GE Healthcare

Recombinant Protein Purification Handbook

Principles and Methods

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Recombinant Protein Purification Handbook

Principles and Methods

Content

Introduction

This handbook is intended for those interested in the expression and purification of recombinant proteins. The use of recombinant proteins has increased greatly in recent years, as has the wealth of techniques and products used for their expression and purification. The advantages of using a protein/peptide tag fused to the recombinant protein to facilitate its purification and detection is now widely recognized. In some cases, tags may improve the stability and solubility of recombinant proteins.

The reader will be introduced to the initial considerations to be made when deciding upon host, vector, and use of a tagged or untagged protein. General guidelines for successful protein expression are also included. Advice is given on harvesting and extraction, handling of inclusion bodies, tag removal, and removal of unwanted salts and small molecules.

Purification of recombinant proteins can be performed manually or by using a chromatography system. The system can be operated manually or it can be automated to save time and effort. The purification can be performed on many scales, in columns of various sizes. Columns can be purchased prepacked with a chromatographic medium, or empty columns can be packed manually. Purification can also be performed in batch, with gravity flow, in SpinTrap™ columns using centrifugation, or in a 96-well plate format using MultiTrap™ products.

Proteins are purified using chromatography techniques that separate them according to differences in their specific properties, as shown in Figure 1. Tags enable recombinant proteins to be purified by affinity chromatography designed to capture the tagged recombinant protein based on biorecognition of the tag. Thus, several different recombinant proteins can be purified by the same affinity technique if they all have the same tag. In the same way, tags also allow the use of a common detection protocol for different recombinant proteins. Consequently, tagged proteins are simple and convenient to work with and, for many applications, a single purification step, using a commercially available chromatography column, is sufficient. This is clearly demonstrated in the specific chapters on the expression, purification, and detection of recombinant proteins fused with the commonly used histidine or glutathione S-transferase (GST) tags. A scheme for the general purification of histidine-tagged proteins is given in Figure 2. In addition, suggestions for the successful purification of untagged recombinant proteins by a single affinity chromatography step are also given in this handbook. When a higher degree of purity is required for either tagged or untagged recombinant proteins, a multistep purification will be necessary. This can become a straightforward task by choosing the right combination of purification techniques.

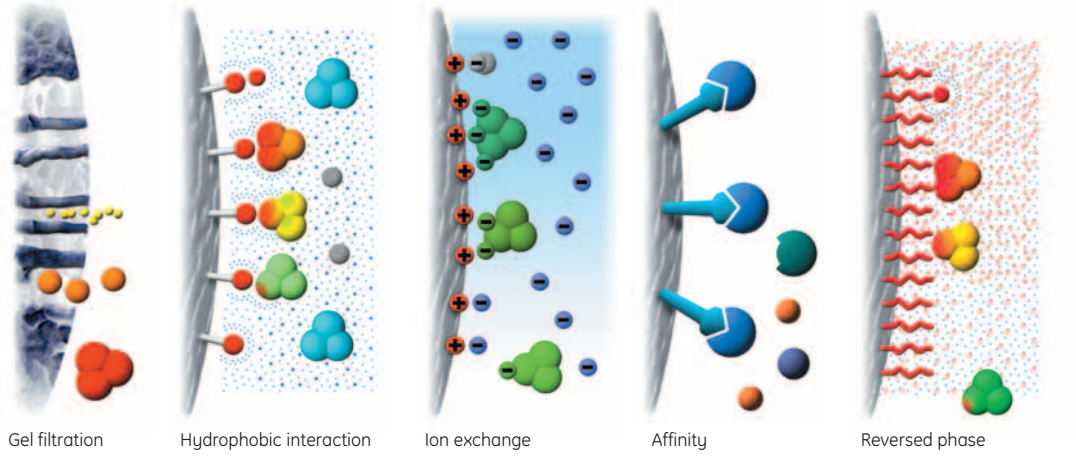

Fig 1. Separation principles in chromatographic purification.

In summary, this handbook aims to help the reader achieve a protein preparation that contains the recombinant protein of interest in the desired quantity and quality required for their particular needs. The quality of the recombinant protein can be reflected in its folding and biological activity.

