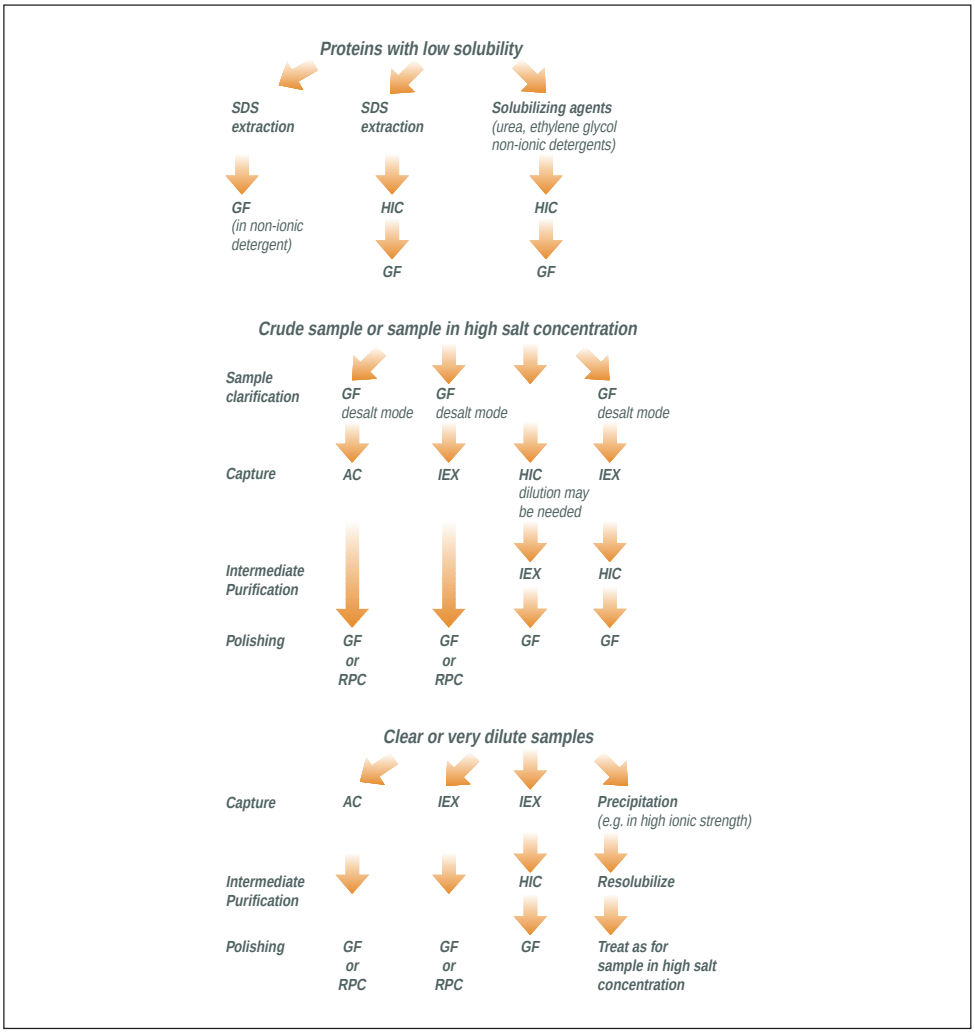


Fig. 19. Logical combinations of chromatographic steps.

For any capture step, select the technique showing the strongest binding to the target protein while binding as few of the contaminants as possible, i.e. the technique with the highest selectivity and/or capacity for the protein of interest.

A sample is purified using a combination of techniques and alternative selectivities. For example, in an IEX-HIC-GF Three Phase Strategy the capture step selects according to differences in charge (IEX), the intermediate purification step according to differences in hydrophobicity (HIC) and the final polishing step according to differences in size (GF).

If nothing is known about the target protein use IEX-HIC-GF. This combination of techniques can be regarded as a standard protocol.

Consider the use of both anion and cation exchange chromatography to give different selectivities within the same purification strategy.

IEX is a technique which offers different selectivities using either anion or cation exchangers. The pH of the separation can be modified to alter the charge characteristics of the sample components. It is therefore possible to use IEX more than once in a purification strategy, for capture, intermediate purification or polishing. IEX can be used effectively both for rapid separation in low resolution mode during capture, and in high resolution mode during polishing in the same purification scheme.

Consider RPC for a polishing step provided that the target protein can withstand the run conditions.

Reversed phase chromatography (RPC) separates proteins and peptides on the basis of hydrophobicity. RPC is a high selectivity (high resolution) technique, requiring the use of organic solvents. The technique is widely used for purity check analyses when recovery of activity and tertiary structure are not essential.

Since many proteins are denatured by organic solvents, the technique is not generally recommended for protein purification where recovery of activity and return to a correct tertiary structure may be compromised. However, in the polishing phase, when the majority of protein impurities have been removed, RPC can be excellent, particularly for small target proteins that are not often denatured by organic solvents.

It should be noted that this Three Phase Strategy does not mean that all strategies must have three purification steps. For example, capture and intermediate purification may be achievable in a single step, as may intermediate purification and polishing. Similarly, purity demands may be so low that a rapid capture step is sufficient to achieve the desired result. For purification of therapeutic proteins a fourth or fifth purification step may be required to fulfil the highest purity and safety demands. The number of steps used will always depend upon the purity requirements and intended use for the protein.

The following example demonstrates the successful application of the Three Phase Strategy in the purification of a recombinant protein.

Three step purification of a recombinant enzyme

This example demonstrates one of the most common purification strategies suitable when high purity levels are required: IEX for capture, HIC for intermediate purification and GF for the polishing step.

The objective was to obtain highly purified deacetoxycephalosporin C synthase (DAOCS), an oxygen-sensitive enzyme that had been produced by over-expression in soluble form in the cytoplasm of *E. coli* bacteria.

A more detailed description of this work can be found in Application Note 18-1128-91.

Sample extraction and clarification

Cells were suspended in lysis buffer and lysed using ultrasonication. Streptomycin sulphate and polyethyleneimine were added to precipitate DNA. The extract was clarified by centrifugation. EDTA, DTT, Benzamidine-HCl and PMSF were used in the lysis buffer to inhibit proteases and minimize damage to the oxygen sensitive-enzyme. Keeping the sample on ice also reduced protease activity.

Capture

The capture step focused on the rapid removal of the most harmful contaminants from the relatively unstable target protein. This, together with the calculated isoelectric point of DAOCS (pI = 4.8), led to the selection of an anion exchange purification. A selection of anion exchange columns, including those from HiTrap IEX Selection Kit, was screened to select the optimum medium (results not shown). Optimization of the capture step (in Figure 21) allowed the use of a step elution at high flow rate to speed up the purification.

