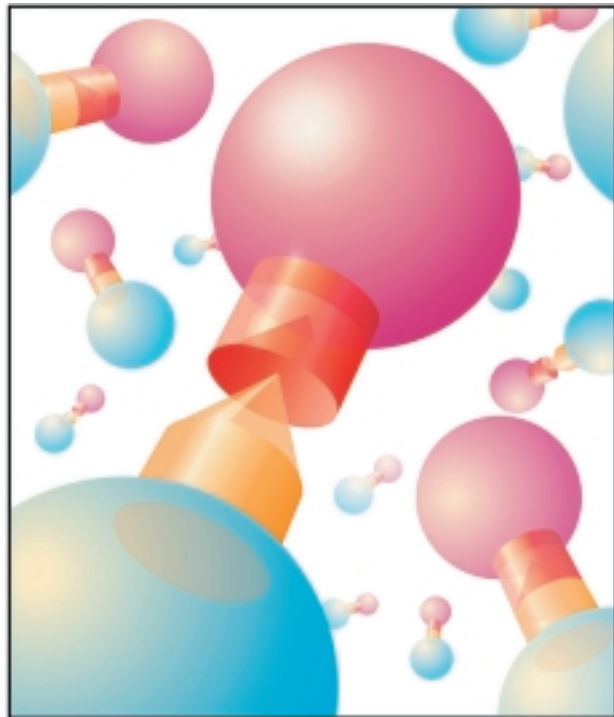


# The Recombinant Protein Handbook

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## Protein Amplification and Simple Purification



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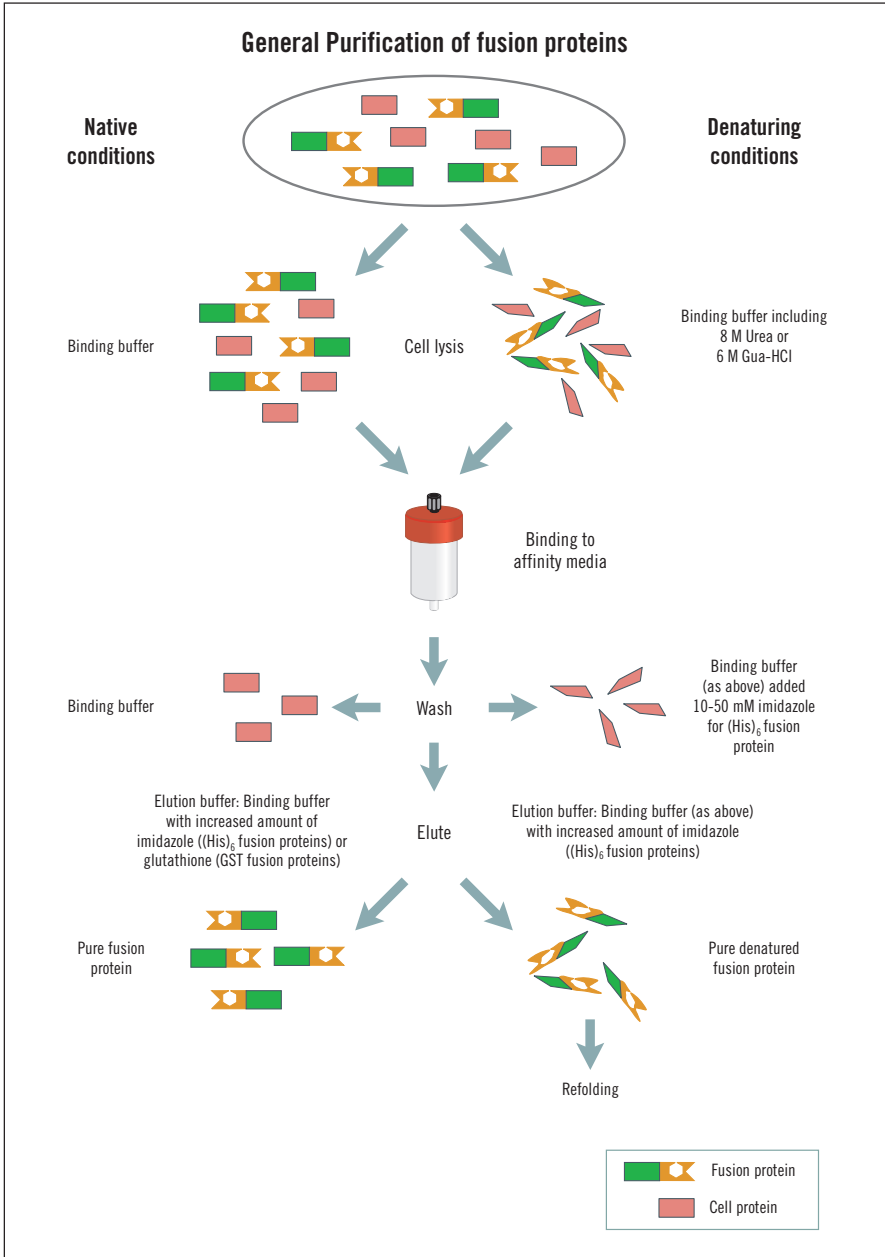
# **The Recombinant Protein Handbook**

Protein Amplification  
and Simple Purification

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# 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 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 unwanted 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)<sub>6</sub> 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 and abbreviations



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



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



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



reagents and equipment required.



experimental protocol.

PBS phosphate buffered saline.

# CHAPTER 1

## Choice of host for protein amplification

Several host systems are available including 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 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 <i>e.g. Escherichia coli</i>	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 <i>e.g. Staphylococcus aureus</i>	Secretes fusion proteins into the growth media	Does not express such high levels as <i>E. coli</i>
		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 <i>Baculovirus vector</i>	Facilitates glycosylation and formation of disulphide bonds	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 <i>e.g. Aspergillus sp.</i>	Well established systems for fermentation of filamentous fungi	High level of expression not yet achieved
	Growth inexpensive	Genetics not well characterized
	<i>A.niger</i> 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
<b>Fusion proteins</b>	
Targetting information can be incorporated into a tag	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	
<b>Non- fusion proteins</b>	
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
pTrc 99 A	<i>E. coli</i> 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 <i>tac</i> promotor in <i>E. coli</i>
pSVK 3	For <i>in vivo</i> expression in mammalian cell lines
PSVL SV40	For high level transient expression in mammalian 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™ FF Glutathione Sepharose™ Fast Flow
PQE	6 x Histidine	His MicroSpin Purification Module HisTrap™ Kit HiTrap™ Chelating HP Chelating Sepharose Fast Flow
pET	6 x Histidine	His MicroSpin Purification Module HisTrap Kit HiTrap Chelating HP 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)<sub>6</sub> fusion proteins.

## Choice of fusion tag

The two most commonly used tags are glutathione S-transferase (GST tag) and 6 x histidine residues (His)<sub>6</sub> tag. As for the selection of host and vectors, the decision to use either a GST or a (His)<sub>6</sub> 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) <sub>6</sub> 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) <sub>6</sub> 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) <sub>6</sub> - 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) <sub>6</sub> fusion proteins*

**Table 5.**

\*Geoghagan, K.F., *et al.*, *Anal Biochem* **267** (1), 169–184 (1999).



Polyhistidine tags such as (His)<sub>4</sub> or (His)<sub>10</sub> are also used. They may provide useful alternatives to (His)<sub>6</sub> if there are specific requirements for purification, as discussed on page 42.

## CHAPTER 2

### Protein amplification



Cell culture conditions are dependent upon the host system. Follow the instructions of the supplier. Before performing a large scale purification, check protein amplification in the culture or do a small pilot experiment to establish optimum conditions for expression.



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.



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 l	20 l
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 6.*

### Troubleshooting protein amplification

*(for specific details on GST or (His)<sub>6</sub> fusion proteins, see page 21 for detection of GST fusion proteins or page 53 for detection of (His)<sub>6</sub> 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, with the pGEX vector system there is a *lac* promoter located upstream between the 3'-end of the *lacI<sup>a</sup>* gene and the *tac* promoter. This *lac* promoter may contribute to the basal level of expression of inserts cloned into the pGEX multiple cloning site, 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, as suggested below. It may be necessary to combine these approaches. Exact conditions must be determined empirically for each fusion protein.
  - Lower the growth temperature (within the range of +20 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)<sub>6</sub> 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. In this case, the fusion protein does not have to be purified for quantification as long as a purified standard is available. 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

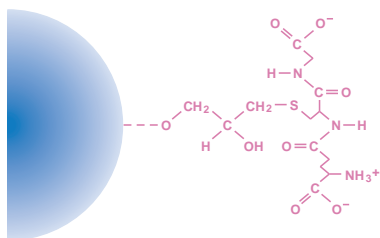
## GST fusion proteins

### 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 <i>E. coli</i> with full enzymatic activity
Properties as determined in pGEX-1N	
Dimer Molecular Weight	$M_r$ 58 500
$K_m$ (glutathione)	$0.43 \pm 0.07$ mM
$K_m$ (CDNB)	$2.68 \pm 0.77$ 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  $\beta$ -D-thiogalactoside (IPTG). Induced cultures should be left to express GST fusion proteins for several hours before the cells are harvested.

### The host

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

Genotype	$F^-$ , <i>ompT</i> , <i>hscS</i> ( $r_B^-$ , $m_B^-$ ), <i>gal</i> (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) as BL21 does not transform well.



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<sup>q</sup>* 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 <i>in vitro</i>	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 (prepacked) or Media**	Amount of GST fusion protein for a single purification	Comment
GST MicroSpin Purification Module	Up to 400 µg	Ready to use, prepacked columns, buffers and chemicals High throughput when used with MicroPlex™ 24 Vacuum (up to 48 samples simultaneously)
GSTrap FF 1 ml	10–12 mg	Prepacked column, ready to use
GSTrap FF 5 ml	50–60 mg	Prepacked 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 FF 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 prepacked 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 FF 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 FF columns (1 ml or 5 ml) in series or, for even larger capacity requirements, pack Glutathione Sepharose 4 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<sub>2</sub>HPO<sub>4</sub>, 18 mM KH<sub>2</sub>PO<sub>4</sub>, 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  $\mu$ l of sample 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 water vacuum).
- Side arm flask, 500 ml or 1 litre.
- Single or double hole rubber stop.
- Vacuum tubing.
- MicroPlex 24 Vacuum apparatus (one or two).

1. Assemble the MicroPlex 24 Vacuum following the instructions supplied.
2. Resuspend the Glutathione Sepharose in each MicroSpin column by vortexing gently.
3. Remove the caps and snap off the bottom closures from the MicroSpin columns. Place the columns in the manifold, filling any unused holes with the plugs provided with MicroPlex 24 Vacuum.
4. 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  $\mu$ l 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  $\mu$ l 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. Additional 600  $\mu$ l washes may be performed if desired.
11. Add 200  $\mu$ l 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.

*Note: Yields of fusion protein may be increased by repeating the elution step two or three times and pooling the eluates.*

### Troubleshooting



See *Purification and Detection Troubleshooting* page 28.



### Alternative 2. Purification of multiple samples using a microcentrifuge

Do not apply more than 600  $\mu$ l of sample 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 g.
4. Discard the buffer from each centrifuge tube and replace the bottom closures.
5. Apply up to 600  $\mu$ l 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 g to collect flow through.
9. Place each column into a clean, pre-labelled 1.5 or 2 ml microcentrifuge tube.
10. Apply 600  $\mu$ l of 1X PBS wash buffer to each column and repeat spin procedure. Additional 600  $\mu$ l washes with 1X PBS may be performed if desired.
11. Add 100–200  $\mu$ l of Glutathione elution buffer to each column. Replace top caps and bottom closures. Incubate at room temperature for 5–10 minutes.
12. Remove and discard top caps and bottom closures and place the column into a clean 1.5 or 2 ml microcentrifuge tube.
13. Spin all columns again to collect eluate. Save for analysis.

*Note: Yields of fusion protein may be increased by repeating the elution step two or three times and pooling the eluates.*

### Troubleshooting



See *Purification and Detection Troubleshooting* page 28.

### Alternative 3. Purification using MicroPlex Centrifugation



Do not apply more than 600  $\mu$ l of sample 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  $\mu$ l of lysate to each column. Incubate at room temperature for 5–10 minutes.
6. Centrifuge the unit for 2 minutes following the instructions supplied. If desired remove the manifold from each collection tray and place on a clean paper towel. Reassemble each unit with a fresh collection tray.
7. Apply 600  $\mu$ l of 1X PBS wash buffer to each column and repeat spin procedure. Additional 600  $\mu$ l washes with 1X PBS may be performed if desired. Remove the manifold from each collection tray and place it on clean paper.
8. Add 100–200  $\mu$ l of glutathione elution buffer to each column. Incubate at room temperature for 5–10 minutes.
9. Centrifuge the unit for 2 minutes following the instructions supplied. Cover the eluted samples with sealing tape until required for analysis.

*Note: Yields of fusion protein may be increased by repeating the elution step two or three times and pooling the eluates.*

### Troubleshooting



See *Purification and Detection Troubleshooting* page 28.

## Purification using GSTrap FF 1 ml or 5 ml columns

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

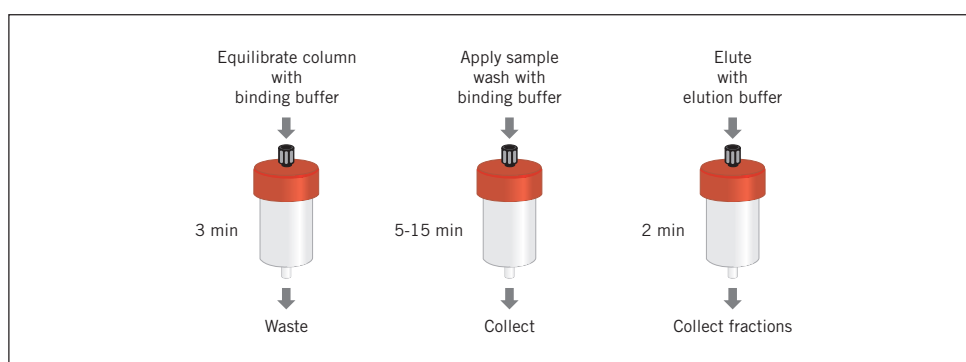


Fig. 2. Simple purification of GST fusion proteins using GSTrap FF.



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 FF columns (1 ml or 5 ml) can be connected in series to increase binding capacity and hence scale of purification. Larger columns can be packed with Glutathione Sepharose 4 Fast Flow (see Appendix 6 for column packing).

### Sample and buffer preparation



Use high quality water and chemicals. Filtration through 0.45  $\mu\text{m}$  filters is recommended.



Samples should be centrifuged immediately before use and/or filtered through a 0.45  $\mu\text{m}$  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<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3).

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



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

1. Fill the syringe with binding buffer.
2. Connect the column to the syringe using the adapter 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.\*

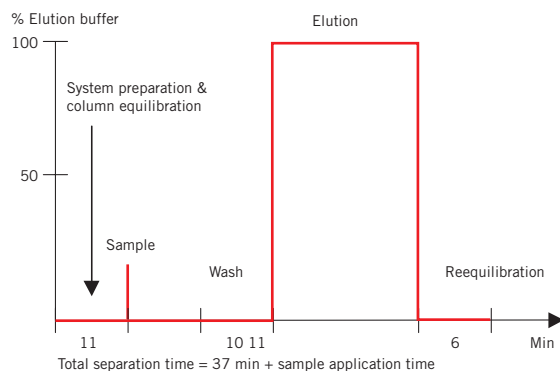
\* One ml/min corresponds to approximately 30 drops/min when using a syringe with a HiTrap 1 ml column and five ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column.



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

## Alternative 2. Simple purification with ÄKTAprime

ÄKTAprime contains a pre-programmed template for purification of GST fusion proteins using a single GSTrap FF column, as shown below. This provides a standard purification protocol which can be followed exactly or optimized as required.



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 AKTAp<sub>ri</sub>me cue card (Code No. 18-1138-06).
2. Select the Application Template.
3. Start the method.
4. Enter the sample volume and press OK to start.



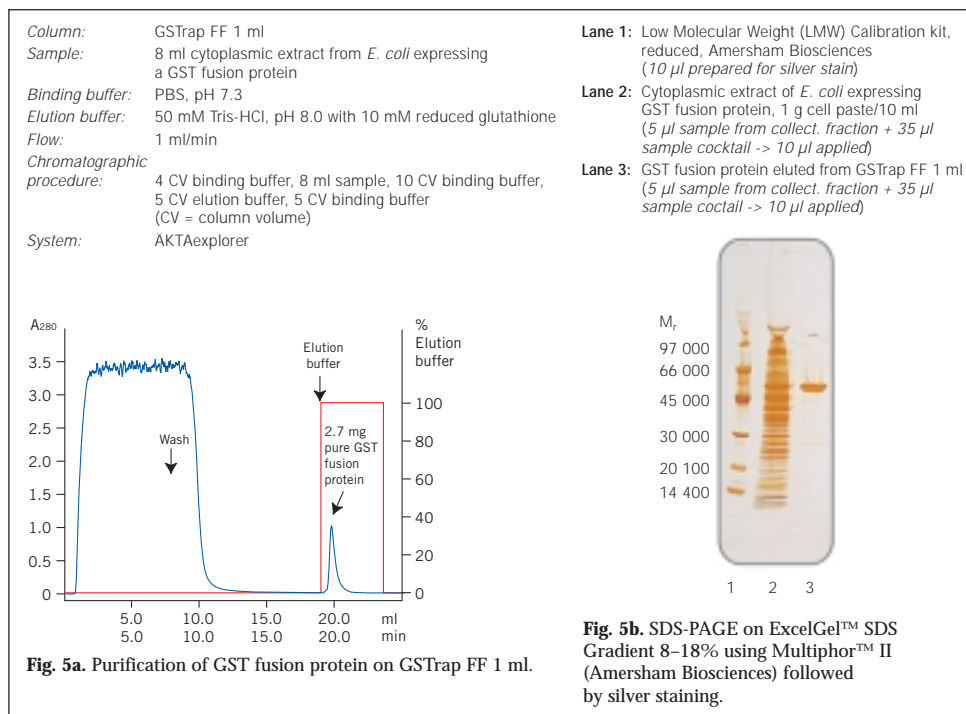
Connecting the column.



Preparing the fraction collector.