Common acronyms and abbreviations

A_{280}	UV absorbance at specified wavelength (in this example, 280 nanometers)
AC	affinity chromatography
BCA	bicinchoninic acid
CDNB	1-chloro-2,4-dinitrobenzene
CF	chromatofocusing
CIPP	Capture, Intermediate Purification, and Polishing
CV	column volume
DAB	3,3'-diaminobenzidine
DNase	deoxyribonuclease
ELISA	enzyme-linked immunosorbent assay
FF	Fast Flow
Gua-HCl	guanidine-HCl
GF	gel filtration
GST	glutathione S-transferase
HIC	hydrophobic interaction chromatography
HMW	high molecular weight
HP	High Performance
HRP	horseradish peroxidase
IEX	ion exchange chromatography
IMAC	immobilized metal ion affinity chromatography
IPTG	isopropyl β-D-thiogalactoside
LMW	low molecular weight
MPa	megaPascal
M_r	relative molecular weight
N/m	column efficiency expressed as theoretical plates per meter
PBS	phosphate buffered saline
pI	isoelectric point, the pH at which a protein has zero net surface charge
psi	pounds per square inch
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene fluoride
r	recombinant, as in rGST and rBCA
RNase	ribonuclease
RPC	reverse phase chromatography
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride

Symbols

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

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

this symbol highlights troubleshooting advice to help analyze and resolve difficulties.

highlights chemicals, buffers and equipment.

outline of experimental protocol.

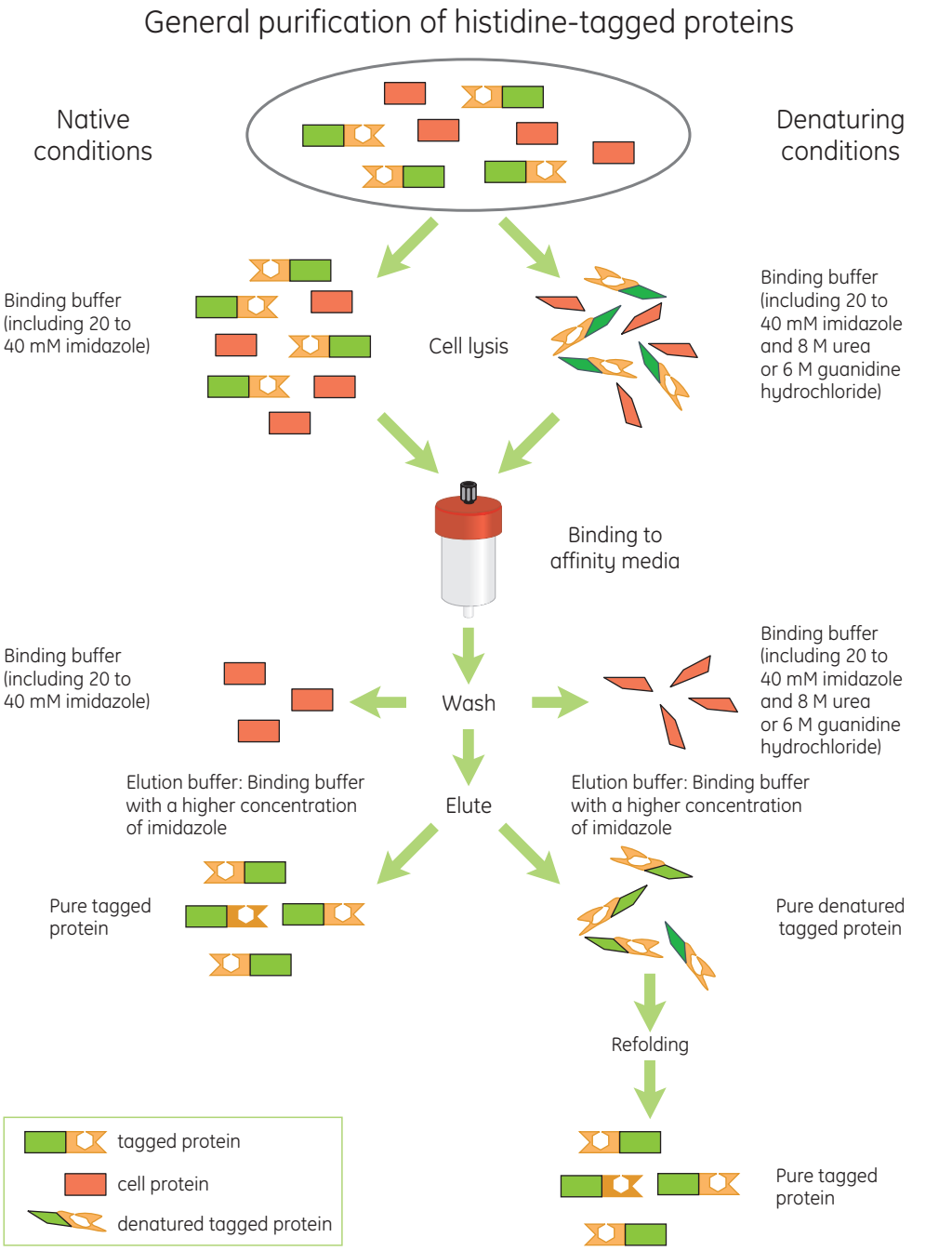

Fig 2. General purification workflow for histidine-tagged proteins (assumes use of Ni^{2+}-charged affinity media, but other metal-ion-charged media follow the same workflow).