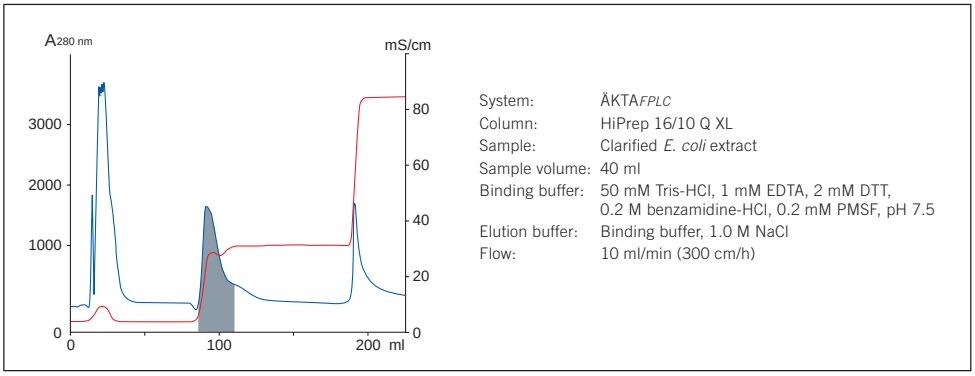


Fig. 20. Capture using IEX. The elution position of DAOCS is shaded.

Intermediate Purification

Hydrophobic interaction chromatography (HIC) was selected because the separation principle is complementary to ion exchange and because a minimum amount of sample conditioning was required. Hydrophobic properties are difficult to predict and it is always recommended to screen different media. After screening RESOURCE™ ISO was selected on the basis of the resolution achieved. In this intermediate step, shown in Figure 21, the maximum possible speed for separation was sacrificed in order to achieve higher resolution and allow significant reduction of remaining impurities.

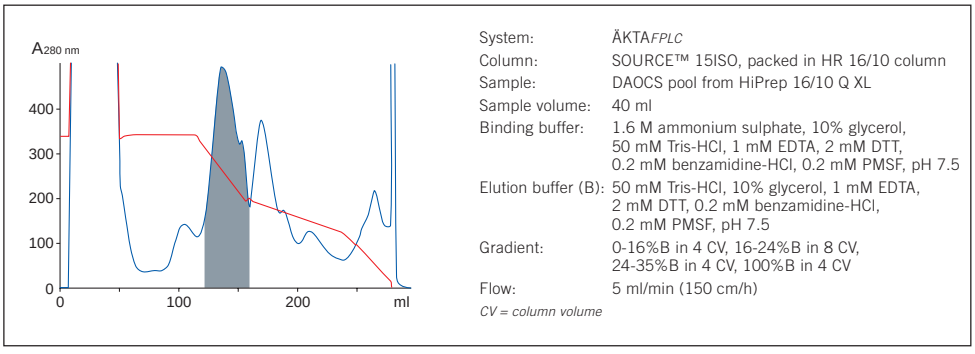
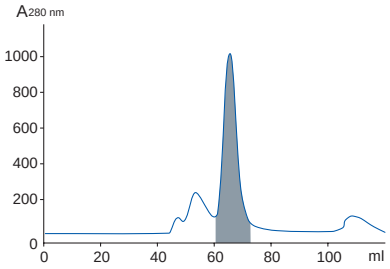


Fig. 21. Intermediate purification using HIC.

Polishing

The main goal of the polishing step, shown in Figure 22, was to remove aggregates and minor contaminants and transfer the purified sample into a buffer suitable for further use in structural studies. The final product was used successfully in X-ray diffraction studies. *Ref: Structure of a cephalosporin synthase. K. Valegard, A.C. Terwisscha van Scheltinga, M. Lloyd, T. Hara, S. Ramaswamy, A. Perrakis, A. Thompson, H.J. Lee, J.E. Baldwin, C.J. Schofield, J. Hajdu and I. Andersson. Nature 394 (1998) 805-809.*

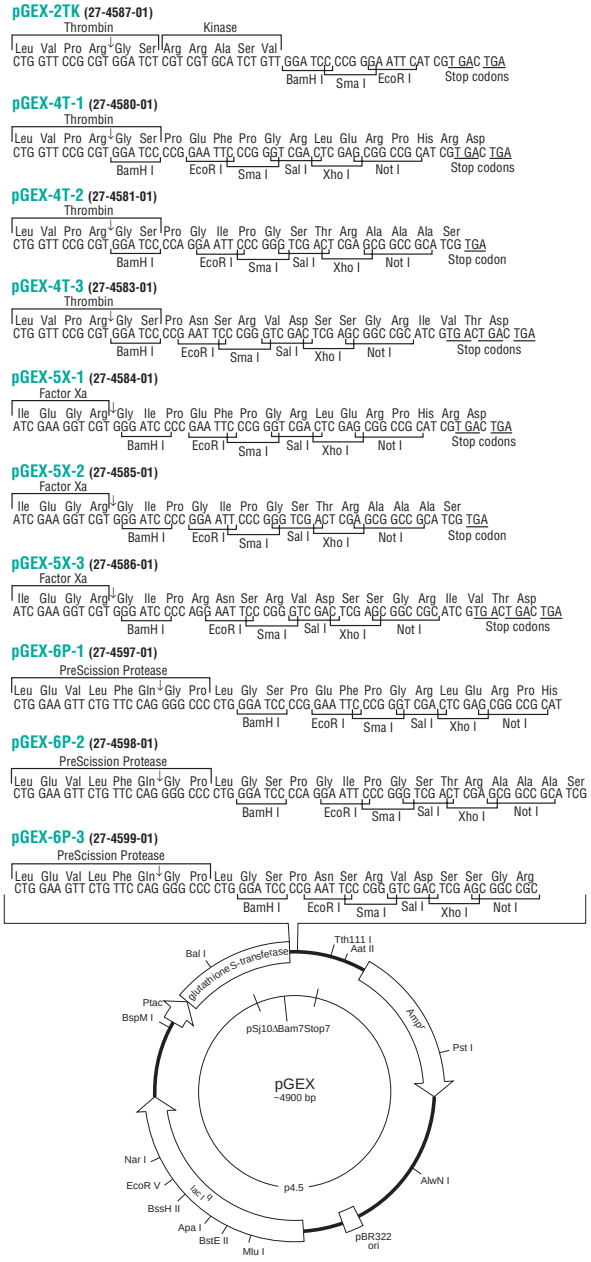


System:	ÄKTA*FPLC*
Column:	HiLoad™ 16/60 Superdex 75 prep grade
Sample:	Concentrated DAOCS pool from SOURCE 15ISO
Sample volume:	3 ml
Buffer:	100 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, 0.2 mM benzamidine-HCl, 0.2 mM PMSF, pH 7.5
Flow:	1 ml/min (30 cm/h)

Fig. 22. Polishing step using gel filtration.