**Fig. 4.** Typical procedures when using AKTAp<sub>ri</sub>me.

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



## Troubleshooting

See *Purification and Detection Troubleshooting* page 28.



## Optimization of GST fusion protein purification

Following the instructions supplied for each prepacked GStrap FF 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 to remove the dimers. A column prepacked with Superdex™ will give the highest possible resolution between two molecules of 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 based on the properties of the protein of interest (and not the GST tag) 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  $\mu$ l of 6X SDS loading buffer to 5–10  $\mu$ l 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 (Coomassie Blue R Tablets) or silver (PlusOne Silver Staining Kit, Protein).



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 ( $M_r \times 10^{-3}$ )
<b>Single percentage:</b>	
5%	36–200
7.5%	24–200
10%	14–200
12.5%	14–100
15%	14–60 <sup>1</sup>
<b>Gradient:</b>	
5–15%	14–200
5–20%	10–200
10–20%	10–150

<sup>1</sup>The larger proteins fail to move significantly into the gel.

**Table 12.**



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



### 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.



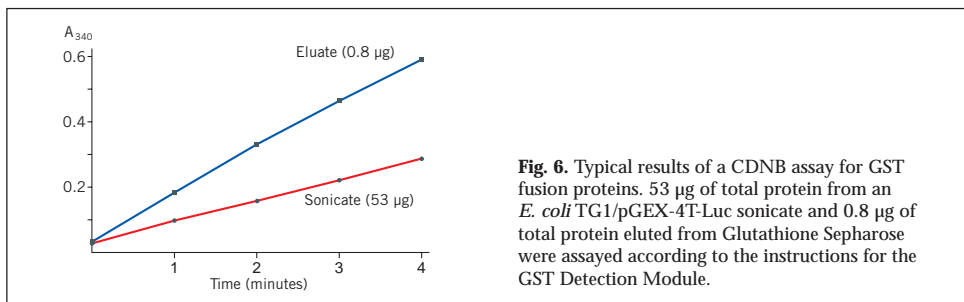
Transformants expressing the fusion protein will be identified by the absence from total cellular proteins of the parental  $M_r$  29 000 GST and by the presence of a novel, larger fusion protein. Parental pGEX vectors produce a 29 kDa GST fusion protein containing amino acids that are coded for by the pGEX multiple cloning site. In some cases both the  $M_r$  29 000 GST and fusion protein may be present. This can be caused by translational pausing at the junction between GST and the fusion partner or a mixed culture between cells with parental plasmid and cells with fusion plasmid.



Interpretation is sometimes complicated when fusion proteins break down and release the GST moiety  $M_r$  26 000. Such cases are usually recognized by the appearance of the  $M_r$  26 000 species, and a 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 is also supplied 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.

- Transfer 500  $\mu\text{l}$  volumes of the above solution into two UV-transparent cuvettes. Add sample (5–50  $\mu\text{l}$ ) to the "sample cuvette". To the "blank cuvette", add a volume of 1X reaction buffer equal to the sample volume in the sample cuvette.
- Cover each cuvette with wax film and invert to mix.
- 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.
- 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.
- 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

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



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



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.

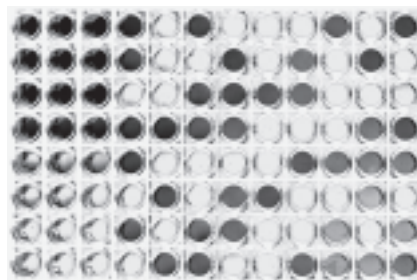


### Troubleshooting

- 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  $\Delta A_{340}$ /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<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 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.

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

### 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 well strips in the pouch with the desiccant 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<sub>450</sub>) or 2',2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS™) (A<sub>410</sub>) have been used successfully.

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

### Troubleshooting

See also *Purification and Detection Troubleshooting* page 28.





### 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.

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<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 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 Biosciences has been cross-adsorbed with *E. coli* proteins, low levels of cross-reacting antibodies may remain. It is recommended always to run samples of *E. coli* sonicates that do not contain a recombinant pGEX plasmid and samples that contain the parental pGEX plasmid as controls.

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 or 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 Biosciences.

### 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 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  $\mu$ l of antibody to 50 ml of buffer. Refer to Amersham Biosciences 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 Biosciences 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.



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

## Troubleshooting

*See also Purification and Detection Troubleshooting page 28.*



### Multiple bands seen on Western blot analysis

- Anti-GST antibody from Amersham Biosciences has been cross-absorbed against *E. coli* proteins and tested for its lack of non-specific background binding in a Western blot. 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. This *E. coli* must not contain the pGEX plasmid.

## 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.
- 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.
- 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  $6.5 < \text{pH} < 8.0$  (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 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  $+4\text{ }^{\circ}\text{C}$  and limit the number of washes.
- Column capacity may have been exceeded. If using GSTrap FF columns (1 ml or 5 ml) link 2 or 3 columns in series to increase capacity or pack a larger column.
- Fusion protein may be in the inclusion bodies, although using a GST-tag reduces the chance of this problem occurring.



### 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.
- Use prepacked GSTrap FF 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 (see Chapter 9). 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).



Serine protease inhibitors must be removed prior to cleavage by thrombin or Factor Xa. Use HiTrap Benzamidine FF (high sub) (see page 39).



### $M_r$ 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 *dnaK* 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 Biosciences and (<http://bionet.hgmp.mrc.ac.uk/hypermil/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 7). 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 8). The protease used for cleavage can be removed using Benzamidine Sepharose, a purification medium with a high specificity for serine proteases (see page 39).



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. For the removal of thrombin and Factor Xa, a GStrap FF and a HiTrap Benzamidine FF (high sub) column can be connected in series so that cleaved product passes directly from the GStrap FF into the HiTrap Benzamidine FF (high sub). Samples are cleaved and proteases removed in a single step (see page 39).



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 <sub>r</sub> 46 000
Bovine thrombin	M <sub>r</sub> 37 000
Bovine Factor Xa	M <sub>r</sub> 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 (the inhibitors will usually be eluted in the flow-through when sample is loaded onto a GStrap FF column).

Enzyme	Inhibitor
PreScission protease	100 mM ZnCl <sub>2</sub> (> 50% inhibition) 100 µM chymostatin 4 mM Pefabloc
Factor Xa and thrombin	AEBSF, APMSF, antithrombin III, Antipain, α <sub>1</sub> -antitrypsin, aprotinin, chymostatin, hirudin, leupeptin, PMSF
Factor Xa only	Pefabloc FXa
Thrombin only	Pefabloc TH Benzamidine

Cleavage of fusion proteins is most commonly performed on milligram quantities of fusion protein suitable for purification on GStrap FF. The following protocols describe a manual cleavage and purification using a syringe and a 1 ml GStrap FF 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 FF 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.

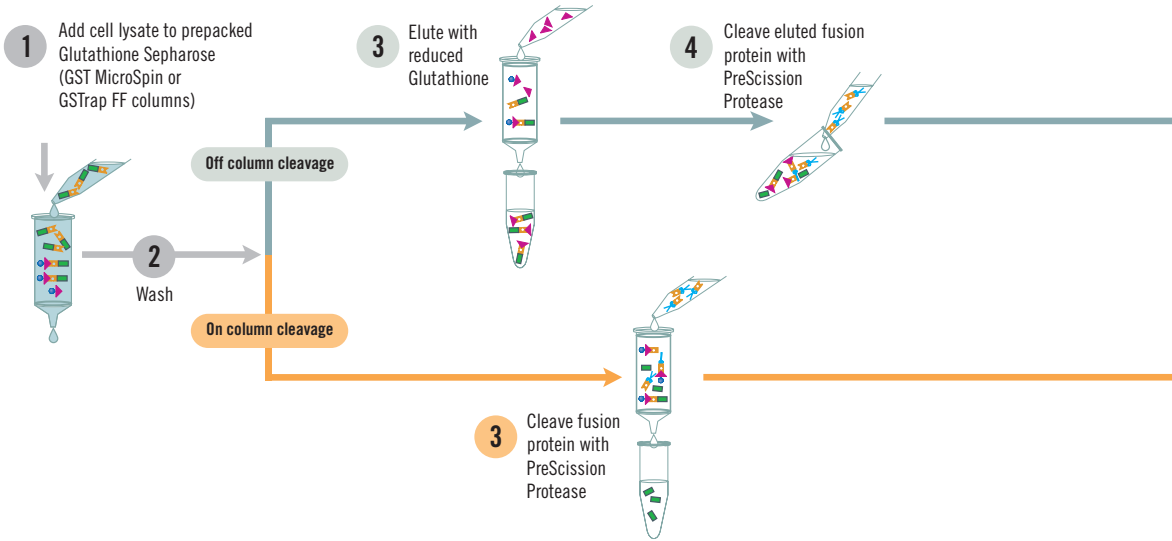


Cleavage should be complete following a 4 hour treatment at +5 °C with at least 10 units/mg of fusion protein.



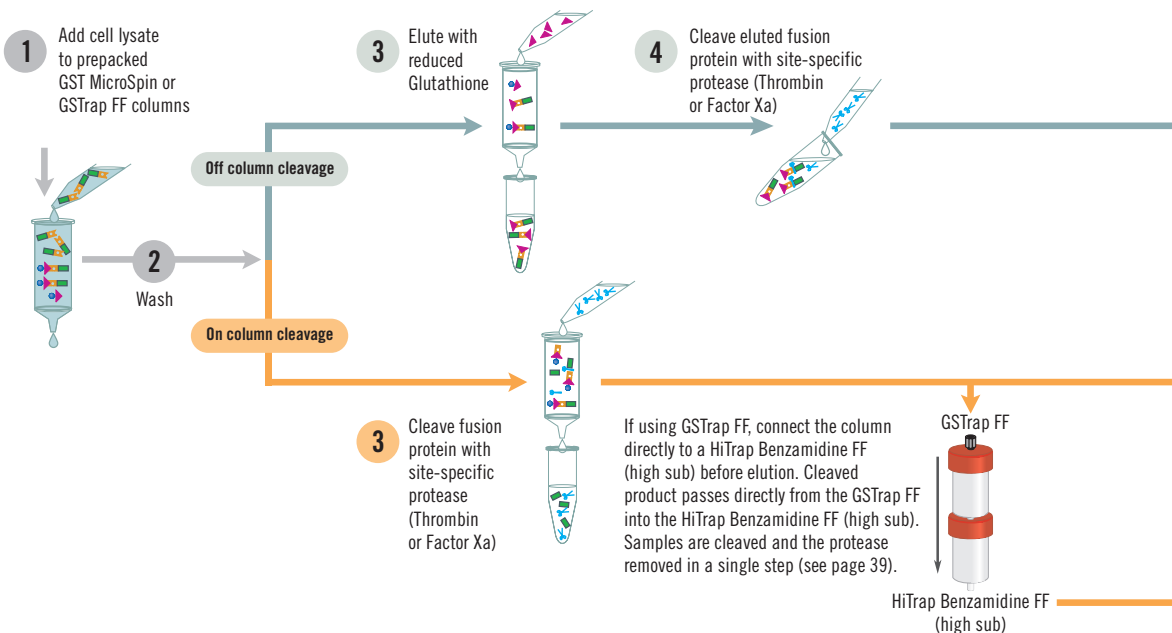
Incubation times may be reduced by adding a greater amount of PreScission Protease.  
*(continued on page 34)*

## Cleavage of GST tag using PreScission Protease

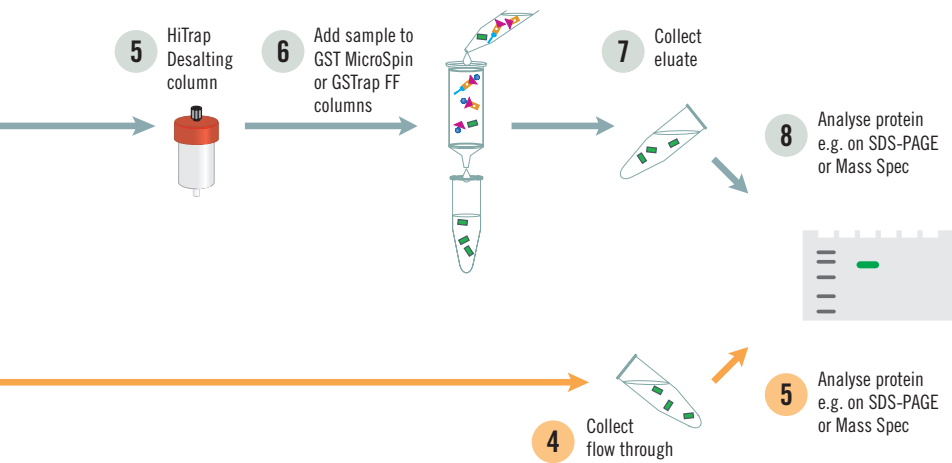


**Fig. 7.** Flow chart of the affinity purification procedure and PreScission Protease cleavage of glutathione S-transferase fusion proteins.

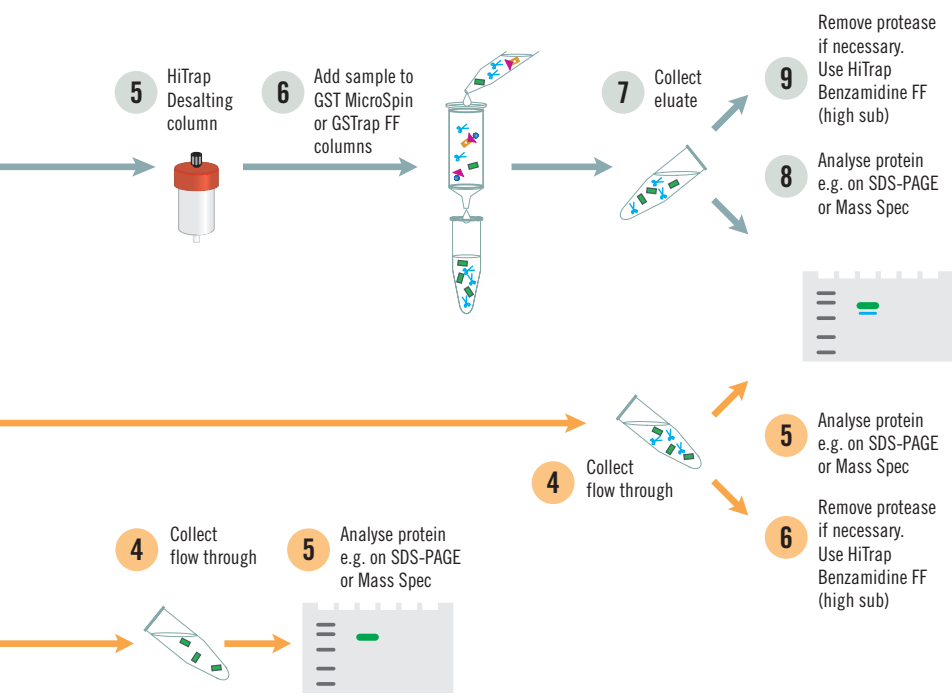
## Cleavage of GST tag using Thrombin or Factor Xa










**Fig. 8.** Flow chart of the affinity purification procedure and Thrombin or Factor Xa cleavage of glutathione S-transferase fusion proteins.



Ettan™ MALDI-ToF mass spectrometer



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

## Alternative 1. On-column cleavage and purification

*Assume: 8 mg GST fusion protein bound/ml gel, using GStrap FF 1 ml column (adjust volumes for larger columns)*

1. Fill the syringe with binding buffer.
2. Connect the column to the syringe using the adapter 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  $\mu$ l (160 units) of PreScission Protease with 920  $\mu$ l of PreScission cleavage buffer at +5 °C.
9. Load the PreScission Protease mix onto the column using a syringe and the adapter supplied. Seal the column with the top cap and the domed nut supplied.
10. Incubate the column at +5 °C for 4 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 FF.*

## Alternative 2. Off-column cleavage and purification

*Assume: 8 mg GST fusion protein bound/ml gel, using GStrap FF 1 ml column (adjust volumes for larger columns)*

1. Follow steps 1–6 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  $\mu$ l (2 units) of PreScission Protease for each 100  $\mu$ g of fusion protein in the eluate. If the amount of fusion protein in the eluate has not been determined, add 80  $\mu$ l (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 FF column to remove the GST moiety of the fusion protein and the PreScission Protease.

*NB. 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<sup>2+</sup> 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<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3.

Thrombin Solution: Dissolve 500 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 > 7 500 units/mg protein one unit of thrombin will digest ≥ 90% of 100 µg of a test fusion protein in 16 hours at +22 °C in elution buffer. A unit is approximately equal to 0.2 NIH units.



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



Thrombin can be removed using Benzamidine Sepharose, a purification medium with a high specificity for serine proteases (see page 39). A GStrap FF and a HiTrap Benzamidine FF (high sub) column can be connected in series so that cleaved product passes directly from the GStrap FF into the HiTrap Benzamidine FF (high sub). Samples are cleaved and the thrombin removed in a single step (see page 39).

## Alternative 1. On-column cleavage and purification

Assume: 8 mg GST fusion protein bound/ml gel, using GStrap FF 1 ml column (adjust volumes for larger columns)

1. Follow steps 1–6 under on-column Prescission Protease cleavage.
2. Prepare the thrombin mix: mix 80  $\mu$ l thrombin solution (1 unit/ $\mu$ l) with 920  $\mu$ l PBS.
3. Load the thrombin mix onto the column using a syringe and the adapter supplied. Seal the column with the top cap and the domed nut supplied.
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. 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 FF.*

## Alternative 2. Off-column cleavage and purification

Assume: 8 mg GST fusion protein bound/ml gel, using GStrap FF 1 ml column (adjust volumes for larger columns)

1. Follow steps 1–6 under on-column Prescission Protease cleavage.
2. Elute with 5–10 column volumes of elution buffer. Maintain flow rates of 1–2 ml/min during elution.
3. Add 10  $\mu$ l (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  $\mu$ l (80 units) thrombin solution.
4. Incubate at room temperature (+22 to +25 °C) for 2–16 hours.
5. Once digestion is complete, remove the reduced glutathione using a quick buffer exchange on HiTrap Desalting or HiPrep 26/10 Desalting depending on the sample volume.
6. Apply the sample to an equilibrated GStrap FF column to remove the GST moiety of the fusion protein.

*N.B. 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 +22 to +25 °C and increase the amount of thrombin used in the reaction.
- Check the DNA sequence of the construct to verify the presence of the thrombin site. Verify that the thrombin site has not been altered.
- Check that protease inhibitors have been removed.