Chapter 1
Expression and sample preparation

Components of the expression system

A protein expression system includes, among other things, a vector with an appropriate promoter and other regulatory sequences, along with the gene encoding the recombinant protein of interest. Vectors are available commercially for the expression of recombinant proteins either fused to a tag or untagged. Such expression vectors are designed with control regions to suit the specific host (for example, *E. coli* versus mammalian cells) and type of expression needed. The presence of resistance markers makes selection of the correct clones more straightforward. Expression of the recombinant protein can be constitutive or regulated, or it can be at a high or low level, depending on the specific requirements. The choice of vector is important because it affects so many of the processes that follow the cloning steps including expression, protein processing, and purification. The completed vector construct is used in a prokaryotic or eukaryotic organism, tissue, or cell line to produce the recombinant protein that may be of academic and/or industrial importance. The recombinant protein may then need to be detected, quantitated, and/or purified. Selection of a suitable expression system depends on the desired scale of production, the time and resources available, and the intended use of the recombinant protein. Several alternative systems for expression may be suitable.

Choice of host

Many host systems are available including bacteria, yeast, plants, filamentous fungi, insect or mammalian cells grown in culture, and transgenic animals or plants. Each host system has its own advantages and disadvantages, and it is important to consider these before final selection of host.

The choice of host affects not only the expression 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. Table 1 summarizes features of several expression systems.

Table 1. Features of several types of expression systems.

Processing	Bacteria	Yeast	Insect cells	Mammalian cells
Inclusion bodies	+/-	(+)/-	–	–
Secretion	+/–	+[1]	+	+
Glycosylation	–	+[2]	+	+
Proteolytic cleavage	+/–	+/–	–	–
Other post-translational modifications	–	+[3]	+	+

+ = Yes

– = No

[1] Constructs are often prepared to allow secretion of the protein. This eliminates the need for cell lysis, which requires more powerful methods for yeast than for E. coli.

[2] Yeast give more extensive glycosylation than insect cells and mammalian cells; this is a drawback of heterologous expression in yeast.

[3] Yeast lack some functions of post-translational modifications that exist in higher eukaryotes.

The location of product within the host will affect the choice of methods for isolation and purification of the product. For example, in addition to expressing the protein cytoplasmically, a bacterial host may secrete the protein into the growth medium, transport it to the periplasmic space, or store it as insoluble inclusion bodies within the cytoplasm (Fig 3). Expression in different parts of the cell will lead to varying amounts of cellular (contaminant) proteins that will need to be removed to obtain a pure target protein.

The main focus of this handbook is purification of soluble proteins from bacterial sources, as these are the most common systems. Purification of proteins expressed as inclusion bodies is also discussed (see Chapter 8).

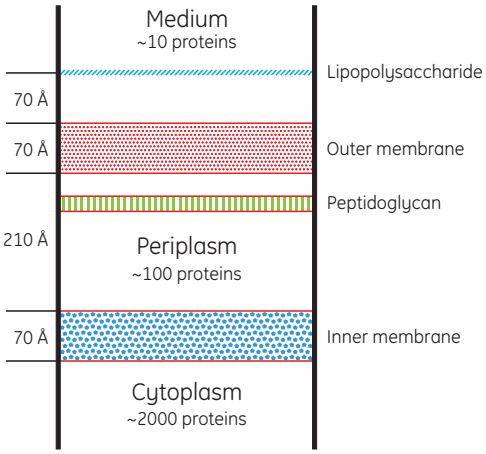

Fig 3. Schematic cross-section of the cell wall and typical number of protein species in *E. coli*.

Choice of vector

The choice of vector family is largely governed by the host. Once the host has been selected, many different vectors are available for consideration, from simple expression vectors to those that contain specialized sequences in order to secrete the recombinant proteins. In order to clone the gene of interest, all engineered vectors have a selection of unique restriction sites downstream of a transcription promoter sequence. Recent developments in cloning technology provide increased flexibility in the choice of host and vector systems, including options allowing the DNA sequence of interest to be inserted into multiple types of expression vectors.