Appendix 1

Map of the GST fusion vectors showing reading frames and main features

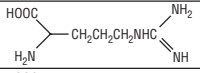
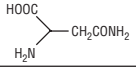
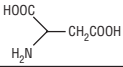
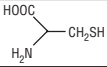
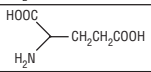
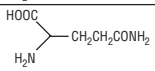
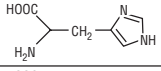
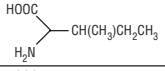
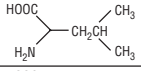
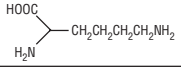
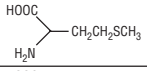
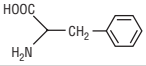
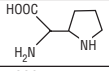
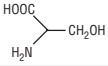
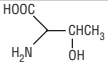
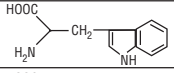
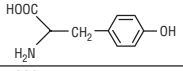
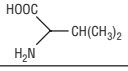


SELECTION GUIDE – pGEX Vector Control Regions	pGEX- 2TK 27- 4587- 01	pGEX- 4T- 1 27- 4580- 01	pGEX- 4T- 2 27- 4581- 01	pGEX- 4T- 3 27- 4583- 01	pGEX- 5X- 1 27- 4584- 01	pGEX- 5X- 2 27- 4585- 01	pGEX- 5X- 3 27- 4586- 01	pGEX- 6P- 1 27- 4597- 01	pGEX- 6P- 2 27- 4598- 01	pGEX- 6P- 3 27- 4599- 01
Glutathione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

Complete DNA sequences and restriction site data are available at the Amersham Pharmacia Biotech Web Site (http:// www. apbiotech. com).

Appendix 2

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	MW	Middle unit residue (-H_2O) Formula	MW	Charge at pH 6.0-7.0	Hydrophobic (non-polar)	Uncharged (polar)	Hydrophilic (polar)
$C_3H_7NO_2$	89.1	C_3H_5NO	71.1	Neutral	■		
$C_6H_{14}N_4O_2$	174.2	$C_6H_{12}N_4O$	156.2	Basic (+ve)			■
$C_4H_8N_2O_3$	132.1	$C_4H_6N_2O_2$	114.1	Neutral		■	
$C_4H_7NO_4$	133.1	$C_4H_5NO_3$	115.1	Acidic(-ve)			■
$C_3H_7NO_2S$	121.2	C_3H_5NOS	103.2	Neutral		■	
$C_5H_9NO_4$	147.1	$C_5H_7NO_3$	129.1	Acidic (-ve)			■
$C_5H_{10}N_2O_3$	146.1	$C_5H_8N_2O_2$	128.1	Neutral		■	
C_2H_5NO	275.1	C_2H_3NO	57.1	Neutral		■	
$C_6H_9N_3O_2$	155.2	$C6H_7N_3O$	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 3

Protein conversion data

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

Appendix 4.

Centrifuges, rotors and carriers for use with MicroPlex 24

Centrifuge model	Manufacturer's number	Manufacturer	Rotor	Separate carriers needed?	Number of places	Maximum g-force and rpm
GS-15 GS-15R	#360908 #360902	Beckman	S2096 (#361111)	no	2	1100 x g, 3000 rpm
Sorvall RT-6000B	#07983	DuPont	H1000B (#11706)	yes #11093	2	1900 x g, 3000 rpm
6K	#83794	Fisher	#04-976-420	yes #04-975-410 MT	2	1506 x g, 3500 rpm
Megafuge 1.0	#C1725-3 (Baxter #)	Heraeus	#C1725-10 #C1725-35	yes	4	1280 x g, 2800 rpm
Z-320	#C-0320	Hermle	#C-0320-50	no	2	1506 x g, 3500 rpm
GP8 GP8R	#3121 #3122	IEC	#216 or #228	yes #5785	2 or 4	2340 x g, 3500 rpm
HN-SII	#2355	IEC	#244	no	2	1450 x g, 3575 rpm
MP4 MP4R	#2437 #2438	IEC	#244	no	2	1450 x g, 3575 rpm
C312 CR312	#11175087 #11175090	Jouan	E4 (#11174153)	yes #11174168	4	2290 x g, 3300 rpm
1130	Not required	Kubota	S11222	no	2	1107 x g, 3000 rpm
1140	Not required	Kubota	S11222	no	2	1107 x g, 3000 rpm

Appendix 5

Characteristics, regeneration and storage of Glutathione Sepharose

Glutathione Sepharose 4B is recommended for packing small columns and other formats. Glutathione Sepharose Fast Flow is excellent for packing high performance columns for use with purification systems and for scaling up.

Characteristics

Ligand density	7-15 µmol glutathione per ml Glutathione Sepharose 4B 120-320 µmole glutathione per ml Glutathione Sepharose 4 Fast Flow
Capacity	>8 mg recombinant GST per ml Glutathione Sepharose 4B >10 mg recombinant GST per ml Glutathione Sepharose 4 Fast Flow
Ligand and spacer arm	Glutathione and 10 carbon linker arm
Molecular weight exclusion limit	~2 x 10^7 daltons
Particle size	45-165 µm (90 µm)

Chemical stability

No significant loss of the capacity when exposed to 0.1 M citrate (pH 4.0), 0.1 M NaOH, 70% ethanol or 6 M guanidinium hydrochloride for 2 hours at room temperature. No significant loss of binding capacity after exposure to 1% SDS for 14 days.

Regeneration

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

If required, Glutathione Sepharose 4B and Glutathione Sepharose 4 Fast Flow can be regenerated for re-use as follows:

1. Wash with 2-3 column volumes of alternating high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5) buffers.
2. Repeat the cycle 3 times.
3. Re-equilibrate with 3-5 column volumes of 1X PBS.

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

To remove precipitated or denatured substances:

1. Wash with 2 column volumes of 6 M guanidinium hydrochloride.
2. Wash with 5 column volumes of 1X PBS.

To remove hydrophobically bound substances:

1. Wash with 3-4 column volumes of 70% ethanol (or 2 bed volumes of a non-ionic detergent (conc. 0.1%)).
2. Wash with 5 column volumes of 1X PBS.

For long-term storage (>1 month):

1. Wash the column twice with 10 column volumes of 1X PBS.
2. Repeat washes using 20% ethanol.
3. Store at +4°C.
4. Re-equilibrate the column with 5-10 column volumes of 1X PBS before re-use.

Characteristics, regeneration and storage of Chelating Sepharose

Characteristics

Capacity	12 mg $(His)_6$ fusion protein (M_r 27 600) per ml Chelating Sepharose High Performance (in HiTrap columns) <100 µg $(His)_6$ fusion protein (M_r 27 600) per column Chelating Sepharose 4 Fast Flow (in MicroSpin columns and for packing larger columns)
Ligand	Iminodiacetic acid
Molecular weight exclusion limit	~2 x 10^7 daltons
Particle size	45-165 µm (90 µm) Chelating Sepharose Fast Flow 24-44 µm (34 µm) Chelating Sepharose High Performance

Chemical stability

Stable in all commonly used aqueous buffers and denaturants such as guanidinium hydrochloride, 8 M urea and chaotropic salts.

Regeneration

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

The column must be recharged with nickel ions after regeneration.

To remove nickel ions prior to recharging

1. Wash column with 5 column volumes 20 mM sodium phosphate, 0.5 M NaCl, 0.05 M EDTA, pH 7.4.

To remove nickel ions prior to storage

1. Wash column with 5 column volumes 20 mM sodium phosphate, 0.5 M NaCl, 0.05 M EDTA, pH 7.4.
2. Wash with 10 column volumes of distilled water followed by 10 column volumes of 20% ethanol.