### Multiple bands seen on SDS-PAGE analysis 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 additional thrombin recognition sites. Optimum cleavage sites for thrombin are given in 1) and 2) below. Ref: Chang, J-Y., *Eur. J. Biochem.* **151**, 217 (1985).

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

Examples:

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

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

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

Examples:

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

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

## Factor Xa cleavage and purification

### Enzymatic cleavage

Factor Xa cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, pH 7.5.

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

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



With a specific activity of > 800 units/mg protein, one unit of Factor Xa will digest ≥ 90% of 100 μg of a test fusion protein in 16 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 at least 1% (w/w).



Factor Xa can be removed using Benzamidine Sepharose, a purification medium with a high specificity for serine proteases (see page 39). A GStrap FF and a HiTrap Benzamidine FF (high sub) column can be connected in series so that cleaved product passes directly from the GStrap FF into the HiTrap Benzamidine FF (high sub). Samples are cleaved and the Factor Xa removed in a single step (see page 39). Heparin Sepharose has been used for this application but, since benzamidine has a higher specificity for Factor Xa, the protease will be removed more effectively.



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

*Assume: 8 mg GST fusion protein bound/ml gel, using GStrap FF 1 ml column (adjust volumes for larger columns)*

1. Follow steps 1–6 under on-column PreScission Protease cleavage.
2. Wash GStrap FF with 10 column volumes of Factor Xa cleavage buffer.
3. Prepare the Factor Xa mix: Mix 80  $\mu$ l Factor Xa solution with 920  $\mu$ l Factor Xa cleavage buffer.
4. Load the Factor Xa mix onto the column using a syringe and the adapter supplied. Seal the column with the top cap and the domed nut.
5. Incubate the column at room temperature (+22 to +25 °C) for 2–16 hours.
6. Fill a syringe with 3 column volumes of Factor Xa 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 and Factor Xa, while the GST moiety of the fusion protein will remain bound to GStrap FF.*

### Alternative 2. Off-column cleavage and purification

*Assume: 8 mg GST fusion protein bound/ml gel, using GStrap FF 1 ml column (adjust volumes for larger columns)*

1. Follow steps 1–6 under on-column PreScission Protease cleavage.
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 sample volume.
4. 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  $\mu$ l (80 units) of Factor Xa solution.
5. Incubate the column at room temperature (+22 to +25 °C) for 2–16 hours.
6. Once digestion is complete, apply the sample to an equilibrated GStrap FF 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

- Factor Xa requires activation of Factor X with Russell's viper venom. Factor Xa from Amersham Biosciences has been preactivated, but other sources may not be activated. 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<sub>2</sub> at +37 °C for 5 minutes.
- Check the DNA sequence of the construct to verify the presence of the Factor Xa site. Verify that the Factor Xa site has not been altered. The recognition sequence for Factor Xa is Ile-Glu-Gly-Arg↓X, where X can be any amino acid except Arg or Pro.
- Check that the Factor Xa to fusion protein ratio is correct.
- Check that glutathione has been removed as recommended.

- In some cases increasing substrate concentration up to 1 mg/ml may improve results.
- Add  $\leq 0.5\%$  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 increase the amount of Factor Xa (for some fusion proteins, Factor Xa can be increased up to 5%).
- Check that protease inhibitors have been removed.



### Multiple bands on SDS-PAGE gel 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 cleavage at the desired Factor Xa site. If adjustment of conditions does not correct the problem, reclon the insert into a pGEX-6P (PreScission) or pGEX T (thrombin) expression vector.

### Removal of thrombin, Factor Xa or other serine proteases

Before enzymatic cleavage it may be necessary to remove proteases from the sample and after enzymatic cleavage it may be necessary to remove thrombin or Factor Xa. Benzamidine 4 Fast Flow (high sub) provides a convenient and highly specific medium for the removal of serine proteases not only from enzymatic digests, but also from cell culture supernatant, bacterial lysate or serum.

### Purification Options

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

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

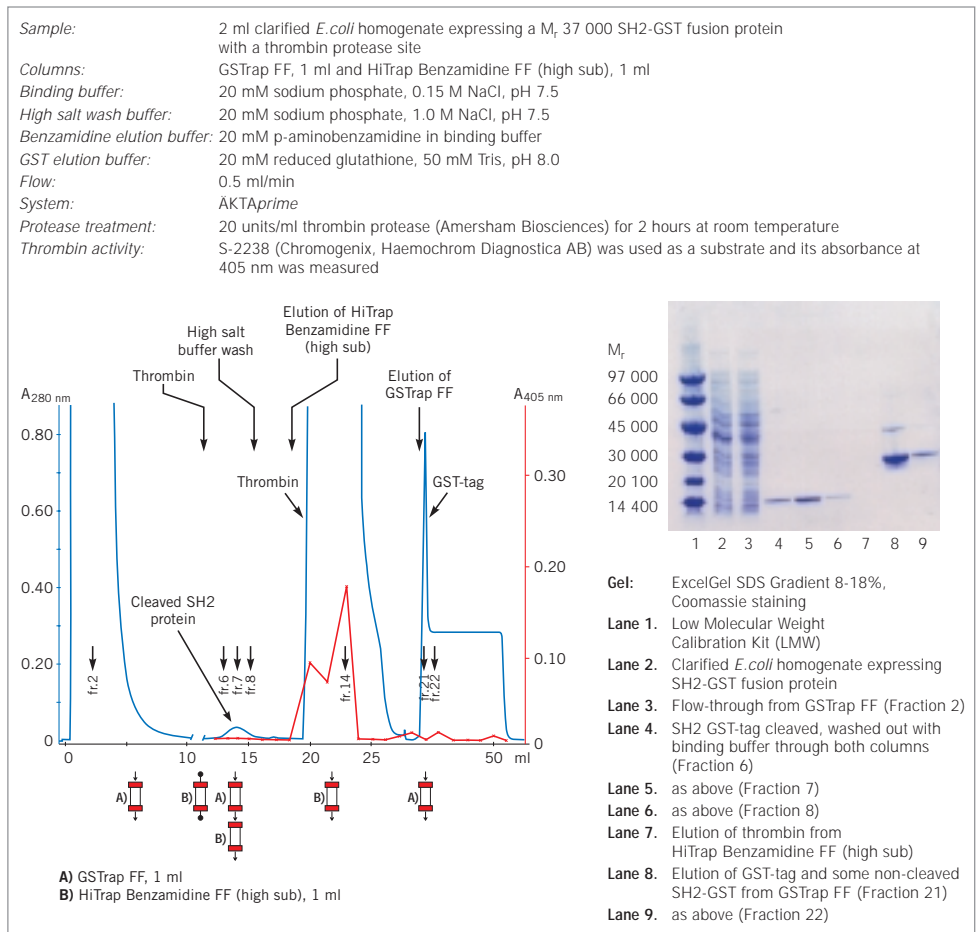
Elution buffer alternatives:

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

1. Equilibrate the column with 5 column volumes of binding buffer.
2. Apply the sample.
3. Wash with 5–10 column volumes of binding buffer or until no material appears in the effluent (monitored by UV absorption at  $A_{280\text{ nm}}$ ).
4. Elute with 5–10 column volumes of elution buffer. Collect fractions in neutralization buffer\*. The purified fractions can be buffer exchanged using desalting columns (see Chapter 7).

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

Figure 9 shows the isolation of a GST fusion protein on a GStrap FF column and on-column cleavage with thrombin (as described previously). After the thrombin incubation a HiTrap Benzamidine FF (high sub) column is placed in series below the GStrap FF column. As binding buffers are passed through the two columns the cleaved fusion protein and thrombin are washed from the GStrap FF column onto the HiTrap Benzamidine FF (high sub) column. This column binds the thrombin enabling the collection of pure thrombin-free protein in the eluent.



**Fig. 9.** On-column cleavage of a GST fusion protein and removal of thrombin after on-column cleavage, using GStrap FF and HiTrap Benzamidine FF (high sub).

# CHAPTER 4

## (His)<sub>6</sub> 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)<sub>6</sub> 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)<sub>6</sub> fusion proteins, including purification under denaturing conditions. On-column purification and refolding of (His)<sub>6</sub> fusion proteins is described in Chapter 5.

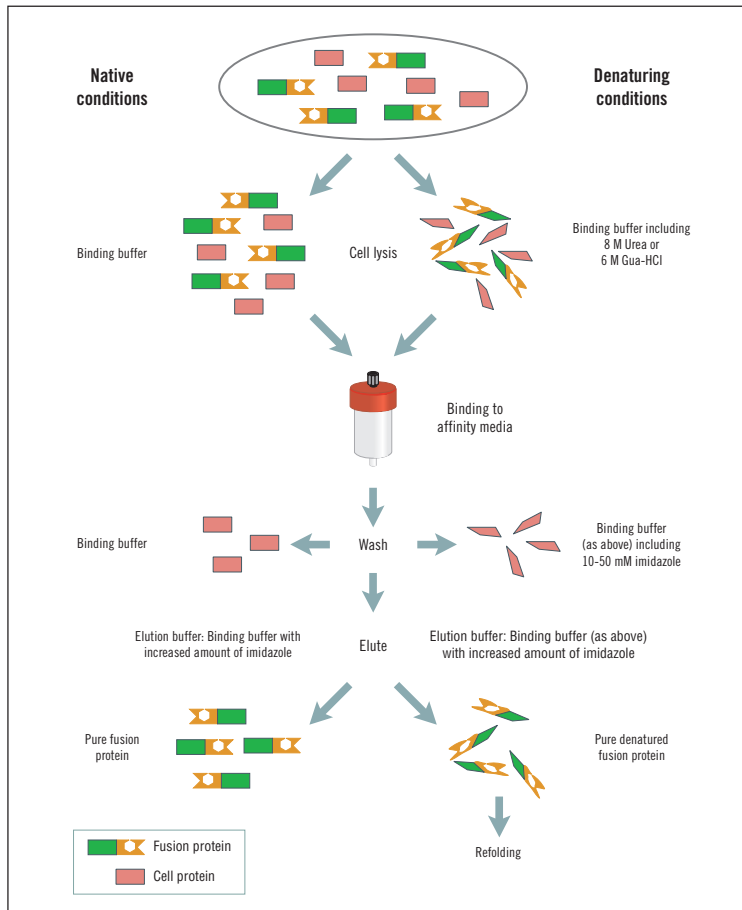


Fig. 10.

For simple, one step purification of (His)<sub>6</sub> fusion proteins, there is a range of products designed to meet specific purification needs, as shown in Table 13. These products can also be used for the purification of fusion proteins containing shorter or longer polyhistidine tags, such as (His)<sub>4</sub> or (His)<sub>10</sub>. Under the standard binding and elution conditions described in this handbook, the shorter (His)<sub>4</sub> will bind more weakly and the longer (His)<sub>10</sub> will bind more strongly as compared to (His)<sub>6</sub>. This difference in binding strength can be used to advantage during purification. For example, since (His)<sub>10</sub> binds more strongly, a higher concentration of imidazole can be used during the washing step before elution. This can facilitate the removal of contaminants which may otherwise be co-purified with a (His)<sub>6</sub> fusion protein.

Chelating Sepharose, when charged with Ni<sup>2+</sup> ions, selectively retains proteins if complex-forming amino acid residues, in particular histidine, are exposed on the protein surface. (His)<sub>6</sub> fusion proteins can be easily desorbed with buffers containing imidazole.



If the (His)<sub>6</sub> fusion proteins are expressed as inclusion bodies, see Chapter 5 for solubilization, refolding and purification information.

Column (prepacked) or Media**	Amount of protein for a single purification	Comment
His MicroSpin Purification Module	Up to 100 µg	Ready to use, prepacked columns, buffers and chemicals High throughput when used with MicroPlex 24 Vacuum (up to 48 samples simultaneously)
HiTrap Chelating HP 1 ml	Up to 12 mg*	Prepacked column, ready to use
HiTrap Kit	Up to 12 mg/column*	As above, but includes buffers for up to 12 purifications using a syringe
HiTrap Chelating HP 5 ml	Up to 60 mg*	Prepacked column, ready to use
Chelating Sepharose Fast Flow	12 mg (His) <sub>6</sub> fusion protein* per ml  *estimate for a protein of M <sub>r</sub> 27 600, binding capacity varies according to specific protein	For packing columns and scale up

**Table 13.** Summary of purification options for (His)<sub>6</sub> fusion proteins.

\*\*Characteristics of HiTrap Chelating HP 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 prepacked 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 HP columns are ready to use with a syringe, a peristaltic pump or a chromatography system.
- To increase capacity use several HiTrap Chelating HP 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<sup>prime</sup> 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 ÄKTA<sup>design</sup> 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<sub>2</sub>HPO<sub>4</sub>, 18 mM KH<sub>2</sub>PO<sub>4</sub>, 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<sub>20</sub> 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<sub>400</sub> 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  $\mu$ l of sample 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 water vacuum).
- Side arm flask, 500 ml or 1 litre.
- Single or double hole rubber stop.
- Vacuum tubing.
- MicroPlex 24 Vacuum apparatus (one or two).

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  $\mu$ l of lysate to the column.



If using < 400  $\mu$ l adjust volume to 400  $\mu$ l 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  $\mu$ l of PNI<sub>20</sub> 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. Additional 600  $\mu$ l washes with PI<sub>20</sub> may be performed if desired. Washes with higher concentrations of imidazole may remove impurities, but decrease yield of the fusion protein.
12. Add 200  $\mu$ l of PNI<sub>400</sub> 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.

*Note: Yields of fusion protein may be increased by repeating the elution step two or three times and pooling the eluates.*

## Troubleshooting

See *Purification and Detection Troubleshooting* page 56.





## Alternative 2. Purification of multiple samples using a microcentrifuge

Do not apply more than 400  $\mu\text{l}$  of sample 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  $\mu\text{l}$  of lysate to the column.



If using < 400  $\mu\text{l}$  adjust volume to 400  $\mu\text{l}$  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 g.
5. Remove the MicroSpin columns and place into a clean 1.5 or 2 ml microcentrifuge tube.
6. Apply 600  $\mu\text{l}$  of PNI<sub>20</sub> wash buffer to each column. Repeat spin procedure. Additional 600  $\mu\text{l}$  washes with PNI<sub>20</sub> may be performed if desired. Washes with higher concentrations of imidazole may remove impurities, but decrease yield of the fusion protein.
7. Replace the bottom closure on each column. Add 100–200  $\mu\text{l}$  of PNI<sub>400</sub> 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.

*Note: Yields of fusion protein may be increased by repeating the elution step two or three times and pooling the eluates.*



## Troubleshooting

See *Purification and Detection Troubleshooting* page 56.



### Alternative 3. Purification using MicroPlex Centrifugation

Do not apply more than 400  $\mu\text{l}$  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  $\mu\text{l}$  of lysate to each column.



If using < 400  $\mu\text{l}$  adjust volume to 400  $\mu\text{l}$  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  $\mu\text{l}$  of  $\text{PNI}_{20}$  wash buffer to each column.
8. Centrifuge the unit for 2 minutes following the instructions supplied. Remove the manifold from each collection tray and place on a clean paper towel. Reassemble each unit with a fresh collection tray. Additional 600  $\mu\text{l}$  washes with  $\text{PI}_{20}$  may be performed if desired. Washes with higher concentrations of imidazole may remove impurities, but decrease yield of the fusion protein.
9. Add 100–200  $\mu\text{l}$  of  $\text{PNI}_{400}$  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.

*Note: Yields of fusion protein may be increased by repeating the elution step two or three times and pooling the eluates.*



### Troubleshooting

See *Purification and Detection Troubleshooting* page 56.

## Purification using HiTrap Chelating HP 1 ml or 5 ml columns

HiTrap Chelating HP columns are prepacked and ready for use. They can be operated with a syringe, a peristaltic pump or a liquid chromatography system such as ÄKTA<sup>prime</sup>. Figure 11 illustrates the simple steps required for purification of a (His)<sub>6</sub> fusion protein.

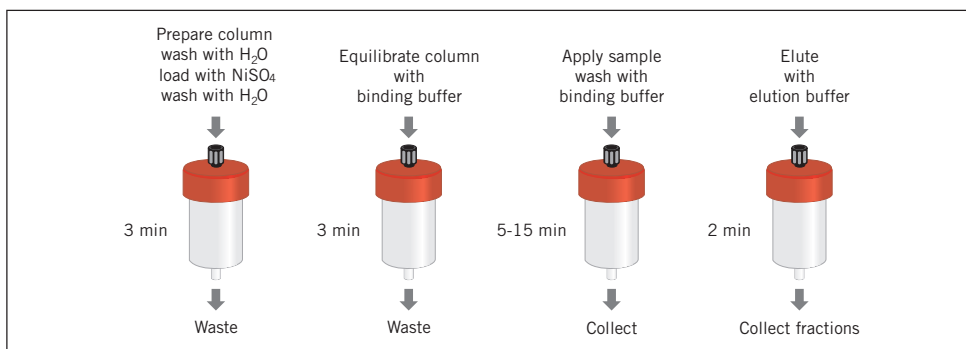


Fig. 11. HiTrap Chelating HP (1 ml) and a schematic overview of (His)<sub>6</sub> fusion protein purification.



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 HP 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 guanidine 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)<sub>6</sub> fusion protein, conditions may need to be optimized.

## Alternative 1. Purification using HisTrap Kit

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



- HiTrap Chelating HP 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<sub>4</sub> 10 ml
- Syringe 5 ml
- All necessary connectors

### Purification protocols

#### Column preparation: loading with nickel ions

1. Fill syringe with distilled water. Remove stopper and connect the column to the syringe with the adapter 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.



Use water, not buffer, to wash away the storage solution (precipitation of nickel salts may occur if the nickel solution comes into contact with the 20% ethanol in the storage solution). If air is trapped in the column, wash the column with distilled water until the air disappears.

4. Disconnect syringe 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, using the syringe.

The column is now ready for use. When high yield is more important than highest purity, follow Protocol A on page 49. To achieve the highest purity, follow Protocol B on page 50.