The expression of a recombinant protein fused to a tag of known size and biological function can greatly simplify subsequent purification and detection (for expression method development and purification). In some cases, the protein yield can also be increased. Table 2 reviews some of the features of tagged protein expression, purification, and detection that may influence the final choice of vector.

Table 2. Advantages and disadvantages of tagged versus untagged protein expression.

Advantages	Disadvantages
Tagged proteins	
Solubility and stability can be improved.	Tag may interfere with protein structure and affect folding and biological activity.
Targeting information can be incorporated into a tag.	If tag needs to be removed, cleavage may not always be achieved at 100%, and sometimes amino acids may be left[1].
A marker for expression is provided.	
Simple purification is possible using affinity chromatography. Generic two-step purification protocols can often be set up for lab-scale protein production platforms.	
Detection of the tag instead of the target protein moiety allows for a generic detection method in, e.g., protein production platforms for structural biology.	
Some tags allow strong binding to chromatography media in the presence of denaturants, making on-column refolding possible.	

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Table 2. Advantages and disadvantages of tagged versus untagged protein expression (continued).

Advantages	Disadvantages
Untagged proteins	
Tag removal is not necessary.	Purification and detection not as simple.
	Problems with solubility and stability may be difficult to overcome, reducing potential yield.

[1] The effectiveness of proteases used for cleavage may be decreased by substances, for example, detergents, in the protein preparation or by inappropriate conditions.

Choice of tag

The two most commonly used tags are $(histidine)_6$ and GST. Other polyhistidine tags consisting of between 4 and 10 histidine residues are also used; they may provide useful alternatives to the $(histidine)_6$ tag if specific requirements exist for purification.

As for the selection of host and vectors, the decision to use either a histidine or a GST tag must be made according to the requirements of the specific application. Table 3 highlights some key features of these tags that should be considered.

Table 3. Choice of tag.

Histidine tag	GST 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. The GST tag may also increase the solubility of expressed proteins.
Selection of purification products available for any scale.	Selection of purification products available for any scale.
Small tag may not need to be removed (e.g., tag is weakly immunogenic so fusion partner can be used directly as an antigen in antibody production). Site-specific proteases enable cleavage of tag if required. TEV protease is often used to cleave off histidine tags. *Note:* Enterokinase sites that enable tag cleavage without leaving behind extra amino acids are preferable.	Site-specific protease (PreScission™ Protease) enables highly specific cleavage at 4ºC. The protease is also easily removed because it is itself GST tagged (see Chapter 5).
Histidine tag is easily detected using an immunoassay.	GST tag easily detected using an enzyme or immunoassay.
Simple purification. Purification can be performed under denaturing conditions if required. Allows on-column refolding. *Note:* Imidazole may cause precipitation in rare cases. Buffer exchange to remove imidazole may be necessary.	Simple purification. Very mild elution conditions minimize risk of damage to functionality and antigenicity of target protein. Buffer exchange may be desirable to remove reduced glutathione.
Histidine-dihydrofolate reductase tag stabilizes small peptides during expression.	GST tag can help stabilize folding of recombinant proteins.
Small tag is less likely to interfere with structure and function of fusion partner.	Tagged proteins form dimers.

GE Healthcare provides a variety of solutions for purification of histidine- and GST-tagged proteins. Chapters 3 to 5 discuss these solutions in detail. GE Healthcare provides purification solutions for other tagged proteins as well, including the calmodulin-binding peptide tag, the protein A tag, and biotinylated peptide tags. Recombinant proteins fused to the calmodulin-binding peptide can be purified by Calmodulin Sepharose™ 4B. Protein A-tagged proteins can be purified using IgG Sepharose Fast Flow. Recombinant proteins with a biotinylated peptide tag can be purified using HiTrap™ Streptavidin HP columns or by using Streptavidin Sepharose High Performance.

Sample preparation

The key to optimizing expression of tagged proteins is the capability to screen crude lysates from many clones so that optimal expression levels and growth conditions can be readily determined. This can easily be accomplished using the prepacked 96-well plates, His MultiTrap HP and His MultiTrap FF, or GST MultiTrap 4B and GST MultiTrap FF (see Chapters 3 and 5). Once conditions are established, the researcher is ready to prepare large-scale cultures of the desired clones. The samples are then processed and prepared for purification. Various methods for the purification of tagged proteins are available, depending on the expression system (host and vector) and the tag used. An overview of the sample preparation process is depicted in Figure 4.