To remove precipitated proteins

1. Fill column with 1 M NaOH and incubate for 2 hours.
2. Wash out dissolved proteins with 5 column volumes of water and a buffer at pH ~7 until the pH of the flow-through reaches pH ~7.0.

The loss of metal ions is more pronounced at lower pH. The column does not have to be stripped between each purification if the same protein is going to be purified. In this case, perform stripping and re-charging of the column after 5-10 purifications.

To store columns and media

Seal columns with fittings provided to avoid dehydration and store at +4° to +8°C.

For long term storage remove nickel ions and store with 20% ethanol and at +4° to +8°C.

Appendix 6

Column packing and preparation

A Column Packing Video is also available to demonstrate how to produce a well-packed column (see ordering information).

1. Equilibrate all materials to the temperature at which the purification will be performed.
2. Eliminate air by flushing column end pieces with recommended buffer. Ensure no air is trapped under the column net. Close column outlet leaving 1-2 cm of buffer in the column.
3. Gently resuspend the purification medium.
4. Estimate the amount of slurry (resuspended medium) required.
5. Pour the required volume of slurry into the column. Pouring down a glass rod held against the wall of the column will minimize the introduction of air bubbles.
6. Immediately fill the column with buffer.
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

   For example: 15 ml/min in an XK 16/20 column

If the recommended flow rate cannot be obtained use the maximum flow rate the pump can deliver.

9. Maintain the packing flow rate for at least 3 bed volumes after a constant bed height is obtained. Mark the bed height on the column.

Do not exceed 75% of the packing flow rate during any purification.

10. Stop the pump and close the column outlet. Remove the top piece and carefully fill the rest of the column with recommended buffer to form an upward meniscus at the top.
11. Insert the adaptor into the column at an angle, ensuring that no air is trapped under the net.
12. Slide the adaptor slowly down the column (the outlet of the adaptor should be open) until the mark is reached. Lock the adaptor in position.
13. Connect the column to the pump and begin equilibration. Re-position the adaptor if necessary.

The medium must be thoroughly washed especially if 20% ethanol has been used as the storage solution. Residual ethanol may interfere with subsequent procedures.

Medium equilibrated with PBS may be stored at +4°C for up to 1 month.

Appendix 7

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

It is often convenient when comparing results for columns of different sizes to express flow rates in linear flow rate (cm/hour). However, flow rates are usually measured in volumetric flow rate (ml/min). To convert between linear and volumetric flow rate use one of the formulae below.

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

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

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

where

Y = linear flow rate in cm/h

d = column inner diameter in cm

Example:

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

Y = linear flow rate = 150 cm/h

d = inner diameter of the column = 1.6 cm

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

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

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

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

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

where

Z = volumetric flow rate in ml/min

d = column inner diameter in cm

Example:

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

Z = Volumetric flow rate = 1 ml/min

d = column inner diameter = 0.5 cm

$$\text{Linear flow rate} = 1 \times 60 \times \frac{4}{\pi \times 1.6 \times 1.6} \text{ cm/h}$$

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

Appendix 8

Selection of purification equipment

Amersham Pharmacia Biotech offers solutions from the simplest purification through to full-scale production. Using this guide will assist in the selection of the most appropriate solution to suit the immediate purification task and possible needs in the future.

Purification Need	MicroSpin Purification Modules	Syringe + HiTrap columns	ÄKTA*prime* + HiTrap columns
Rapid, high throughput screening (GST or $(His)_6$ fusion proteins)	•		
Very fast, one-step purification (most suited to fusion proteins)	•	•	
One-step purification (most suited to fusion proteins)		•	•
Optimization to increase purity			•
Routine, reproducible results			•
Protein refolding after purification			•

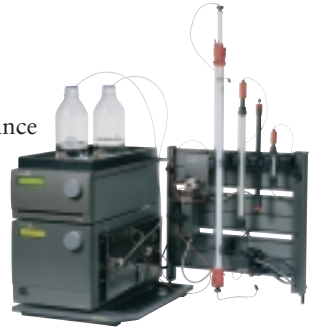
Use ÄKTA*FPLC* or ÄKTAexplorer chromatography systems for:

- Multi-step purification (for non-fusion proteins or to achieve higher purity)
- Method development and optimization
- System control and data handling to follow regulatory requirements e.g. GLP
- Scale up of purification methods
- Easy transfer of methods to production scale

ÄKTA*prime*: **simple** purification of proteins

ÄKTAexplorer: **fast** method and process development and scale-up for proteins, peptides and nucleic acids

ÄKTA*FPLC*: **high** performance purification of proteins



Appendix 9

Principles and standard conditions for purification techniques

Affinity Chromatography (AC)

AC separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand attached to a chromatographic matrix. The technique is ideal for a capture or intermediate step and can be used whenever a suitable ligand is available for the protein(s) of interest. AC offers high selectivity, hence high resolution, and usually high capacity (for the protein(s) of interest).

The target protein(s) is specifically and reversibly bound by a complementary binding substance (ligand). The sample is applied under conditions that favour specific binding to the ligand. Unbound material is washed away, and the bound target protein is recovered by changing conditions to those favouring desorption. Desorption is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Samples are concentrated during binding and protein is collected in purified, concentrated form. The key stages in a separation are shown in Figure 23. Affinity chromatography is also used to remove specific contaminants, for example Benzamidine Sepharose 6B can remove serine proteases.

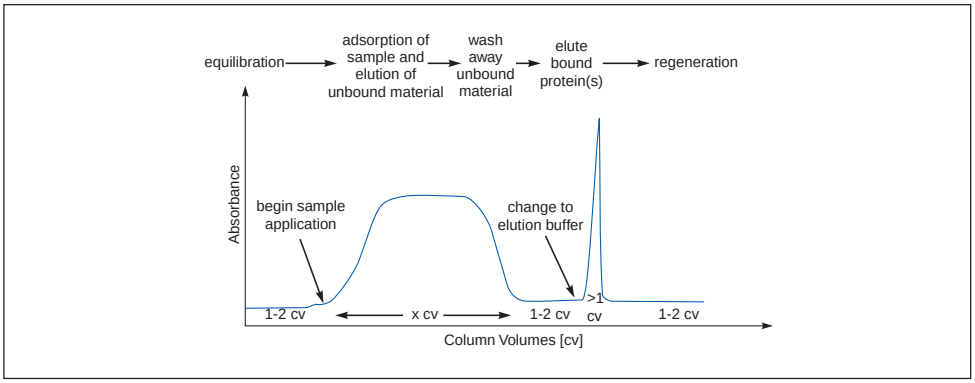


Fig. 23. Typical affinity separation.

Further information

Protein Purification Handbook
Affinity Chromatography Handbook: Principles and Methods

Ion Exchange (IEX)

IEX separates proteins with differences in charge to give a very high resolution separation with high sample loading capacity. The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. Proteins bind as they are loaded onto a column. Conditions are then altered so that bound substances are eluted differentially. Elution is usually performed by increasing salt concentration or changing pH. Changes are made stepwise or with a continuous gradient. Most commonly, samples are eluted with salt (NaCl), using a gradient elution (Figure 24). Target proteins are concentrated during binding and collected in a purified, concentrated form.