## A. Basic protocol for high yield

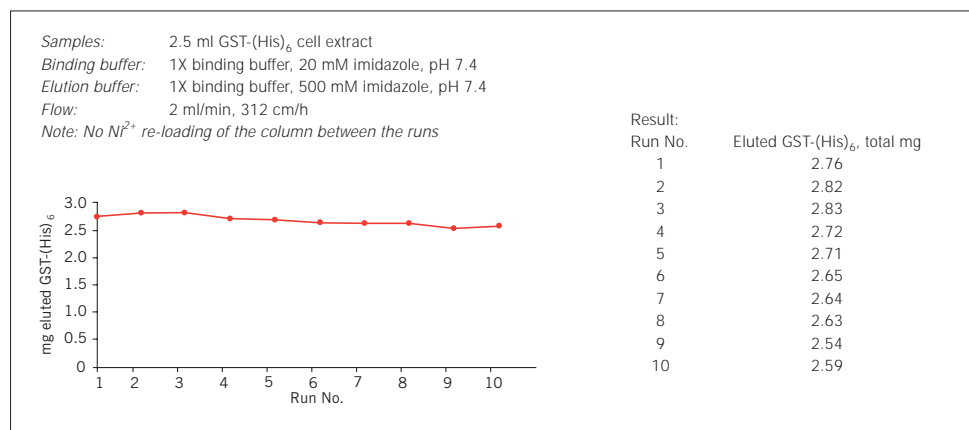
In all steps use the syringe supplied. Note that 1 ml/min corresponds to approximately 30 drops/min when using a syringe with a HiTrap 1 ml column and 5 ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column.

1. Prepare 24 ml binding 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 contains 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 contains 20 mM phosphate, 0.5 M NaCl and 500 mM imidazole.
3. Equilibrate the column with 10 ml binding buffer. Ensure that the column has been loaded with nickel ions before this step.
4. Apply sample at a flow rate 1–3 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 binding buffer. Collect wash fraction.
6. Elute with 5 ml elution buffer. Avoid dilution of the purified product 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 the 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 binding buffer. The column is now ready for a new purification if the same (His)<sub>6</sub> protein is to be purified and there is rarely a need to reload with metal (see Figure 12).



**Fig. 12.** 10 repetitive purifications of GST-(His)<sub>6</sub> without reloading the HiTrap Chelating HP 1 ml column with Ni<sup>2+</sup> between the runs.

## Troubleshooting



See *Purification and Detection Troubleshooting* page 56.

## B. Optimization protocol for high purity

In all steps use the syringe supplied. Note that 1 ml/min corresponds to approximately 30 drops/min when using a syringe with a HiTrap 1 ml column and 5 ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column.

1. Prepare buffers according to Table 14. Use 1X phosphate buffer including 10 mM imidazole as binding 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 (mM)	Phosphate buffer 8X stock solution pH 7.4 (ml)	2 M Imidazole pH 7.4 (ml)	Final volume (ml)*
10	3.0	0.12	24
20	1.0	0.08	8
40	1.0	0.16	8
60	1.0	0.24	8
100	1.0	0.40	8
300	1.0	1.20	8
500	1.0	2.00	8

\*Use distilled water to adjust to final volume

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

2. Load the column with nickel ions according to "Column preparation" (page 48).
3. Equilibrate the column with 10 ml binding buffer (1X phosphate buffer, 10 mM imidazole, pH 7.4).
4. Apply the sample. Collect the flow-through fraction.
5. Wash with 10 ml binding 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 binding buffer.

From the results select the optimal binding and elution buffers. The optimum elution buffer is the one which eluted the (His)<sub>6</sub> fusion protein. The optimum binding buffer is the elution buffer used in the step before, with a lower concentration of imidazole. Using the highest possible concentration of imidazole in the binding buffer will give the highest purity. These buffers can be used for any purification of an identical protein.



Imidazole absorbs at 280 nm. Use elution buffer as the 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 binding buffer followed by 5 column volumes elution buffer. Re-equilibrate the column with 5–10 column volumes of binding buffer before sample application.



For large sample volumes a simple peristaltic pump or an ÄKTA design chromatography system can be used to apply sample and buffers.

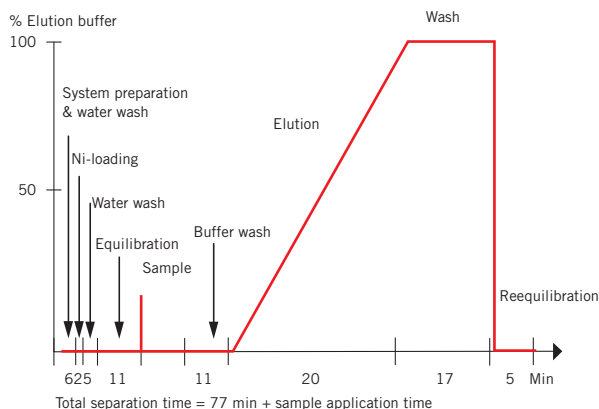
### Troubleshooting



See *Purification and Detection Troubleshooting* page 56.

## Alternative 2. Simple purification with ÄKTAprime

ÄKTAprime contains a pre-programmed template for automatic column preparation and purification of (His)<sub>6</sub> fusion proteins on a single HiTrap Chelating HP column, as shown below. This provides a standard purification protocol which can be followed exactly or optimized as required.



Binding 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<sub>4</sub>  
Prepare at least 500 ml of each buffer.

1. Follow the instructions supplied on ÄKTAprime cue card Code No: 18-1138-02.
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. 13.** Typical procedures when using ÄKTAprime.

**Sample:** 5 ml cytoplasmic extract containing (His)-tagged Glutathione S-transferase, GST-(His)<sub>6</sub>. The clone was a kind gift from Dr. J. Lidholm, Amersham Biosciences, Sweden

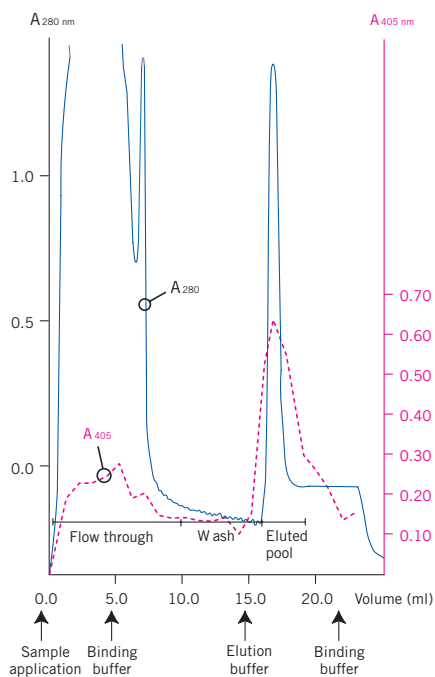
**Column:** HiTrap Chelating HP 1 ml, Ni<sup>2+</sup>-loaded according to the instructions

**Binding buffer:** 1X phosphate buffer, 20 mM imidazole, pH 7.4

**Elution buffer:** 1X phosphate buffer, 500 mM imidazole, pH 7.4

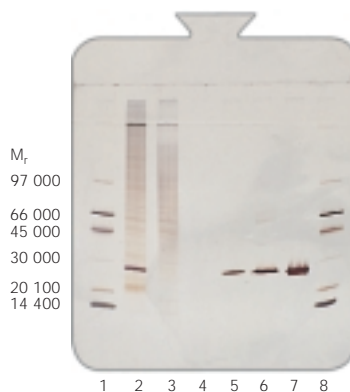
**Flow:** 2 ml/min, 312 cm/h

**Result:** Eluted GST-(His)<sub>6</sub>, 4 ml, A<sub>280</sub>: 1.65  
Total amount: 4.46 mg



**Fig. 14a.** 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)<sub>6</sub>, dil 1:20  
**Lane 6:** Eluted GST-(His)<sub>6</sub>, dil 1:10  
**Lane 7:** GST standard, 0.5 mg/ml  
**Lane 8:** LMW



**Fig. 14b.** SDS electrophoresis on PhastSystem™ using PhastGel™ 10–15, silver staining.

**Fig. 14.**

Figure 14 shows an example of the purification of a (His)<sub>6</sub> fusion protein using a HiTrap Chelating HP 1 ml column.

## Troubleshooting

See *Purification and Detection Troubleshooting* page 56.



An example of the purification and refolding of an insoluble (His)<sub>6</sub> fusion protein is shown on page 61 and clearly demonstrates the advantage of using a chromatography system for this type of work.

## Detection of (His)<sub>6</sub> fusion proteins

Table 15 reviews the methods available for detection of (His)<sub>6</sub> 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 anti-His antibody	Highly specific, detects only (His) <sub>6</sub> fusion protein.
Western blot analysis using anti-His antibody and ECL detection systems	Highly specific, detects only (His) <sub>6</sub> 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) <sub>6</sub> fusion protein is active. Not always available. May require development and optimization.

**Table 15.** Detection methods for (His)<sub>6</sub> 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 (Coomassie Blue R Tablets) or silver (PlusOne Silver Staining Kit, Protein).



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 <sub>r</sub> x 10 <sup>-3</sup> )
<b>Single percentage:</b>	
5%	36–200
7.5%	24–200
10%	14–200
12.5%	14–100
15%	14–60 <sup>1</sup>
<b>Gradient:</b>	
5–15%	14–200
5–20%	10–200
10–20%	10–150

<sup>1</sup>The larger proteins fail to move significantly into the gel.

**Table 16.**



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.



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



### 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.

### 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<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 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 antibody to mouse Ig, HRP-linked Whole Ab, NA931).

1. Separate the protein samples by SDS-PAGE.



Anti-His antibody from Amersham Biosciences 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)<sub>6</sub> 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 or 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 the *Hybond ECL instruction manual* from Amersham Biosciences.

### 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  $\mu$ l of antibody to 50 ml of buffer. Refer to Amersham Biosciences 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 Biosciences 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.



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



## Troubleshooting

See also *Purification and Detection Troubleshooting* page 56.



## Multiple bands seen on Western blot analysis

Anti-His antibody from Amersham Biosciences 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. This *E. coli* should not contain a His-tag encoding plasmid.

## 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

- Solubilizing agents include: 2 M NaCl, 50 mM CHAPS, 50% glycerol, 8 M urea, 6 M guanidine hydrochloride, 0.1–2% Triton X-100, 0.1–2% Tween 20. Mix gently for 30 minutes. Reducing agents such as 2-mercaptoethanol may also help the solubilization process.



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



### Fusion protein does not bind to the 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.
- 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 in the sample.
- If re-using a 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 prepacked 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 guanidine hydrochloride and imidazole concentrations in the range 10–500 mM, pH 7.4.
- Tag may be degraded. Check that tag is not associated with any part of the protein of interest.
- Column capacity is exceeded. Join 2 or 3 HiTrap Chelating HP 1 ml or 5 ml columns in series or use an even larger column.
- Fusion protein may be in inclusion bodies. Add solubilization agents.
- Column may not have been correctly charged with nickel ions. Repeat column preparation steps.



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



Do not go below pH 3.5, as metal ions will be stripped off the column.



### **Purification needs to be optimized**

- Following the instructions supplied with HiTrap Chelating HP 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 binding and elution buffers: Follow instructions on page 50 (HiTrap Kit protocols). Higher concentrations of imidazole in the binding buffer will remove more impurities from the column resulting in a higher purity of fusion protein in the elution buffer.
- Yields of (His)<sub>6</sub> 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.



Increasing the purity by increasing the imidazole concentration may decrease the yield of protein as some of the (His)<sub>6</sub> fusion proteins may be washed away. Always determine the optimum combination of purity and yield for the specific application.



### **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.
- Over-sonication can lead to co-purification of host proteins with the fusion protein. Check conditions as described earlier. Avoid frothing. Decrease sonication times. Add lysozyme.
- Follow optimization instructions on page 50 (HiTrap 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 binding 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 binding buffer.
- Use further chromatographic purification steps (see Chapter 9).
- Use a protease deficient host or add protease inhibitors.

### Tag removal by enzymatic cleavage

In most cases, functional tests can be performed using the intact (His)<sub>6</sub> fusion protein. If removal of the tag is necessary, 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)<sub>6</sub> fusion proteins.



rTEV protease (Life Technologies Cat. No. 10127-017) has a (His)<sub>6</sub> 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)<sub>6</sub> 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)<sub>6</sub> tag and the (His)<sub>6</sub> 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 <sub>r</sub> 29 000
Carboxypeptidase A*	M <sub>r</sub> 94 000

\* for the removal of C-terminal (His)<sub>6</sub> tags.



Some cleavage procedures will require a second purification step to be performed to remove the protease or other contaminants. Conventional chromatographic separation techniques such as ion exchange or hydrophobic interaction 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 or out into the culture medium. While secretion is advantageous in terms of protein folding, solubility and disulphide bonding, 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 and 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 which can be difficult to separate
	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 guanidine 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 reducing 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 guanidine hydrochloride or urea. Other denaturants (up to 2% Tween 20; 1% CTAB; or 0.03% SDS) have also been used.

## Refolding of solubilized recombinant proteins

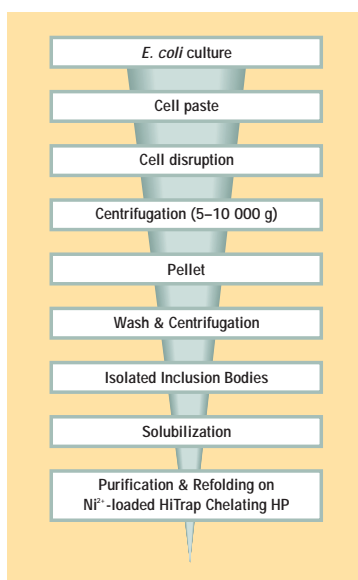
Following solubilization, proteins must be properly refolded to regain function. Denaturing agents must always be removed to allow refolding of the protein and formation of the correct intramolecular associations. Critical parameters during refolding include pH, presence of reducing reagents, the speed 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)<sub>6</sub> 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 15 has been used successfully for several different (His)<sub>6</sub> fusion proteins.



**Fig. 15.** General scheme for the extraction, solubilization and refolding of (His)<sub>6</sub> fusion proteins produced as inclusion bodies in *E. coli* cells.

High concentrations of chaotropic agents (such as urea or guanidine hydrochloride) enhance the binding of the histidine tract to immobilized divalent metal ions. Consequently, (His)<sub>6</sub> 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, the 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)<sub>6</sub> 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
Binding buffer:	6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0
Wash buffer:	6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 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
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 <sub>4</sub>



Phosphate buffers can also be used instead of Tris-HCl (see earlier purification protocols for (His)<sub>6</sub> 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 buffer when using ÄKTA<sub>prime</sub>.



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. See the optimization protocol for high purity on page 50.

### Disruption, wash and isolation of inclusion bodies

1. Resuspend the cell paste from 100 ml culture in 4 ml resuspension buffer.
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 binding buffer.
2. Stir for 30–60 min. at room temperature.
3. Centrifuge for 15 min. at high speed, +4 °C.
4. Remove remaining 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<sup>prime</sup>, select the Application Template for automatic refolding and purification of His-tagged proteins and follow the instructions on the cue card (code no: 18-1139-43). The following steps will be performed automatically.

## Preparation of the column

1. Wash HiTrap Chelating HP 1 ml column with 5 ml distilled water.
2. Load 0.5 ml 0.1 M NiSO<sub>4</sub> solution and continue to wash with 5 ml distilled water.
3. Equilibrate column with 5–10 ml binding buffer.

## Loading and washing

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

## Refolding

1. Refolding of the bound protein is performed by the use of a linear 6–0 M urea gradient, starting with the wash buffer above and finishing with the refolding 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 refolding 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 refolding buffer and ending with the elution buffer.
2. Check collected fractions with, for example, SDS-PAGE and pool as suitable. Figure 16 illustrates an example of this on-column refolding and purification procedure.

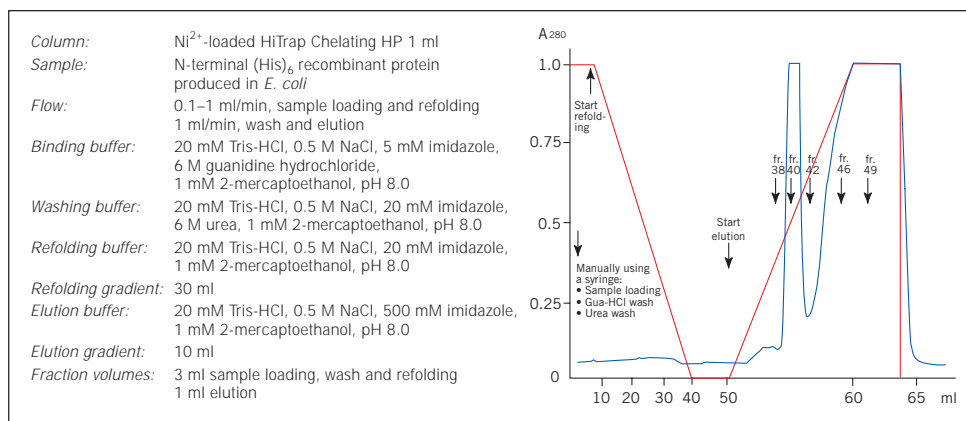


Fig. 16.



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 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
<b>Gentle</b> Cell lysis (osmotic shock)	2 volumes water to 1 volume packed pre-washed cells	<i>E. coli</i> periplasm: intracellular proteins	lower product yield but reduced protease release
Enzymatic digestion	lysozyme 0.2 mg/ml, +37 °C, 15 mins.	bacteria: intracellular proteins and periplasm	lab scale only, often combined with mechanical disruption
<b>Moderate</b> Grinding with abrasive e.g. sand	follow equipment instructions	bacteria, cells/tissues	
<b>Vigorous</b> 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, cells/tissues	
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:

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

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

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

Additives such as 8 M urea or 6 M guanidine 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 extraction buffer when working with (His)<sub>6</sub> fusion proteins.

1. Harvest cells by centrifugation (e.g. at 7 000–8 000 g for 10 minutes or 1 000–1 500 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 extraction 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 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 guanidine hydrochloride is present, this must be exchanged for 8 M urea by using a prepacked 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 frequently used at laboratory scale to remove gross impurities from small sample volumes, and occasionally used in small-scale commercial production.

When using a HiTrap affinity purification column (such as GStrap FF or HiTrap Chelating HP) at laboratory scale, it is unlikely that fractional precipitation will be required.

Precipitation techniques separate fractions by the principle of differential solubility. Because protein species differ in their degree of hydrophobicity, increased salt concentrations can enhance hydrophobic interactions between the proteins and cause precipitation. Fractional precipitation can be applied to remove gross impurities in three different ways, as shown in Figure 17.



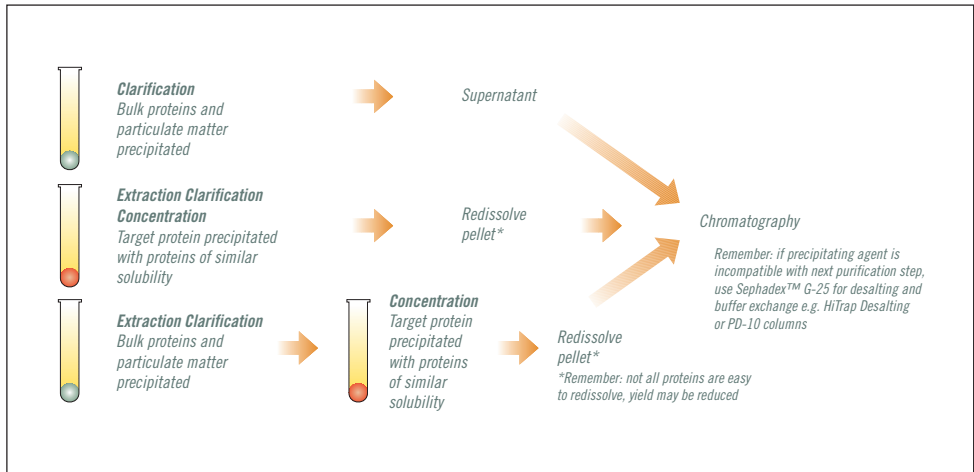
Precipitation techniques are affected by temperature, pH and sample concentration. These parameters must be controlled to ensure reproducible results.



Not all proteins are easy to redissolve, yield may be reduced.



Most precipitation techniques are not suitable for large-scale preparation.



**Fig. 17.** Three ways to use precipitation.

Precipitation agents 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 on page 66	> 1 mg/ml proteins especially immunoglobulins	stabilizes proteins, no denaturation, supernatant can go directly to HIC
Dextran sulphate	Add 0.04 ml 10% dextran sulphate and 1 ml 1 M CaCl <sub>2</sub> per ml sample, mix 15 min., centrifuge 10 000 g, discard pellet	samples with high levels of lipoprotein e.g. ascites	precipitates lipoprotein
Polyvinylpyrrolidone	Add 3% (w/vol), stir 4 hours, centrifuge, discard pellet	as above	alternative to dextran sulphate
Polyethylene glycol (PEG, M <sub>w</sub> > 4 000)	up to 20% w/vol	plasma proteins	no denaturation, supernatant goes direct to IEX or AC, complete removal may be difficult
Acetone (cold)	up to 80% vol/vol at ±0 °C collect pellet after centrifugation at full speed in an Eppendorf centrifuge	useful for peptide precipitation or concentration of sample for electrophoresis	may denature protein irreversibly
Polyethyleneimine	0.1% w/vol		precipitates aggregated nucleoproteins
Protamine sulphate	1% w/vol		as above
Streptomycin sulphate	1% w/vol		precipitation of nucleic acids

**Table 19.** Examples of precipitation techniques.

Details taken from:

Protein Purification, Principles and Practice. Scopes, R.K., *Springer* (1994).

Protein Purification, Principles, High Resolution Methods and Applications. Janson, J.C. and Rydén, L., *2nd ed. Wiley Inc.* (1998).

Other personal communications.



For general advice on buffer selection and the use of detergents please refer to the Sample Preparation chapter in the *Affinity Chromatography, Principles and Methods Handbook* from Amersham Biosciences.

## Ammonium sulphate precipitation

### Solutions required

Saturated ammonium sulphate: 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  $\mu\text{m}$ ) or centrifuge (10 000 g, refrigerated at +4 °C) sample.
2. Add 1 part 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently. Add saturated 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 10 000 g.
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, as in step 4.
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 (see Chapter 7) 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.

Starting percent saturation	Final percent saturation to be obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	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 the 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, transferred into a new buffer, and low molecular weight materials are removed.

Desalting columns are used not only for the removal of low molecular weight contaminants such as salt, but also to transfer the sample into a different buffer before or after purification. They are also used 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. 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. Figure 18 shows a typical desalting and buffer exchange separation, monitored by following changes in UV absorption and conductivity.

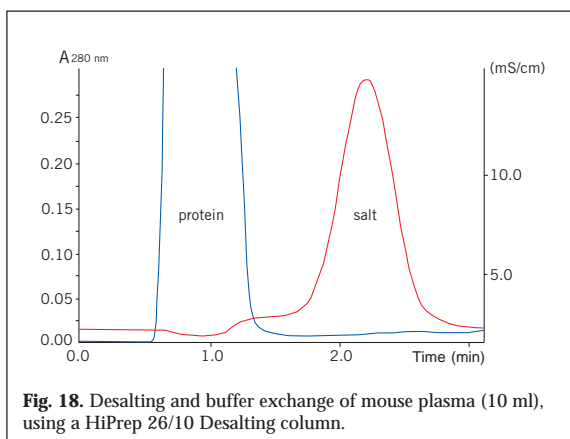


Table 21 shows a selection guide for prepacked, ready to use desalting and buffer exchange columns.

Column	Column volume	Sample volume	Volume after elution
MicroSpin G-25	0.5 ml	0.1–0.15 ml	0.1–0.15 ml
HiTrap Desalting	5 ml	0.25–1.5 ml	1.0–2.0 ml
HiPrep 26/10 Desalting	53 ml	2.5–15 ml	7.5–20 ml

**Table 21.** Selection guide for desalting columns.



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 can take less than 5 minutes per sample with greater than 95% recovery for most proteins.



To prevent possible ionic interactions the presence of a low salt concentration (25 mM NaCl) is recommended during desalting and in the final sample buffer.



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 stop-plug. To avoid introducing air into the column, connect the column "drop to drop" to the syringe (via the adapter provided).
2. Remove the twist-off end.
3. Wash the column with 25 ml buffer at 5 ml/min to completely remove the storage buffer which contains 20% ethanol. 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.

*Note: 5 ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column.*



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 adapter by piercing a hole through the bottom-end 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.

4. Apply the sample using a 2–5 ml syringe or a Multipipette at a flow rate between 1–10 ml/min. Discard the liquid eluted from the column. If the sample volume is less than 1.5 ml, change to buffer and proceed with the injection until a total of 1.5 ml has been eluted. Discard the eluted liquid.
5. Elute the protein with the appropriate volume selected from Table 22. Collect the desalted protein in the volume indicated.



The maximum recommended sample volume is 1.5 ml. See Table 22 for the effect of reducing the sample volume applied to the column.

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	0.0	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 ÄKTAprime

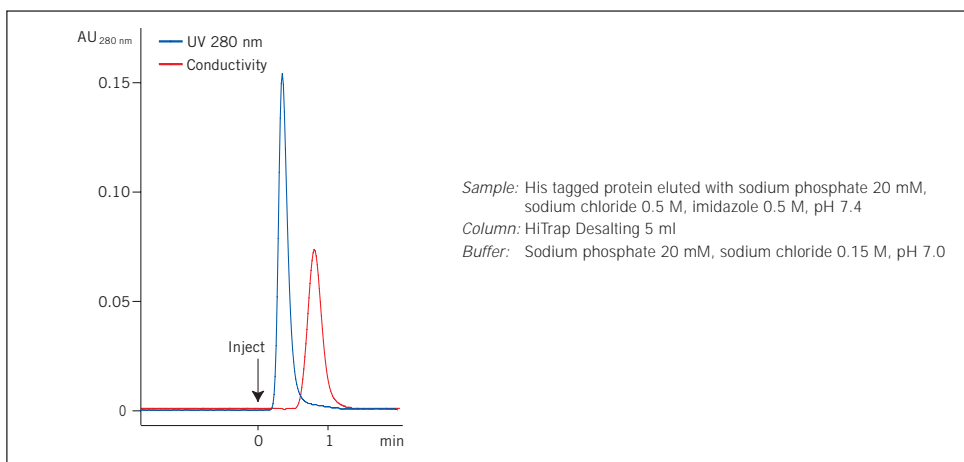
ÄKTAprime contains pre-programmed templates for individual HiTrap Desalting and HiPrep 26/10 Desalting columns.



Prepare at least 500 ml of the required buffer.

1. Follow the instructions supplied on ÄKTAprime cue cards, Desalting on HiTrap Desalting (code no: 18-1138-03) or Desalting on HiPrep 26/10 Desalting (code no: 18-1138-04).
2. Select the Application Template.
3. Start the method.
4. Enter the sample volume (up to 1.5 ml) and press OK.

Figure 19 shows a typical desalting procedure using ÄKTAprime. The UV and conductivity traces enable the appropriate desalted fractions to be pooled.



**Fig. 19.** Desalting of a (His)<sub>6</sub> fusion protein on ÄKTAprime.



## CHAPTER 8

### Simple purification of other recombinant proteins

GST MicroSpin and His MicroSpin Purification Modules, GSTrap FF, HiTrap Chelating HP and HisTrap Kit 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.



Single step purification using specific 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 in series to increase the capacity of a single purification step. Media are also available for packing larger columns.

Application	Column	Capacity (mg/ml affinity medium)
<b>Isolation of immunoglobulins</b>		
IgG, fragments and subclasses from all sources	HiTrap rProtein A FF	Human IgG, 50 mg/ml
IgG, fragments and subclasses from all sources	HiTrap Protein A HP	Human IgG, 20 mg/ml
IgG, fragments and subclasses including human IgG <sub>3</sub> . Strong affinity for monoclonal mouse IgG <sub>1</sub> and rat IgG	HiTrap Protein G HP	Human IgG, 25 mg/ml
IgG, fragments and subclasses including human IgG <sub>3</sub> . Strong affinity for monoclonal mouse IgG <sub>1</sub> and rat IgG	MABTrap™ Kit (HiTrap Protein G HP column (1 ml), accessories, pre-made buffers)	Human IgG, 25 mg/ml
IgY from egg yolk	HiTrap IgY Purification HP	IgY, 20 mg/ml
Monoclonal and human IgM	HiTrap IgM Purification HP	Mouse IgM, 5 mg/ml
<b>Group Specific Media</b>		
Albumin, various nucleotide-requiring enzymes	HiTrap Blue HP	HSA, 20 mg/ml
Proteins and peptides with exposed amino acids: His (Cys, Trp) His-tagged proteins	HiTrap Chelating HP	(His) <sub>6</sub> fusion protein (M <sub>r</sub> 27 600), 12 mg/ml
Biotin and biotinylated substances	HiTrap Streptavidin HP	Biotinylated BSA, 6 mg/ml
Coagulation factors, lipoprotein lipases, steroid receptors, hormones, DNA binding proteins, interferon, protein synthesis factors	HiTrap Heparin HP	Antithrombin III (bovine), 3 mg/ml
Removal of proteolytic activity or purification of trypsin or trypsin-like serine proteases	HiTrap Benzamidine FF (high sub)	Trypsin, 35 mg/ml
Proteins and peptides with exposed amino acids: His (Cys, Trp). His-tagged proteins	HisTrap Kit (HiTrap Chelating HP 1 ml, buffers for up to 12 purifications using a syringe)	(His) <sub>6</sub> fusion protein (M <sub>r</sub> 27 600), 12 mg/ml
GST-tagged proteins	GSTrap FF	Glutathione-S-transferase, 10–12 mg/ml
<b>Matrix for preparation of affinity columns</b>		
Coupling of primary amines	HiTrap NHS-activated HP	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 that are 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 Biosciences. A detailed account of the principles of affinity chromatography can be found in the *Affinity Chromatography, Principles and Methods Handbook* also available from Amersham Biosciences.

### Preparation of HiTrap NHS-activated HP 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 10-atom spacer arms attached to the matrix by epichlorohydrine and activated by N-hydroxysuccinimide. The substitution level is ~10  $\mu\text{mol}$  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 prepacked HiTrap NHS-activated HP 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<sup>prime</sup>.



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

### Buffer preparation

Acidification solution: 1 mM HCl (ice-cold)

Coupling buffer: 0.2 M NaHCO<sub>3</sub>, 0.5 M NaCl, pH 8.3



Use high quality water and chemicals. Filtration through 0.45  $\mu\text{m}$  filters is recommended.



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

## Ligand and 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 (maximum recommended flow rates are 1 ml/min (equivalent to approximately 30 drops/min when using a syringe) with HiTrap 1 ml and 5 ml/min (equivalent to approximately 120 drops/min when using a syringe) with HiTrap 5 ml). The column contents can be irreversibly compressed.

2. Immediately 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 HP 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<sub>2</sub>HPO<sub>4</sub>, 0.1% NaN<sub>3</sub>, pH 7.



Sodium azide can interfere with many coupling methods and some biological assays. It can be removed by using a desalting column (see Chapter 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.



Use high quality water and chemicals. Filtration through 0.45  $\mu\text{m}$  filters is recommended.



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



Samples should be centrifuged immediately before use and/or filtered through a 0.45  $\mu\text{m}$  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 (blank run)

1. Wash with 3 column volumes of binding buffer.
2. Wash with 3 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding 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, 0.2–1 ml/min on a HiTrap 1 ml column.
2. Wash with 5–10 column volumes of binding 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 1–3 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 prepacked desalting columns (see Chapter 7).

### Re-equilibrate the column

Re-equilibrate the column by washing with 5–10 column volumes of binding 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, a purification strategy of Capture, Intermediate Purification and Polishing (CIPP) can be applied (Figure 20). 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 Biosciences) is highly recommended as a guide to planning efficient and effective protein purification strategies.

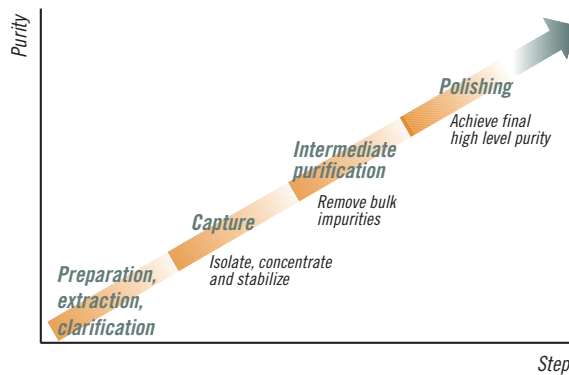


Fig. 20. Preparation and CIPP.

CIPP 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.

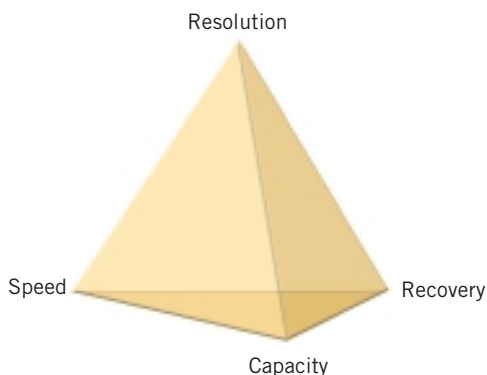
### Selection and combination of purification techniques

Proteins are purified using 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)

**Table 24.**

\*Expanded bed adsorption is a technique used for large-scale purification. Proteins can be purified from crude sample without the need for separate clarification, concentration and initial purification to remove particulate matter. The STREAMLINE adsorbents, used for expanded bed adsorption, capture the target molecules using the same principles as affinity, ion exchange or hydrophobic interaction chromatography.



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

**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.

**Capacity**, in the simple model shown, refers to the amount of target protein that can be 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 reduced by destructive processes in the sample and unfavourable conditions on the column.



Select a chromatographic 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 CIPP is shown in Table 25.

Technique	Main features	Capture	Intermediate	Polishing	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 <b>concentrated sample</b>
HIC	good resolution good capacity high speed	★★	★★★★	★	high ionic strength sample volume not limiting	low ionic strength <b>concentrated sample</b>
AC	high resolution high capacity high speed	★★★★	★★★★	★★	specific binding conditions sample volume not limiting	specific elution conditions <b>concentrated sample</b>
GF	high resolution using Superdex		★	★★★★	limited sample volume (<5% total column volume) and flow rate range	buffer exchanged (if required) diluted sample
RPC	high resolution		★	★★★★	sample volume usually not limiting additives may be required	in organic solvent, risk loss of biological activity

**Table 25.** Suitability of purification techniques for the CIPP.



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 a buffer suitable for the start conditions required for the next technique (see Table 25).

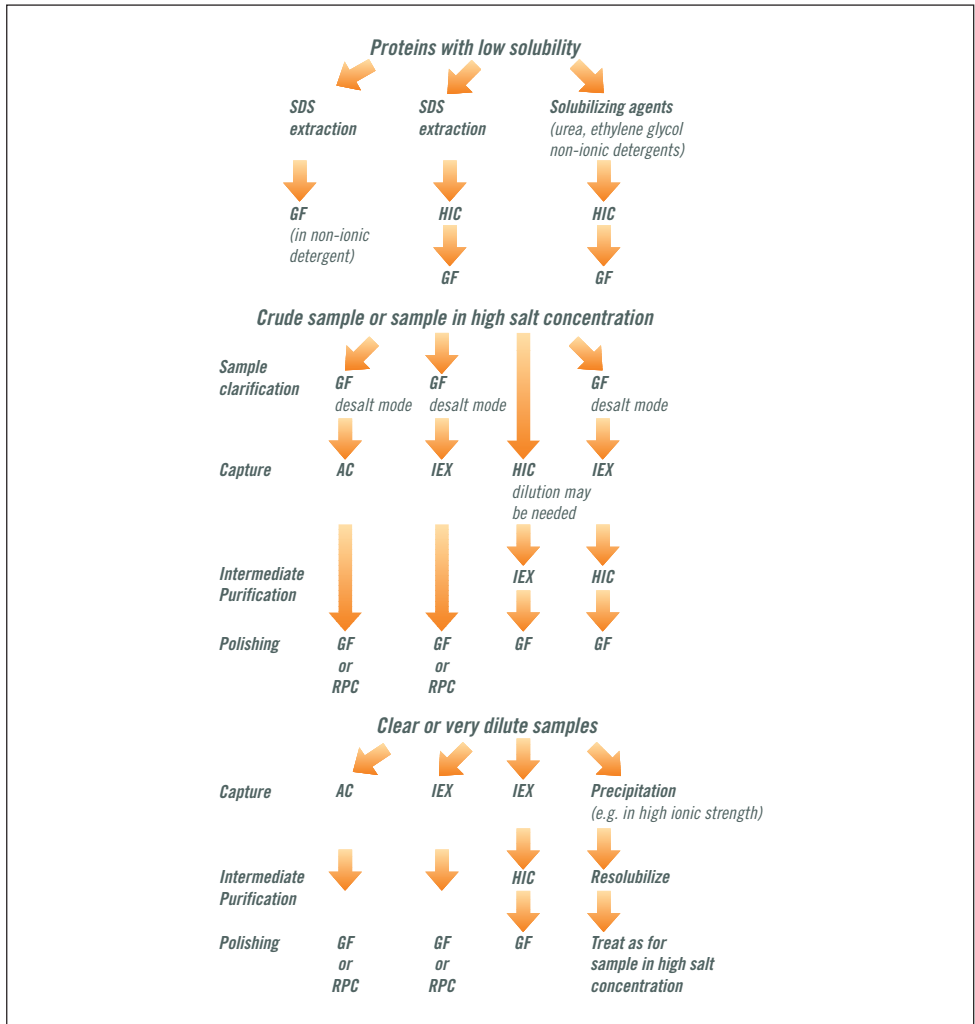


Ammonium sulphate precipitation is a common sample clarification and concentration step and so HIC (which requires high salt to enhance binding to the media) is ideal as the capture step. The salt concentration and the total sample volume will be significantly reduced after elution from the HIC column. 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) 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 21.