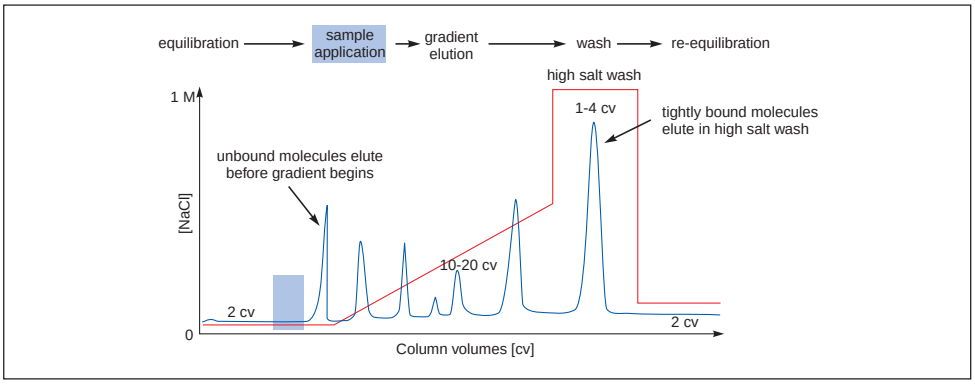


Fig. 24. Typical IEX gradient elution.

The net surface charge of proteins varies according to the surrounding pH. When above its isoelectric point (pI) a protein will bind to an anion exchanger, when below its pI a protein will behind to a cation exchanger. Typically IEX is used to bind the target molecule, but it can also be used to bind impurities if required. IEX can be repeated at different pH values to separate several proteins which have distinctly different charge properties, as shown in Figure 25.

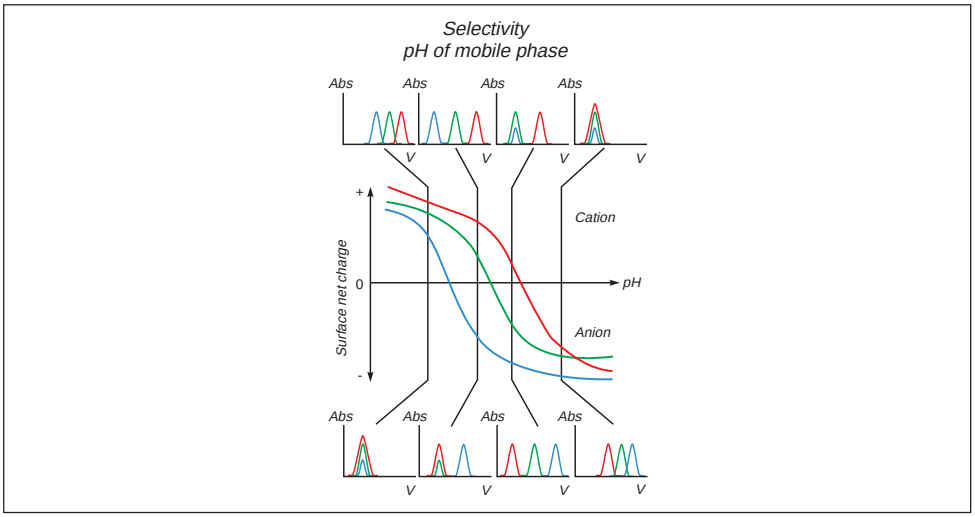


Fig. 25. Effect of pH on protein elution patterns.

Method development (in priority order)

1. Select optimum ion exchanger using small columns as in the HiTrap IEX Selection Kit to save time and sample.
2. Scout for optimum pH. Begin 0.5-1 pH unit away from the isoelectric point of the target protein if known.
3. Select the steepest gradient to give acceptable resolution at the selected pH.
4. Select the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.

To reduce separation times and buffer consumption transfer to a step elution after method optimization as shown in Figure 26. It is often possible to increase sample loading when using step elution.

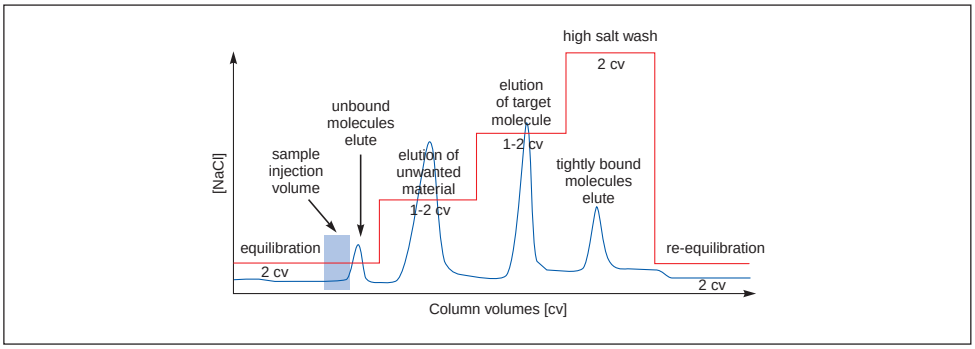


Fig. 26. Step elution.

Further information

Protein Purification Handbook
Ion Exchange Chromatography Handbook: Principles and Methods

Hydrophobic Interaction Chromatography (HIC)

HIC separates proteins with differences in hydrophobicity. The technique is ideal for the capture or intermediate steps in a purification. Separation is based on the reversible interaction between a protein and the hydrophobic surface of a chromatographic medium. This interaction is enhanced by high ionic strength buffer which makes HIC an ideal "next step" after precipitation with ammonium sulphate or elution in high salt during IEX. Samples in high ionic strength solution (e.g. 1.5 M ammonium sulphate) bind as they are loaded onto a column. Conditions are then altered so that the bound substances are eluted differentially.

Elution is usually performed by decreases in salt concentration (Figure 27). Changes are made stepwise or with a continuous decreasing salt gradient. Most commonly, samples are eluted with a decreasing gradient of ammonium sulphate. Target proteins are concentrated during binding and collected in a purified, concentrated form. Other elution procedures include reducing eluent polarity (ethylene glycol gradient up to 50%), adding chaotropic species (urea, guanidinium hydrochloride) or detergents, changing pH or temperature.

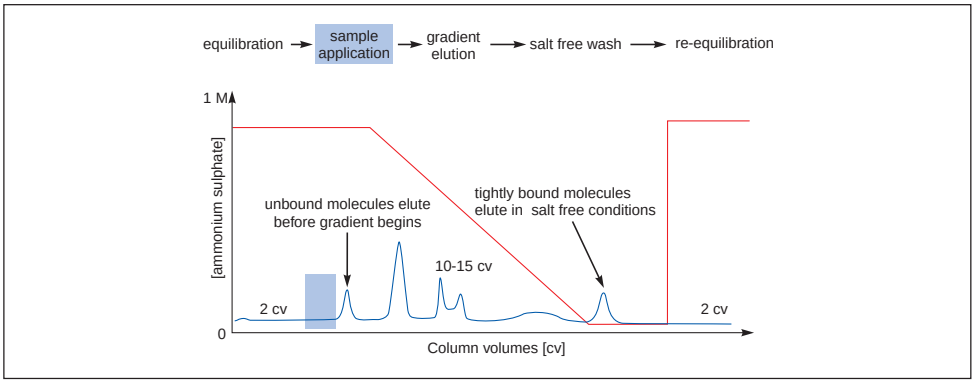


Fig. 27. Typical HIC gradient elution.