**Fig. 21.** 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 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 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 native 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.

CIPP 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 CIPP in the purification of a recombinant protein.

### Three step purification of a recombinant enzyme using ÄKTA<sup>FPLC</sup>

This example demonstrates one of the most common purification strategies used 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 Tris-based lysis buffer, pH 7.5 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 the HiTrap IEX Selection Kit, was screened to find the optimum medium (results not shown). Optimization of the capture step (in Figure 22) allowed the use of a step elution at high flow rate to speed up the purification.

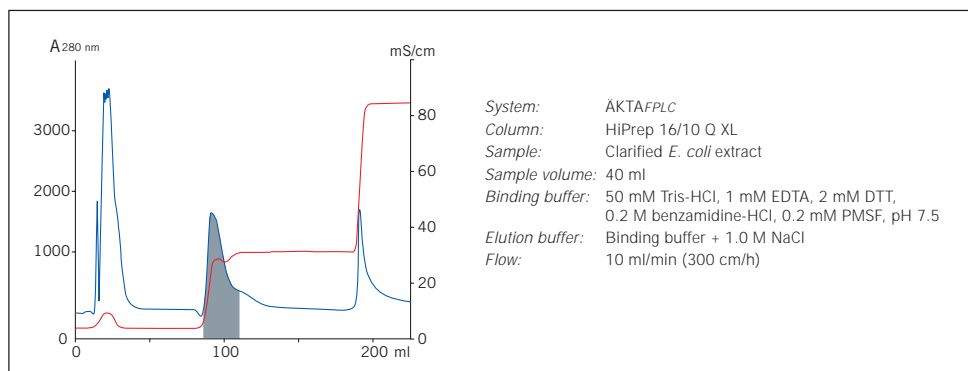


Fig. 22. Capture using IEX. The elution position of DAOCS is shaded.

## 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<sup>™</sup> ISO was selected on the basis of the resolution achieved. In this intermediate step, shown in Figure 23, the maximum possible speed for separation was sacrificed in order to achieve higher resolution and allow significant reduction of impurities.

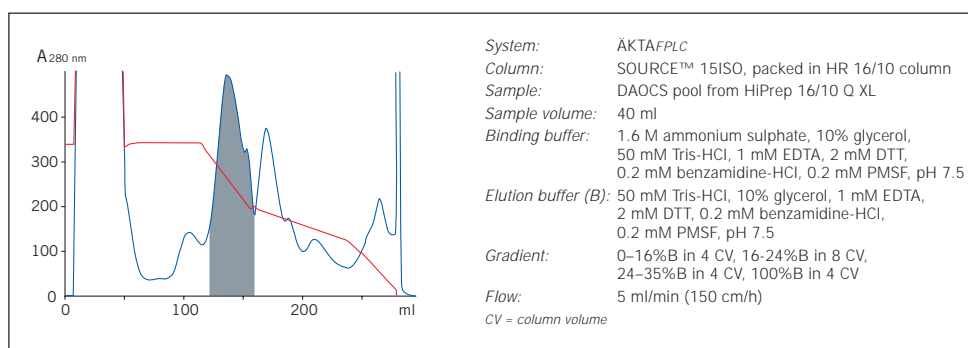
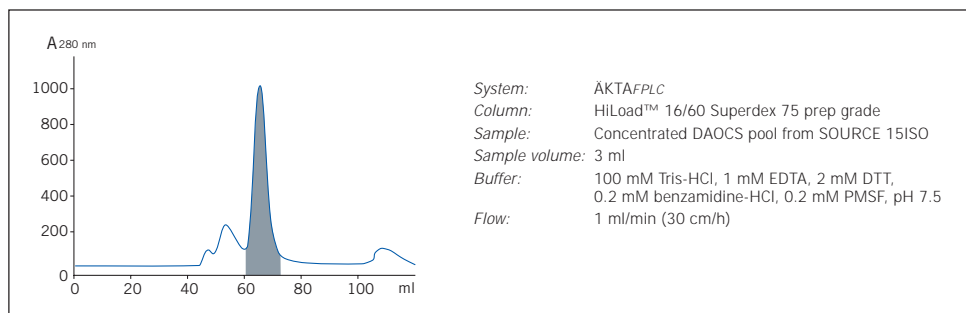


Fig. 23. Intermediate purification using HIC. The elution position of DAOCS is shaded.

## Polishing

The main goal of the polishing step, shown in Figure 24, was to remove aggregates and minor contaminants and transfer the purified sample into a buffer suitable for use in structural studies. The final product was used successfully in X-ray diffraction studies.

**Ref:** Structure of a cephalosporin synthase. Valegard, K., Terwisscha van Scheltinga, A.C., Lloyd, M., Hara, T., Ramaswamy, S., Perrakis, A., Thompson, A., Lee, H.J., Baldwin, J.E., Schofield, C.J., Hajdu, J. and Andersson, I. *Nature* **394**, 805–809 (1998).



**Fig. 24.** Polishing step using gel filtration. The elution position of DAOCS is shaded.

## Three step purification of a recombinant phosphatase using ÄKTAPrime

The objective of this application was to produce a pure phosphatase (rPhosphatase) with retained biological activity. The phosphatase gene was overexpressed and the protein was produced in soluble form in the cytoplasm of *Escherichia coli*. Using the pre-programmed method templates of ÄKTAPrime with prepacked HiPrep and HiLoad columns ensured quick and easy method development. The purification strategy consisted of a capture step by ion exchange chromatography, intermediate purification by hydrophobic interaction chromatography and polishing by gel filtration. Active rPhosphatase (35 mg) was purified within 8 hours.

A more detailed description of this work can be found in Application Note 18-1142-32.

### Sample preparation and extraction

The *E. coli* cells were suspended in lysis buffer, 1 g cells to every 10 ml lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, pH 7.4). The suspended cells were lysed by ultra-sonication, 6 x 20 bursts with 60 seconds cooling between each burst. DNA was removed by precipitation with 1% w/v streptomycin sulphate. The sample was clarified by centrifugation, 15 minutes at 22 000 g, before it was applied to the first chromatography column.

## Capture

The main purpose of the capture step was to concentrate the rPhosphatase and remove most of the contaminants. The ÄKTA<sup>prime</sup> system pump was used to apply 200 ml of the clarified *E. coli* supernatant, diluted 1:2 with water, to a HiPrep 16/10 DEAE FF column. A pre-programmed method template for ion exchange chromatography was used for the separation.

Fractions of the eluate were collected and analysed with an enzyme immunoassay detecting alkaline phosphatase activity at an absorbance of 405 nm. The purity of the fractions containing rPhosphatase was determined by SDS-PAGE.

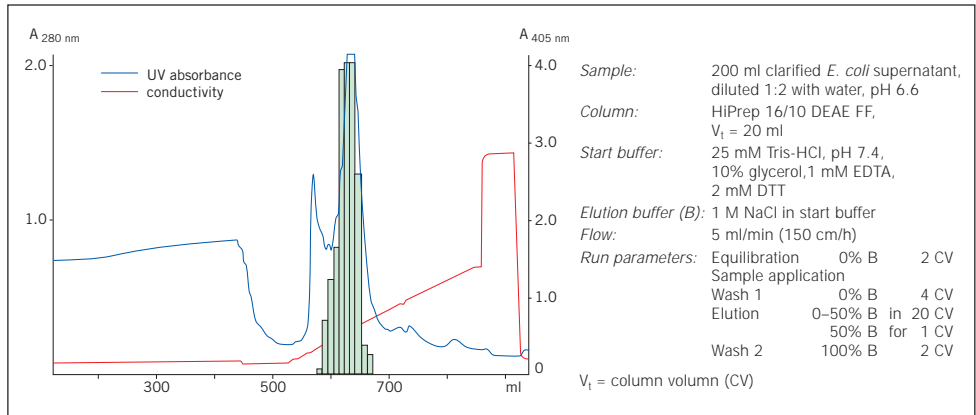


Fig. 25. Capture step using ion exchange. The phosphatase activity is represented by the green bars (absorbance at 405 nm).

## Intermediate purification

Hydrophobic interaction chromatography was used for intermediate purification because of its compatibility with samples containing a high salt concentration. The pooled fractions from the ion exchange column were purified on HiLoad 16/10 Phenyl Sepharose High Performance, using a pre-programmed method template in ÄKTA<sup>prime</sup>. The fractions containing rPhosphatase were pooled and concentrated to 10 ml on an Amicon<sup>TM</sup> 50 ml stirred-cell using a Diaflow<sup>TM</sup> PM10 filter. Reducing the sample volume enables a smaller gel filtration column to be used for the final polishing step.

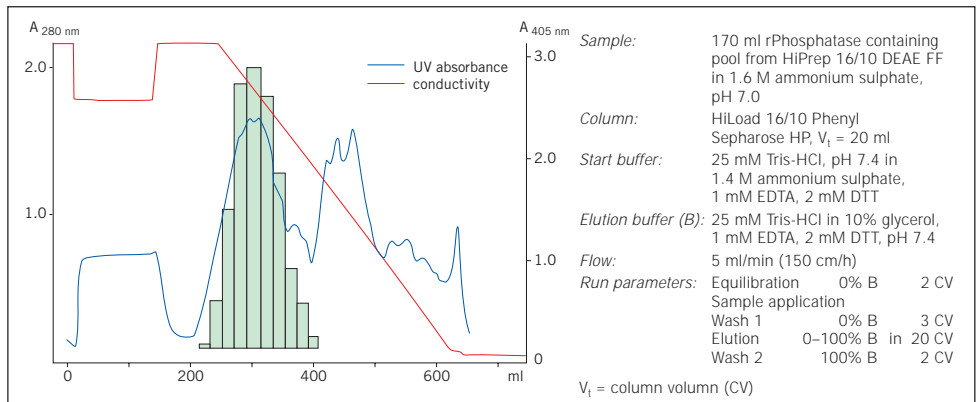
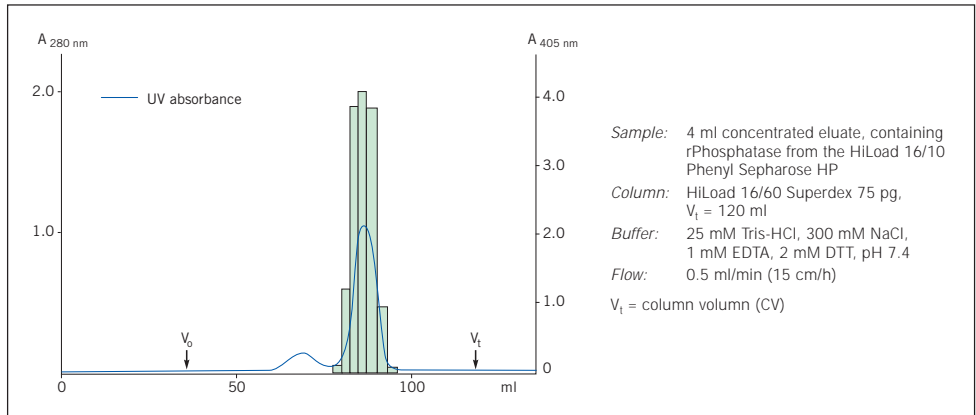


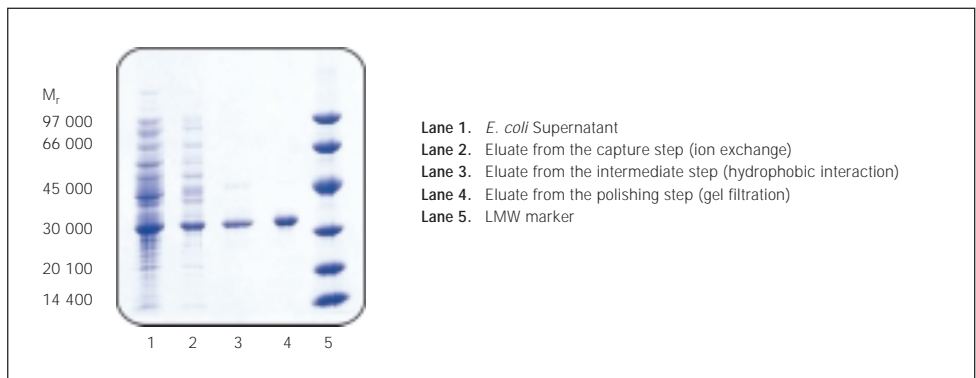
Fig. 26. Intermediate purification step using hydrophobic interaction. The phosphatase activity is represented by the green bars (absorbance at 405 nm).

## Polishing

The final polishing step used a pre-programmed method template to run a gel filtration separation on a HiLoad 16/60 Superdex 75 prep grade column. The purity of the fractions containing rPhosphatase was checked with SDS-PAGE (Figure 28) and by mass spectrometry (results not shown).



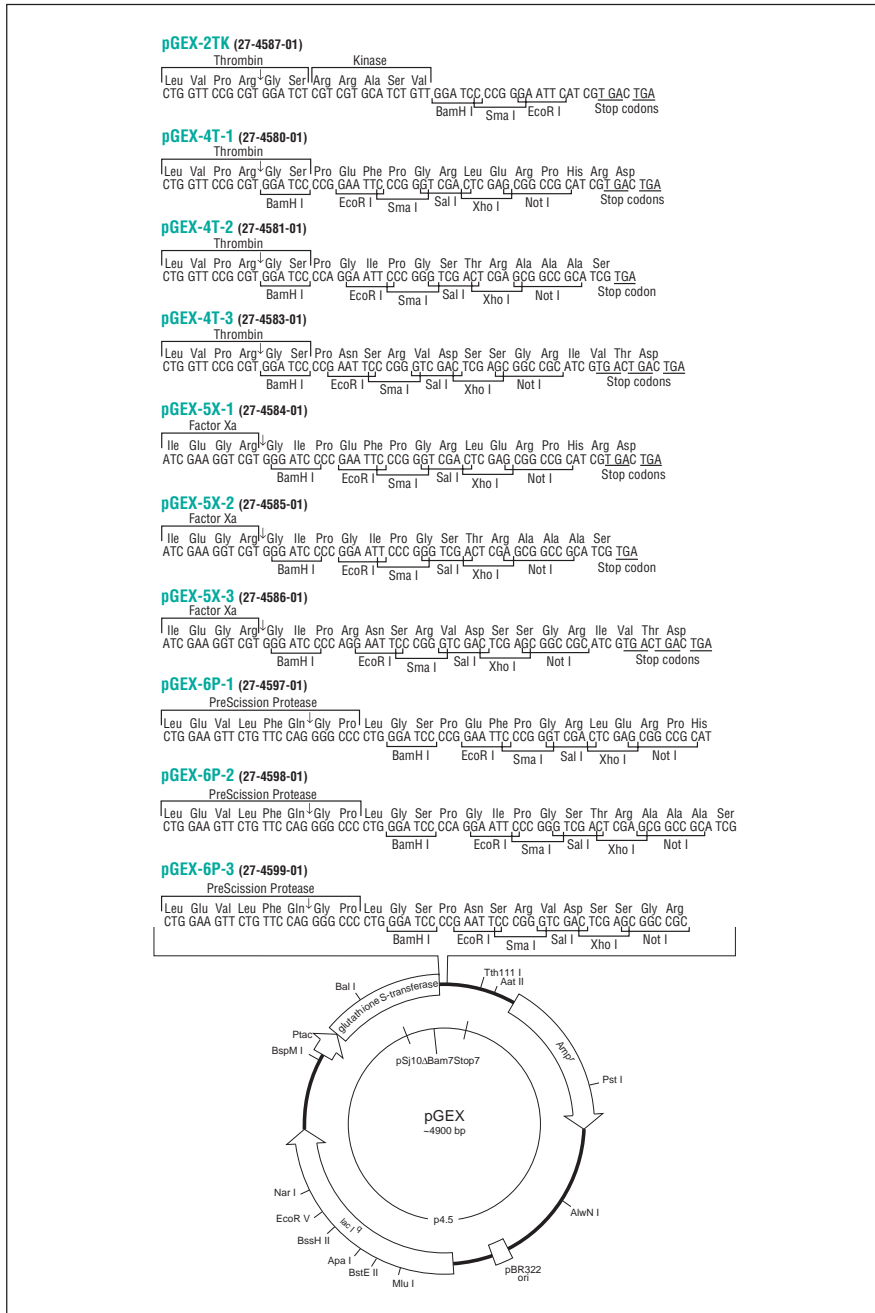
**Fig. 27.** Polishing step using gel filtration. The phosphatase activity is represented by the green bars (absorbance at 405 nm).



**Fig. 28.** Purity check by SDS-PAGE using Multiphor II and ExcelGel SDS Gradient 8–18%. The proteins were stained with Coomassie Brilliant Blue.