Method development (in priority order)

1. The hydrophobic behaviour of a protein is difficult to predict and binding conditions must be studied carefully. Use the HiTrap HIC Selection Kit or the RESOURCE HIC Test Kit to select the medium that gives optimum binding and elution over the required range of salt concentration. For proteins with unknown hydrophobic properties begin with 0-100%B (0%B=1M ammonium sulphate).
2. Select the gradient that gives acceptable resolution.
3. Select the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.
4. If samples adsorb strongly to a medium then conditions that cause conformational changes, such as pH, temperature, chaotropic ions or organic solvents can be altered. Conformational changes caused by these agents are specific to each protein. Use screening procedures to investigate the effects of these agents. Alternatively, change to a less hydrophobic medium.

To reduce separation times and buffer consumption, transfer to a step elution after method optimization, as shown in Figure 28. It is often possible to increase sample loading when using step elution, an additional benefit for larger scale purification.

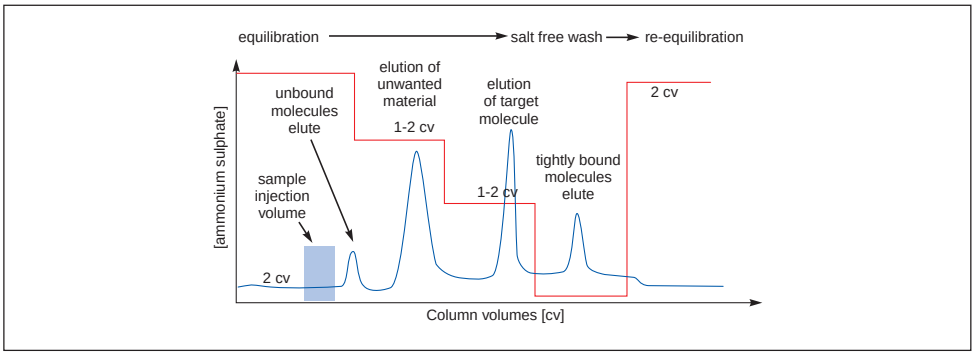


Fig. 28. Step elution.

Further information

Protein Purification Handbook
Hydrophobic Interaction Chromatography Handbook: Principles and Methods

Gel Filtration (GF) Chromatography

GF separates proteins with differences in molecular size. The technique is ideal for the final polishing steps in purification when sample volumes have been reduced (sample volume significantly influences speed and resolution in gel filtration). Samples are eluted isocratically (single buffer, no gradient Figure 29). Buffer conditions are varied to suit the sample type or the requirements for further purification, analysis or storage step, since buffer composition does not directly affect resolution. Proteins are collected in purified form in the chosen buffer.

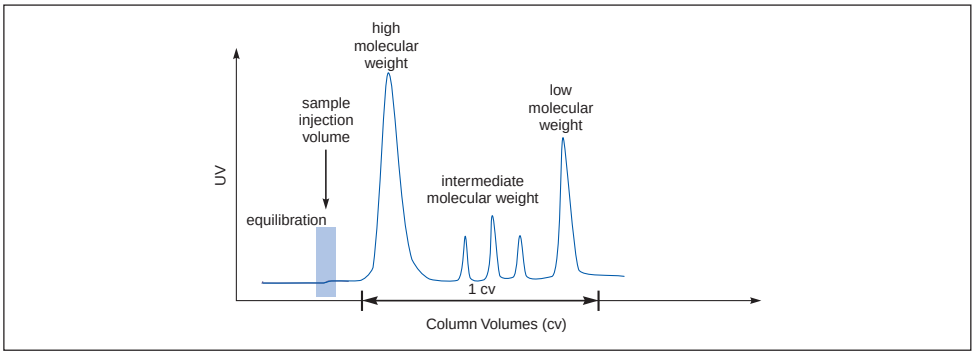


Fig. 29. Typical GF elution.

Further information

Protein Purification Handbook
Gel Filtration Handbook: Principles and Methods

Reversed Phase Chromatography (RPC)

RPC separates proteins and peptides with differing hydrophobicity based on their reversible interaction with the hydrophobic surface of a chromatographic medium. Samples bind as they are loaded onto a column. Conditions are then altered so that the bound substances are eluted differentially. Due to the nature of the reversed phase matrices, the binding is usually very strong and requires the use of organic solvents and other additives (ion pairing agents) for elution. Elution is usually performed by increases in organic solvent concentration, most commonly acetonitrile. Samples, which are concentrated during the binding and separation process, are collected in a purified, concentrated form. The key stages in a separation are shown in Figure. 30.

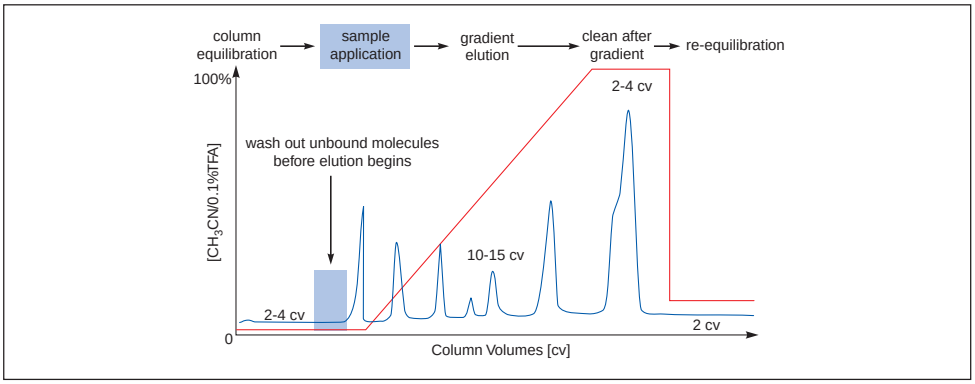


Fig. 30. Typical RPC gradient elution.

RPC is often used in the final polishing of oligonucleotides and peptides and is ideal for analytical separations, such as peptide mapping.

RPC is not recommended for protein purification if recovery of activity and return to a correct tertiary structure are required, since many proteins are denatured in the presence of organic solvents.

Method development

1. Select medium from screening results.
2. Select optimum gradient to give acceptable resolution. For unknown samples begin with 0-100% elution buffer.
3. Select highest flow rate which maintains resolution and minimizes separation time.
4. For large scale purification transfer to a step elution.
5. Samples that adsorb strongly to a medium are more easily eluted by changing to a less hydrophobic medium.

Further information

Protein Purification Handbook
Reversed Phase Chromatography Handbook: Principles and Methods

Expanded Bed Adsorption (EBA)

EBA is a single pass operation in which target proteins are purified from crude sample, without the need for separate clarification, concentration and initial purification to remove particulate matter. Crude sample is applied to an expanded bed of STREAMLINE™ adsorbent particles within a specifically designed STREAMLINE column. Target proteins are captured on the adsorbent. Cell debris, cells, particulate matter, whole cells, and contaminants pass through and target proteins are then eluted. Figure 31 shows a representation of the steps involved in an EBA purification and Figure 32 shows a typical EBA elution.

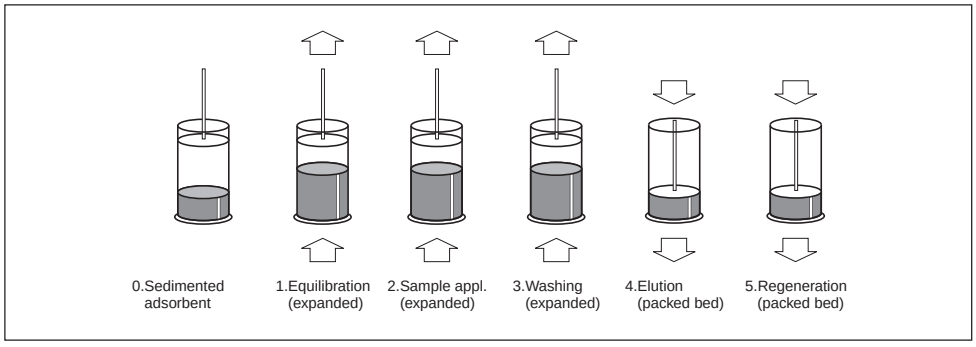


Fig. 31. Steps in an EBA purification process.

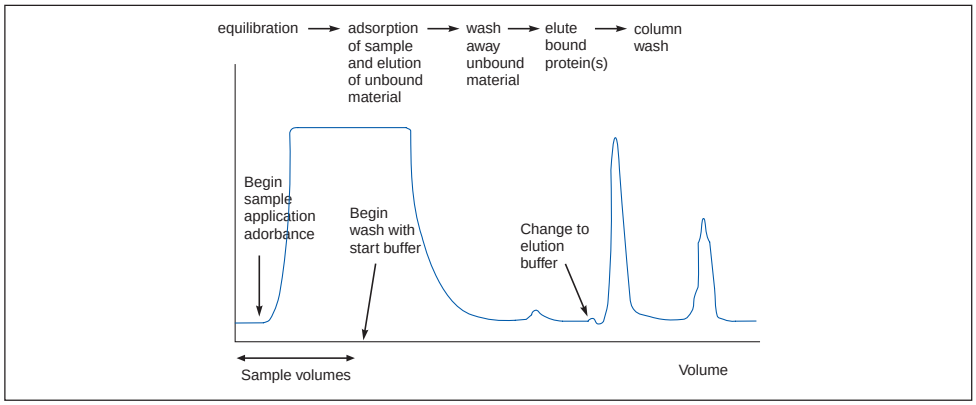


Fig. 32. Typical EBA elution.

Method development

1. Select suitable ligand to bind the target protein.
2. Scout for optimal binding and elution conditions using clarified material in a packed column (0.02 - 0.15 litres bed volume of media). Gradient elution may be used during scouting, but the goal is to develop a step elution.
3. Optimize binding, elution, wash and cleaning-in-place procedures using unclarified sample in expanded mode at small scale (0.02 - 0.15 litres bed volume of media).
4. Begin scale up process at pilot scale (0.2 - 0.9 litres bed volume of media).
5. Full scale production (up to several hundred litres bed volume of media).

Further information

Protein Purification Handbook
Expanded Bed Adsorption Handbook: Principles and Methods

Additional reading and reference material

	Code No.
Gel Media Guide (electrophoresis)	18-1129-79
2D Electrophoresis Handbook	80-6429-60
Protein Electrophoresis Technical Manual	80-6013-88
Protein Purification Handbook	18-1132-29
Protein and Peptide Purification Technique Selection	18-1128-63
Fast Desalting and Buffer Exchange of Proteins and Peptides	18-1128-62
Gel Filtration Handbook Principles and Methods	18-1022-18
Ion Exchange Chromatography Handbook Principles and Methods	18-1114-21
Chromatofocusing with Polybuffer and PBE	50-01-022PB
Hydrophobic Interaction Chromatography Handbook Principles and Methods	18-1020-90
Affinity Chromatography Handbook Principles and Methods	18-1022-29
Reversed Phase Chromatography Handbook Principles and Methods	18-1134-16
Expanded Bed Adsorption Handbook Principles and Methods	18-1124-26
Antibody Purification Handbook	18-1037-46
HiTrap Guide	18-1128-81
ÄKTAdesign Brochure	18-1129-05
GST fusion proteins	
GST Gene Fusion Manual	18-1123-20
Data File, GSTrap and Glutathione Sepharose 4 Fast Flow	18-1136-89
Miniposter, "Rapid Purification of GST-fusion proteins from large sample volumes"	18-1139-51
$(His)_6$ fusion proteins	
Data File, HisTrap	18-1212-00
Data File, HiTrap Chelating 1 ml and 5 ml	18-1134-78
Miniposter, "Purification of Poly(His)-tagged Recombinant Proteins using HisTrap"	18-1116-26
On-column refolding of $(His)_6$ fusion proteins	
Application Note, "Rapid and efficient purification and refolding of a $(His)_6$-tagged recombinant protein produced in *E. coli* as inclusion bodies"	18-1134-37
Multi-step purification	
Application note, "Purification of a labile, oxygen sensitive enzyme for crystallization and 3D structural determination"	18-1128-91
Detection	
Application Note, "ECL Western and ECL Plus Western blotting"	18-1139-13

Ordering information

Product	Quantity	Code No.
GST fusion proteins		
Protein Amplification		
PGEX- 4T-1	25 µg	27-4580-01
PGEX- 4T-2	25 µg	27-4581-01
PGEX- 4T-3	25 µg	27-4583-01
PGEX- 5X-1	25 µg	27-4584-01
PGEX- 5X-2	25 µg	27-4585-01
PGEX- 5X-3	25 µg	27-4586-01
PGEX- 6P-1	25 µg	27-4597-01
PGEX- 6P-2	25 µg	27-4598-01
PGEX- 6P-3	25 µg	27-4599-01
All vectors include *E. coli* B21		
Cleavage		
Thrombin	500 units	27-0846-01
Factor Xa	400 units	27-0849-01
PreScission Protease	500 units	27-0843-01
Purification		
MicroSpin GST Purification Module	50 purifications	27-4570-03
MicroPlex 24 Vacuum	1 system	27-3567-01
Bulk GST Purification Module	5 purifications	27-4570-01
GSTrap	2 x 1 ml	17-5130-02
GSTrap	5 x 1 ml	17-5130-01
GSTrap	1 x 5 ml	17-5131-01
Glutathione Sepharose 4B	10 ml	17-0756-01
Glutathione Sepharose 4B	100 ml	27-4574-01
Glutathione Sepharose 4 Fast Flow	25 ml* *(*larger quantities available on request)*	17-5132-01
Detection		
GST Detection Module	50 reactions	27-4590-01
GST 96 Well Detection Module	96 reactions	27-4592-01
Anti-GST antibody	0.5 ml	27-4577-01
$(His)_6$ fusion proteins		
Purification		
His MicroSpin Purification Module	50 purifications	27-4770-01
MicroPlex 24 Vacuum	1 system	27-3567-01
HisTrap Kit	HiTrap Chelating columns (3 x 1 ml), accessories, pre-made buffers for up to 12 purifications	17-1880-01
HiTrap Chelating	5 x 1 ml	17-0408-01
HiTrap Chelating	1 x 5 ml	17-0409-01
Chelating Sepharose Fast Flow	50 ml	17-0575-01
Chelating Sepharose Fast Flow	500 ml	17-0575-02
Chelating Sepharose Fast Flow	5 l	17-0575-04
Detection		
Anti-His antibody	170 µl	27-4710-01
HRP conjugated (anti-mouse) IgG	1 ml	NA931
Companion Products		
E. coli B21	1 vial	27-1542-01
Isopropyl β-D-thiogalactoside (IPTG)	1 g	27-3054-03
	5 g	27-3054-04
	10 g	27-3054-05
Column Packing Video	PAL	17-0893-01
Column Packing Video	NTSC	17-0894-01
Benzamidine Sepharose 6B	25 ml	17-0568-01