# Appendix 1

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


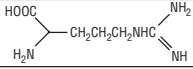
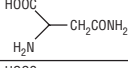
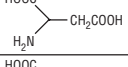

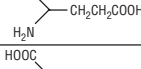
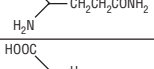

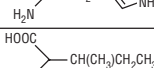
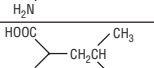
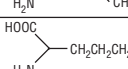
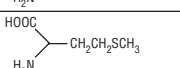
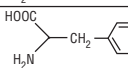
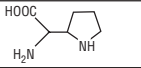
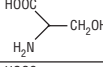
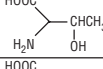
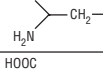
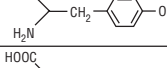
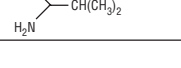



SELECTION GUIDE – pGEX Vector Control Regions										
	pGEX- 2TK 27- 4587- 01	pGEX- 4T- 1 27- 4580- 01	pGEX- 4T- 2 27- 4581- 01	pGEX- 4T- 3 27- 4583- 01	pGEX- 5X- 1 27- 4584- 01	pGEX- 5X- 2 27- 4585- 01	pGEX- 5X- 3 27- 4586- 01	pGEX- 6P- 1 27- 4597- 01	pGEX- 6P- 2 27- 4598- 01	pGEX- 6P- 3 27- 4599- 01
<b>Glutathione S- Transferase Region</b>										
<i>lac</i> promoter- 10	205- 211	205- 211	205- 211	205- 211	205- 211	205- 211	205- 211	205- 211	205- 211	205- 211
<i>lac</i> promoter- 35	183- 188	183- 188	183- 188	183- 188	183- 188	183- 188	183- 188	183- 188	183- 188	183- 188
<i>lac</i> 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
<b>Multiple Cloning Site</b>	951- 966	930- 966	930- 967	930- 965	934- 969	934- 970	934- 971	945- 981	945- 982	945- 980
<b>β -lactamase (Amp<sup>r</sup>) Gene Region</b>										
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
<b><i>Lac</i><sup>z</sup> Gene Region</b>										
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
<b>Plasmid Replication Region</b>										
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
<b>Sequencing Primers</b>										
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
<b>GenBank Accession Number</b>	U13851	U13853	U13854	U13855	U13856	U13857	U13858	U78872	U78873	U78874

Complete DNA sequences and restriction site data are available at the Amersham Biosciences Web Site ( [www.amershambiosciences.com](http://www.amershambiosciences.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	M <sub>r</sub>	Middle unit residue (-H <sub>2</sub> O)		Charge at pH 6.0–7.0	Hydrophobic (non-polar)	Uncharged (polar)	Hydrophilic (polar)
		Formula	M <sub>r</sub>				
C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub>	89.1	C <sub>3</sub> H <sub>5</sub> NO	71.1	Neutral	■		
C <sub>6</sub> H <sub>14</sub> N <sub>4</sub> O <sub>2</sub>	174.2	C <sub>6</sub> H <sub>12</sub> N <sub>4</sub> O	156.2	Basic (+ve)			■
C <sub>4</sub> H <sub>8</sub> N <sub>2</sub> O <sub>3</sub>	132.1	C <sub>4</sub> H <sub>6</sub> N <sub>2</sub> O <sub>2</sub>	114.1	Neutral		■	
C <sub>4</sub> H <sub>7</sub> NO <sub>4</sub>	133.1	C <sub>4</sub> H <sub>5</sub> NO <sub>3</sub>	115.1	Acidic(-ve)			■
C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub> S	121.2	C <sub>3</sub> H <sub>5</sub> NOS	103.2	Neutral		■	
C <sub>5</sub> H <sub>9</sub> NO <sub>4</sub>	147.1	C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub>	129.1	Acidic (-ve)			■
C <sub>5</sub> H <sub>10</sub> N <sub>2</sub> O <sub>3</sub>	146.1	C <sub>5</sub> H <sub>8</sub> N <sub>2</sub> O <sub>2</sub>	128.1	Neutral		■	
C <sub>2</sub> H <sub>5</sub> NO <sub>2</sub>	75.1	C <sub>2</sub> H <sub>3</sub> NO	57.1	Neutral		■	
C <sub>6</sub> H <sub>9</sub> N <sub>3</sub> O <sub>2</sub>	155.2	C <sub>6</sub> H <sub>7</sub> N <sub>3</sub> O	137.2	Basic (+ve)			■
C <sub>6</sub> H <sub>13</sub> NO <sub>2</sub>	131.2	C <sub>6</sub> H <sub>11</sub> NO	113.2	Neutral	■		
C <sub>6</sub> H <sub>13</sub> NO <sub>2</sub>	131.2	C <sub>6</sub> H <sub>11</sub> NO	113.2	Neutral	■		
C <sub>6</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	146.2	C <sub>6</sub> H <sub>12</sub> N <sub>2</sub> O	128.2	Basic(+ve)			■
C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub> S	149.2	C <sub>5</sub> H <sub>9</sub> NOS	131.2	Neutral	■		
C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>	165.2	C <sub>9</sub> H <sub>9</sub> NO	147.2	Neutral	■		
C <sub>5</sub> H <sub>9</sub> NO <sub>2</sub>	115.1	C <sub>5</sub> H <sub>7</sub> NO	97.1	Neutral	■		
C <sub>3</sub> H <sub>7</sub> NO <sub>3</sub>	105.1	C <sub>3</sub> H <sub>5</sub> NO <sub>2</sub>	87.1	Neutral		■	
C <sub>4</sub> H <sub>9</sub> NO <sub>3</sub>	119.1	C <sub>4</sub> H <sub>7</sub> NO <sub>2</sub>	101.1	Neutral		■	
C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	204.2	C <sub>11</sub> H <sub>10</sub> N <sub>2</sub> O	186.2	Neutral	■		
C <sub>9</sub> H <sub>11</sub> NO <sub>3</sub>	181.2	C <sub>9</sub> H <sub>9</sub> NO <sub>2</sub>	163.2	Neutral		■	
C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>	117.1	C <sub>5</sub> H <sub>9</sub> NO	99.1	Neutral	■		

## Appendix 3

### Protein conversion data

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

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

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

Protein	A <sub>280</sub> 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

## 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	1 100 g, 3 000 rpm
Sorvall RT-6000B	#07983	DuPont	H1000B (#11706)	yes #11093	2	1 900 g, 3 000 rpm
6K	#83794	Fisher	#04-976-420	yes #04-975-410 MT	2	1 506 g, 3 500 rpm
Megafuge 1.0	#C1725-3 (Baxter #)	Heraeus	#C1725-10 #C1725-35	yes	4	1 280 g, 2 800 rpm
Z-320	#C-0320	Hermle	#C-0320-50	no	2	1 506 g, 3 500 rpm
GP8 GP8R	#3121 #3122	IEC	#216 or #228	yes #5785	2 or 4	2 340 g, 3 500 rpm
HN-SII	#2355	IEC	#244	no	2	1 450 g, 3 575 rpm
MP4 MP4R	#2437 #2438	IEC	#244	no	2	1 450 g, 3 575 rpm
C312 CR312	#11175087 #11175090	Jouan	E4 (#11174153)	yes #11174168	4	2 290 g, 3 300 rpm
1130	Not required	Kubota	S11222	no	2	1 107 g, 3 000 rpm
1140	Not required	Kubota	S11222	no	2	1 107 g, 3 000 rpm

# Appendix 5

## Characteristics, cleaning 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 $\mu\text{mole}$ glutathione per ml Glutathione Sepharose 4B 120–320 $\mu\text{mole}$ glutathione per ml Glutathione Sepharose 4 Fast Flow
Capacity	up to 8 mg recombinant GST per ml Glutathione Sepharose 4B up to 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	$\sim 2 \times 10^7$
Particle size	45–165 $\mu\text{m}$ (90 $\mu\text{m}$ )

### Chemical stability

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



### Cleaning

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

If 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 PBS, pH 7.4.

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 guanidine hydrochloride.
2. Immediately wash with 5 column volumes of PBS, pH 7.4.

### To remove hydrophobically bound substances:

1. Wash with 3–4 column volumes of 70% ethanol (or 2 column volumes of 1% Triton X-100).
2. Immediately wash with 5 column volumes of PBS, pH 7.4.

### For long-term storage (>1 month):

1. Wash the column twice with 10 column volumes of PBS, pH 7.4.
2. Repeat washes using 20% ethanol.
3. Store at +4 °C.
4. Re-equilibrate the column with 5–10 column volumes of PBS, pH 7.4 before re-use.

## Characteristics, cleaning and storage of Chelating Sepharose

### Characteristics

Capacity	up to 12 mg (His) <sub>6</sub> fusion protein per ml Chelating Sepharose 4 Fast Flow 12 mg (His) <sub>6</sub> fusion protein (M <sub>r</sub> 27 600) per ml Chelating Sepharose High Performance (in HiTrap columns)
Ligand	Iminodiacetic acid
Molecular weight exclusion limit	~2 x 10 <sup>7</sup>
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 6 M guanidine hydrochloride, 8 M urea and chaotropic agents.



### Cleaning

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

### 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.

### To remove nickel ions prior before recharging or 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.
3. For storage wash with 5 column volumes of 20% ethanol.
4. Seal columns with the fittings provided and store at +4 to +8 °C.



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.



The column must be recharged with nickel ions after regeneration.

## 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 column 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.**



**Do not exceed the maximum operating pressure of the medium or column.**

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 adapter into the column at an angle, ensuring that no air is trapped under the net.
12. Slide the adapter slowly down the column (the outlet of the adapter should be open) until the mark is reached. Lock the adapter in position.
13. Connect the column to the pump and begin equilibration. Re-position the adapter 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.**



**Many media, when equilibrated with PBS containing an anti-microbial agent, may be stored at +4° C for up to 1 month, but always follow the specific storage instructions supplied with the product.**

## Column selection

XK columns are fully compatible with the high flow rates achievable with modern media and a broad range of column dimensions are available. For a complete listing refer to the *Amersham Biosciences BioDirectory* or *web catalogue*.

Columns	Volume (ml)	Code no
XK 16/20 column	2-34	18-8773-01
XK 26/20 column	0-80	18-1000-72
XK 16/70 column	102-135	18-8775-01
XK 26/70 column	281-356	18-8769-01

HR columns can be used for small scale chromatography applications.

Columns	Volume (ml)	Code no
HR 5/2 column	0.2-0.59	18-0382-01
HR 5/5 column	0.8-1.2	18-0383-01
HR 10/2 column	0.08-2.43	18-1000-97
HR 10/10 column	6.4-8.7	19-7402-01

## Appendix 7

### Converting from linear flow (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 as linear flow (cm/hour). However, flow is usually measured in volumetric flow rate (ml/min). To convert between linear flow and volumetric flow rate use one of the formulae below.

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

$$\begin{aligned}\text{Volumetric flow rate (ml/min)} &= \frac{\text{Linear flow (cm/h)}}{60} \times \text{column cross sectional area (cm}^2\text{)} \\ &= \frac{Y}{60} \times \frac{\pi \times d^2}{4}\end{aligned}$$

where

Y = linear flow in cm/h

d = column inner diameter in cm

*Example:*

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

Y = linear flow = 150 cm/h

d = inner diameter of the column = 1.6 cm

$$\begin{aligned}\text{Volumetric flow rate} &= \frac{150 \times \pi \times 1.6 \times 1.6}{60 \times 4} \text{ ml/min} \\ &= 5.03 \text{ ml/min}\end{aligned}$$

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

$$\begin{aligned}\text{Linear flow (cm/h)} &= \frac{\text{Volumetric flow rate (ml/min)} \times 60}{\text{column cross sectional area (cm}^2\text{)}} \\ &= Z \times 60 \times \frac{4}{\pi \times d^2}\end{aligned}$$

where

Z = volumetric flow rate in ml/min

d = column inner diameter in cm

*Example:*

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

Z = Volumetric flow rate = 1 ml/min

d = column inner diameter = 0.5 cm

$$\begin{aligned}\text{Linear flow} &= 1 \times 60 \times \frac{4}{\pi \times 0.5 \times 0.5} \text{ cm/h} \\ &= 305.6 \text{ cm/h}\end{aligned}$$

#### From ml/min to using a syringe

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

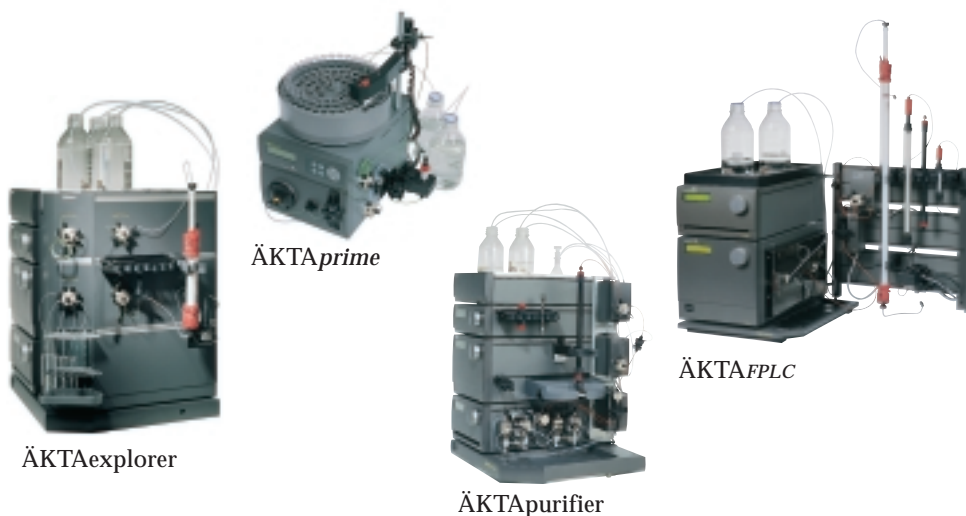
5 ml/min = approximately 120 drops/min on a HiTrap 5 ml column

## Appendix 8

### Selection of purification equipment

Many simple purification steps may be carried out using the simplest methods and equipment, for example step-gradient elution using a syringe together with prepacked HiTrap columns. When more complex elution methods are necessary or the same column is to be used for many runs in series, it is wise to use a dedicated system.

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



# 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 purification are shown in Figure 29. Affinity chromatography is also used to remove specific contaminants, for example Benzamidine Sepharose 4 Fast Flow can remove serine proteases.

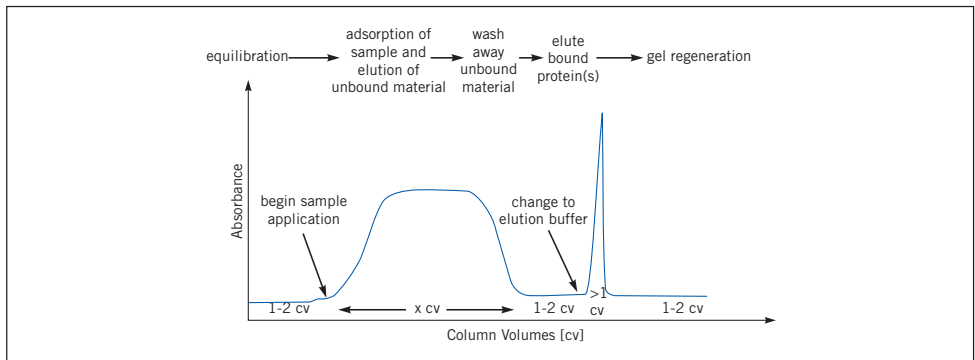


Fig. 29. Typical affinity purification.

### Further information

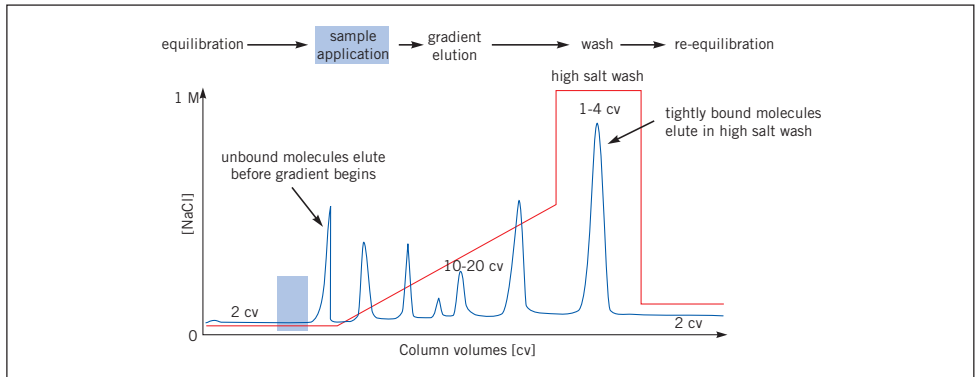
*Protein Purification Handbook*

*Affinity Chromatography Handbook: Principles and Methods*

Chapters 3 and 4 in this handbook for the purification of GST and His tagged proteins, respectively.

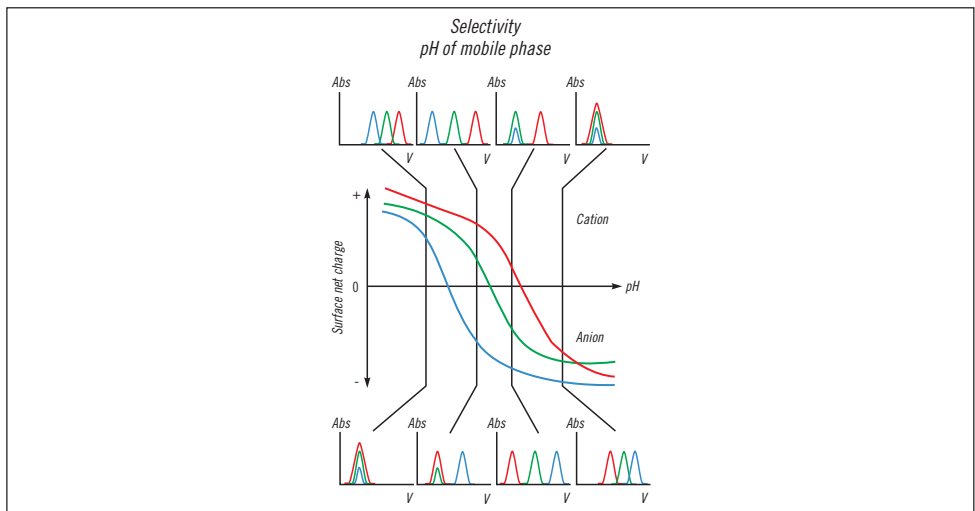
### Ion Exchange (IEX)

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 30). Target proteins are concentrated during binding and collected in a purified, concentrated form.



**Fig. 30.** 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 bind 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 that have distinctly different charge properties, as shown in Figure 31.