Product	Quantity	Code No.
HiTrap Columns: Affinity		
HiTrap rProtein A	5 x 1 ml	17-5079-01
	1 x 5 ml	17-5080-01
	2 x 1 ml	17-5029-02
HiTrap Protein A	5 x 1 ml	17-0402-01
	2 x 1 ml	17-0402-03
	1 x 5 ml	17-0403-01
HiTrap Protein G	5 x 1 ml	17-0404-01
	2 x 1 ml	17-0404-03
	1 x 5 ml	17-0405-01
MAbTrap GII	HiTrap Protein G (1 x 1 ml), accessories, premade buffers for 10 purifications	17-1128-01
HiTrap IgY Purification	1 x 5 ml	17-5111-01
HiTrap IgM Purification	5 x 1 ml	17-5110-01
HiTrap Con A	5 x 1 ml	17-5105-01
HiTrap Lentil Lectin	5 x 1 ml	17-5106-01
HiTrap Peanut Lectin	5 x 1 ml	17-5108-01
HiTrap Wheat Germ Lectin	5 x 1 ml	17-5107-01
HiTrap Lectin Test Kit 4 x 1 ml	HiTrap Con A 1 ml HiTrap Lentil Lectin 1 ml HiTrap Peanut Lectin 1 ml HiTrap Wheat Germ Lectin 1 ml	17-5109-01
HiTrap Blue	5 x 1 ml	17-0412-01
	1 x 5 ml	17-0413-01
HiTrap Chelating	5 x 1 ml	17-0408-01
	1 x 5 ml	17-0409-01
HisTrap Kit	HiTrap Chelating (3 x 1 ml), accessories, pre-made buffers for up to 12 purifications	17-1880-01
HiTrap Streptavidin	5 x 1 ml	17-5112-01
HiTrap Heparin	5 x 1 ml	17-0406-01
	1 x 5 ml	17-0407-01
HiTrap NHS-activated	5 x 1 ml	17-0716-01
	1 x 5 ml	17-0717-01
HiTrap Columns: IEX		
HiTrap IEX Selection Kit 7 x 1 ml	HiTrap Q XL 1 ml HiTrap SP XL 1 ml HiTrap ANX Sepharose 4 Fast Flow (high sub) 1 ml HiTrap DEAE Sepharose Fast Flow 1 ml HiTrap CM Sepharose Fast Flow 1 ml HiTrap Q Sepharose Fast Flow 1 ml HiTrap SP Sepharose Fast Flow 1 ml	17-6002-33
HiTrap Q High Performance	5 x 1 ml	17-1153-01
	5 x 5 ml	17-1154-01
HiTrap SP High Performance	5 x 1 ml	17-1151-01
	5 x 5 ml	17-1152-01
HiTrap Q XL	5 x 1 ml	17-5158-01
HiTrap Q XL	5 x 5 ml	17-5159-01
HiTrap SP XL	5 x 1 ml	17-5160-01
HiTrap SP XL	5 x 5 ml	17-5161-01
HiTrap ANX Sepharose 4 Fast Flow (high sub)	5 x 1 ml	17-5162-01
HiTrap ANX Sepharose 4 Fast Flow (high sub)	5 x 5 ml	17-5163-01

Product	Quantity	Code No.
Continuing		
HiTrap DEAE Sepharose Fast Flow	5 x 1 ml	17-5055-01
HiTrap DEAE Sepharose Fast Flow	5 x 5 ml	17-5154-01
HiTrap CM Sepharose Fast Flow	5 x 1 ml	17-5056-01
HiTrap CM Sepharose Fast Flow	5 x 5 ml	17-5155-01
HiTrap Q Sepharose Fast Flow	5 x 1 ml	17-5053-01
HiTrap Q Sepharose Fast Flow	5 x 5 ml	17-5156-01
HiTrap SP Sepharose Fast Flow	5 x 1 ml	17-5054-01
HiTrap SP Sepharose Fast Flow	5 x 5 ml	17-5157-01
HiTrap Columns: HIC		
HiTrap HIC Selection Kit 5 x 1 ml	Butyl Sepharose 4 Fast Flow 1 ml Octyl Sepharose 4 Fast Flow 1 ml Phenyl Sepharose 6 Fast Flow (low sub) 1 ml Phenyl Sepharose 6 Fast Flow (high sub) 1 ml Phenyl Sepharose High Performance 1 ml	17-1349-01
HiTrap Phenyl Sepharose Fast Flow (high sub)	5 x 1 ml	17-1355-01
HiTrap Phenyl Sepharose Fast Flow (high sub)	5 x 5 ml	17-5193-01
HiTrap Phenyl Sepharose Fast Flow (low sub)	5 x 1 ml	17-1353-01
HiTrap Phenyl Sepharose Fast Flow (low sub)	5 x 5 ml	17-5194-01
HiTrap Phenyl Sepharose High Performance	5 x 1 ml	17-1351-01
HiTrap Phenyl Sepharose High Performance	5 x 5 ml	17-5195-01
HiTrap Butyl Sepharose Fast Flow	5 x 1 ml	17-1357-01
HiTrap Butyl Sepharose Fast Flow	5 x 5 ml	17-5197-01
HiTrap Octyl Sepharose Fast Flow	5 x 1 ml	17-1359-01
HiTrap Octyl Sepharose Fast Flow	5 x 5 ml	17-5186-01
Desalting and Buffer Exchange		
MicroSpin G-25 Columns	50 columns	27-5325-01
HiTrap Desalting	5 x 5 ml	17-1408-01
HiPrep 26/10 Desalting	1 x 53 ml	17-5087-01
Western Blotting		
Hybond-P	10 sheets	RPN2020F
Hybond-ECL	10 sheets	RPN2020D
ECL Western Blotting Detection Reagents	for 1000 cm^2	RPN2109
ECL Plus Western Blotting Detection System	for 1000 cm^2	RPN2132

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