**Fig. 31.** 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 32. It is often possible to increase sample loading when using step elution.

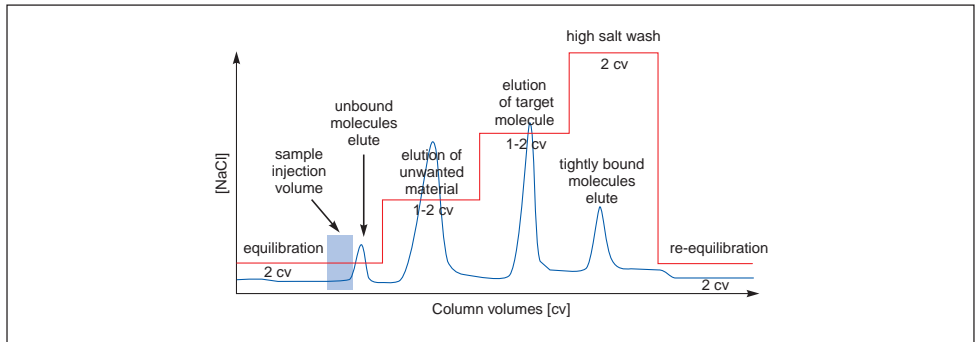


Fig. 32. Step elution.

## 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 33). 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, guanidine hydrochloride) or detergents, changing pH or temperature.

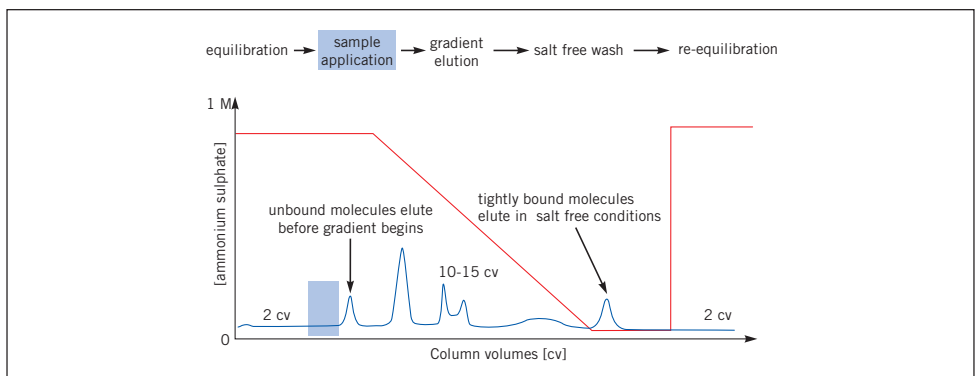


Fig. 33. Typical HIC gradient elution.

## Method development (in priority order)

1. The hydrophobic behaviour of a protein is difficult to predict and binding conditions must be studied carefully. Use HiTrap HIC Selection Kit or 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 34. It is often possible to increase sample loading when using step elution.

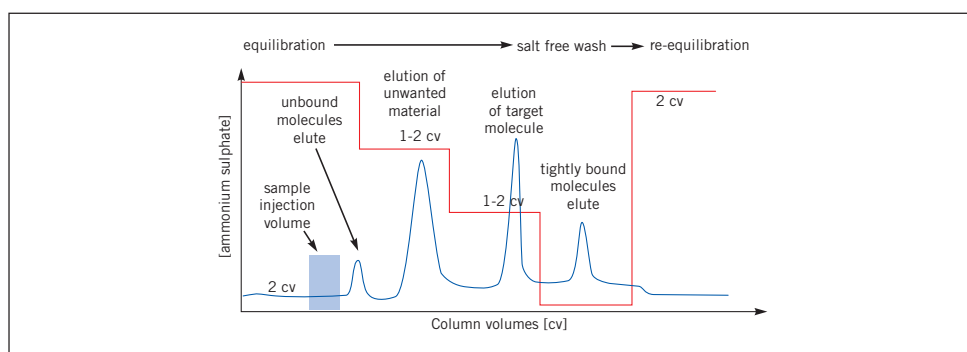


Fig. 34. Step elution.

## 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 35). Buffer conditions are varied to suit the sample type or the requirements for further purification, analysis or storage, since buffer composition does not directly affect resolution. Proteins are collected in purified form in the chosen buffer.

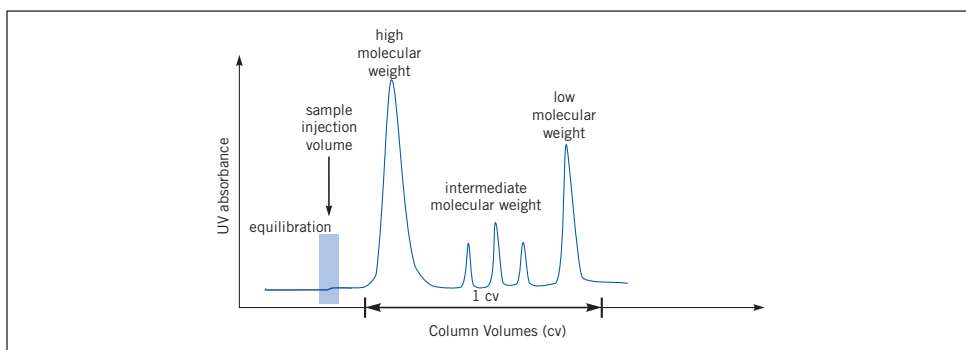


Fig. 35. Typical GF elution.

### 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). 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 36.

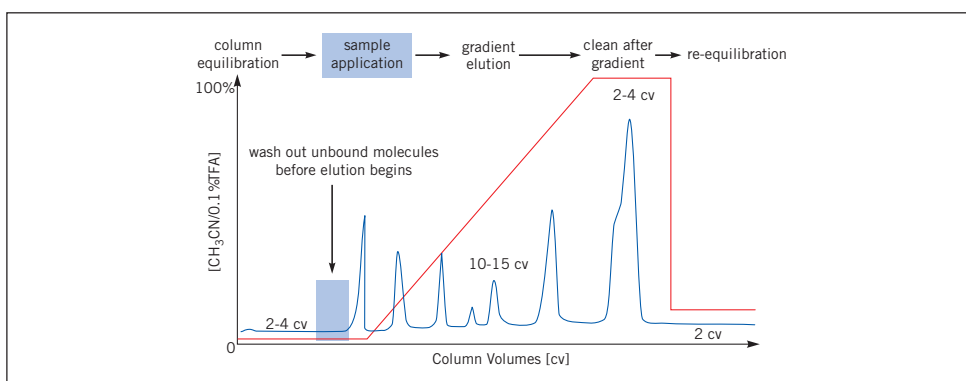


Fig. 36. Typical RPC gradient elution.

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.

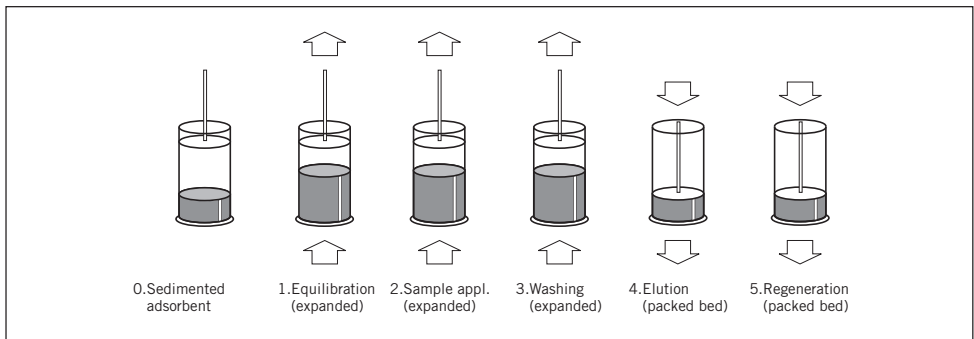
## Further information

*Protein Purification Handbook*

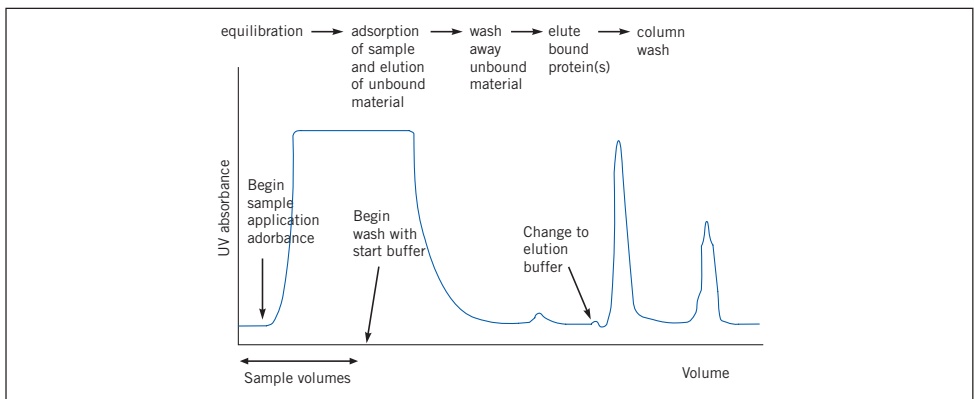
*Reversed Phase Chromatography Handbook: Principles and Methods*

## 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 37 shows a representation of the steps involved in an EBA purification and Figure 38 shows a typical EBA elution.



**Fig. 37.** Steps in an EBA purification process.



**Fig. 38.** 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
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
Convenient Purification, HiTrap Column Guide	18-1129-81
ÅKTAdesign Brochure	18-1129-05
Protein Purification, Principles, High Resolution Methods and Applications, J.C. Janson and L. Rydén, 1998, 2nd ed. Wiley VCH	18-1128-68
<b>GST fusion proteins</b>	
GST Gene Fusion Manual	18-1123-20
Data File, GSTrap FF and Glutathione Sepharose 4 Fast Flow	18-1136-89
Miniposter, "Rapid Purification of GST-fusion proteins from large sample volumes"	18-1139-51
<b>Purification and on-column cleavage of GST-tagged proteins</b>	
Application Note, "Efficient, rapid protein purification and on-column cleavage using GSTrap FF columns"	18-1146-70
<b>(His)<sub>6</sub> fusion proteins</b>	
Data File, HisTrap Kit	18-1212-00
Data File, HiTrap Chelating HP 1 ml and 5 ml	18-1134-78
Miniposter, "Purification of Poly(His)-tagged Recombinant Proteins using HisTrap Kit"	18-1116-26
<b>On-column refolding of (His)<sub>6</sub> fusion proteins</b>	
Application Note, "Rapid and efficient purification and refolding of a (His) <sub>6</sub> -tagged recombinant protein produced in <i>E. coli</i> as inclusion bodies"	18-1134-37
<b>Multi-step purification</b>	
Application Note, "Purification of a labile, oxygen sensitive enzyme for crystallization and 3D structural determination"	18-1128-91
Application Note, "Purification of a recombinant phosphatase using pre-programmed generic templates for different chromatographic techniques"	18-1142-32
<b>Detection</b>	
Application Note, "ECL Western and ECL Plus Western blotting"	18-1139-13
<b>Column Packing</b>	
Column Packing Video PAL	17-0893-01
Column Packing Video NTSC	17-0894-01

## Ordering information

Product	Quantity	Code No.
<b>GST fusion proteins</b>		
<b>Protein Amplification</b>		
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 <i>E. coli</i> B21		
<b>Purification</b>		
GST MicroSpin Purification Module	50 purifications	27-4570-03
MicroPlex 24 Vacuum	1 system	27-3567-01
GSTrap FF	2 x 1 ml	17-5130-02
	5 x 1 ml	17-5130-01
	1 x 5 ml	17-5131-01
Glutathione Sepharose 4 Fast Flow	25 ml	17-5132-01
	100 ml	17-5132-02
	500 ml	17-5132-03
Glutathione Sepharose 4B	10 ml	17-0756-01
	100 ml	27-4574-01
<b>Detection</b>		
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
<b>(His)<sub>6</sub> fusion proteins</b>		
<b>Purification</b>		
His MicroSpin Purification Module	50 purifications	27-4770-01
MicroPlex 24 Vacuum	1 system	27-3567-01
HisTrap Kit	HiTrap Chelating HP columns (3 x 1 ml), accessories, pre-made buffers for up to 12 purifications	17-1880-01
HiTrap Chelating HP	5 x 1 ml	17-0408-01
	1 x 5 ml	17-0409-01
Chelating Sepharose Fast Flow	50 ml	17-0575-01
	500 ml	17-0575-02
	5 l	17-0575-04
<b>Detection</b>		
Anti-His antibody	170 µl	27-4710-01
HRP conjugated (anti-mouse) IgG	1 ml	NA931
<b>Cleavage</b>		
<b>Enzymes</b>		
Thrombin	500 units	27-0846-01
Factor Xa	400 units	27-0849-01
PreScission Protease	500 units	27-0843-01
<b>Removal of thrombin and Factor Xa</b>		
HiTrap Benzamidine FF (high sub)	2 x 1 ml	17-5143-02
	5 x 1 ml	17-5143-01
	1 x 5 ml	17-5144-01
Benzamidine Sepharose 4 Fast Flow (high sub)	25 ml	17-5123-10

Product	Quantity	Code No.
<b>Companion Products</b>		
<i>E. coli</i> B21	1 vial	27-1542-01
Isopropyl $\beta$ -D-thiogalactoside (IPTG)	1 g	27-3054-03
	5 g	27-3054-04
	10 g	27-3054-05
<b>Western Blotting</b>		
Hybond-P	10 sheets	RPN2020F
Hybond-ECL	10 sheets	RPN2020D
ECL Western Blotting		
Detection Reagents	for 1000 cm <sup>2</sup>	RPN2109
ECL Plus Western Blotting		
Detection System	for 1000 cm <sup>2</sup>	RPN2132
<b>HiTrap Columns: Affinity</b>		
HiTrap rProtein A FF	2 x 1 ml	17-5079-02
	5 x 1 ml	17-5079-01
	1 x 5 ml	17-5080-01
HiTrap Protein A HP	2 x 1 ml	17-0402-03
	5 x 1 ml	17-0402-01
	1 x 5 ml	17-0403-01
HiTrap Protein G HP	2 x 1 ml	17-0404-03
	5 x 1 ml	17-0404-01
	1 x 5 ml	17-0405-01
MABTrap Kit	HiTrap Protein G HP (1 x 1 ml), accessories, pre-made buffers for 10 purifications	17-1128-01
HiTrap IgY Purification HP	1 x 5 ml	17-5111-01
	5 x 1 ml	17-5110-01
HiTrap Blue HP	5 x 1 ml	17-0412-01
	1 x 5 ml	17-0413-01
GSTrap FF	2 x 1 ml	17-5130-02
	5 x 1 ml	17-5130-01
	1 x 5 ml	17-5131-01
HiTrap Chelating HP	5 x 1 ml	17-0408-01
	1 x 5 ml	17-0409-01
HisTrap Kit	HiTrap Chelating HP (3 x 1 ml), accessories, pre-made buffers for up to 12 purifications	17-1880-01
HiTrap Streptavidin HP	5 x 1 ml	17-5112-01
HiTrap Heparin HP	5 x 1 ml	17-0406-01
	1 x 5 ml	17-0407-01
HiTrap Benzamidine FF (high sub)	2 x 1 ml	17-5143-02
	5 x 1 ml	17-5143-01
	1 x 5 ml	17-5144-01
HiTrap NHS-activated HP	5 x 1 ml	17-0716-01
	1 x 5 ml	17-0717-01
<b>HiTrap Columns: IEX</b>		
HiTrap IEX Selection Kit 7 x 1 ml	HiTrap Q XL, 1 ml HiTrap SP XL, 1 ml HiTrap ANX FF (high sub), 1 ml HiTrap DEAE FF, 1 ml HiTrap CM FF, 1 ml HiTrap Q FF, 1 ml HiTrap SP FF, 1 ml	17-6002-33
HiTrap Q HP	5 x 1 ml	17-1153-01
	5 x 5 ml	17-1154-01

Product	Quantity	Code No.
<b>HiTrap Columns: IEX</b>		
HiTrap SP HP	5 x 1 ml	17-1151-01
	5 x 5 ml	17-1152-01
HiTrap Q XL	5 x 1 ml	17-5158-01
	5 x 5 ml	17-5159-01
HiTrap SP XL	5 x 1 ml	17-5160-01
	5 x 5 ml	17-5161-01
HiTrap ANX FF (high sub)	5 x 1 ml	17-5162-01
	5 x 5 ml	17-5163-01
HiTrap DEAE FF	5 x 1 ml	17-5055-01
	5 x 5 ml	17-5154-01
HiTrap CM FF	5 x 1 ml	17-5056-01
	5 x 5 ml	17-5155-01
HiTrap Q FF	5 x 1 ml	17-5053-01
	5 x 5 ml	17-5156-01
HiTrap SP FF	5 x 1 ml	17-5054-01
	5 x 5 ml	17-5157-01
<b>HiTrap Columns: HIC</b>		
HiTrap HIC Selection Kit 5 x 1 ml	HiTrap Butyl FF, 1 ml HiTrap Octyl FF, 1 ml HiTrap Phenyl FF (low sub), 1 ml HiTrap Phenyl FF (high sub), 1 ml HiTrap Phenyl HP, 1 ml	17-1349-01
HiTrap Phenyl FF (high sub)	5 x 1 ml	17-1355-01
	5 x 5 ml	17-5193-01
HiTrap Phenyl FF (low sub)	5 x 1 ml	17-1353-01
	5 x 5 ml	17-5194-01
HiTrap Phenyl HP	5 x 1 ml	17-1351-01
	5 x 5 ml	17-5195-01
HiTrap Butyl FF	5 x 1 ml	17-1357-01
	5 x 5 ml	17-5197-01
HiTrap Octyl FF	5 x 1 ml	17-1359-01
	5 x 5 ml	17-5196-01
<b>Desalting and Buffer Exchange</b>		
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



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**Amersham Biosciences** Björkgatan 30, SE-751 84 Uppsala, Sweden

**Amersham Biosciences UK Limited** Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England

**Amersham Biosciences Inc** 800 Centennial Avenue, PO Box 1327, Piscataway, NJ 08855, USA

**Amersham Biosciences Europe GmbH** Munzinger Strasse 9, D-79111 Freiburg, Germany

**Amersham Biosciences K.K.** Sanken Building, 3-25-1, Hyakunincho, Shinjuku-ku, Tokyo 169-0073, Japan

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