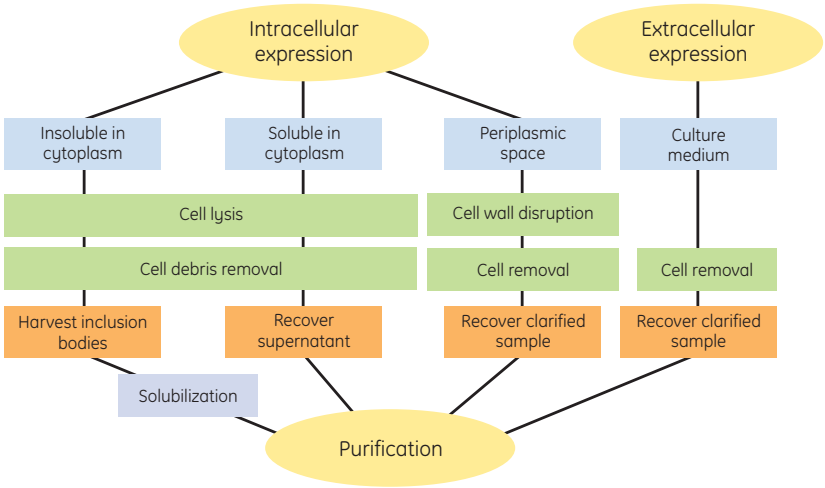

Fig 4. Overview of sample preparation.

Yield of recombinant proteins is highly variable and is affected by the nature of the tagged protein, the host cell, and the culture conditions. Recombinant protein yields can range from 0 to 10 mg/l. Table 4 can be used to approximate culture volumes based on an average yield of 2.5 mg/l.

Table 4. Recombinant protein yields.

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 lysate	0.5 ml	1 ml	20 ml	200 ml	1000 ml

For specific sample preparation steps, see Chapter 3 for histidine-tagged proteins and Chapter 5 for GST-tagged proteins.

Cell harvesting and extraction

Cell harvesting and extraction procedures should be selected according to the source of the protein, such as bacterial, plant, or mammalian, intracellular or extracellular. Harvesting, in which the cells are separated from the cell culture media, generally involves either centrifugation or filtration. Refer to standard protocols for the appropriate methodology based on the source of the target protein.

Selection of an extraction technique depends as much on the equipment available and scale of operation as on the type of sample. Examples of common extraction processes for recombinant proteins are shown in Table 5. In many situations, researchers may select a combination of these methods to achieve optimal results.

Table 5. Common sample extraction processes for recombinant proteins.

Extraction process	Typical conditions	Comment
Gentle		
Cell lysis (osmotic shock)	2 volumes water to 1 volume packed prewashed cells.	Lower product yield but reduced protease release.
Enzymatic digestion	Lysozyme 0.2 mg/ml, 37°C, 15 min.	Lab scale only, often combined with mechanical disruption.
Moderate		
Grinding with abrasive, e.g., glass beads	Add glass beads to prewashed cells, vortex, centrifuge, repeat up to five times, pooling supernatants.	Physical method. Chemical conditions are less important for cell lysis but may be important for subsequent removal of cell debris and purification steps.
Freeze/thaw	Freeze cells, thaw, resuspend pellet by pipetting or gentle vortexing in room-temperature lysis buffer. Incubate, centrifuge, retain supernatant.	Several cycles.
Vigorous		
Ultrasonication or bead milling	Follow equipment instructions.	Small scale; release of nucleic acids may cause viscosity problems (may add DNase to decrease viscosity); inclusion bodies must be resolubilized.
Manton-Gaulin homogenizer	Follow equipment instructions.	Large scale.
French press	Follow equipment instructions.	Lab scale.
Fractional precipitation	See Appendix 3.	Precipitates must be resolubilized.

The results obtained from cell lysis depend on several factors, including sample volume, cell concentration, time, temperature, energy input (speed of agitation, pressure, etc.), and physical properties of the cell lysis device.

- Use procedures that are as gentle as possible because too vigorous cell or tissue disruption may denature the target protein or lead 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.
- The release of nucleic acids may cause viscosity problems (addition of DNase may decrease viscosity). Frequently, protease inhibitors are needed to reduce protein breakdown during extraction. Fractional precipitation (see Appendix 3) may reduce the presence of proteases.
- In bacterial and yeast expression systems, the recombinant protein may often be contained in inclusion bodies. Extraction requires solubilization of the inclusion bodies, usually in the presence of denaturants, followed by refolding before or after purification. Refer to Chapter 8 for more information.

Preparation for chromatographic purification

Samples for chromatographic purification should be clear and free from particulate matter. Simple steps to clarify a sample before beginning purification will avoid clogging the column, may reduce the need for stringent washing procedures, and can extend the life of the chromatographic medium. An exception to this rule is when purifying a histidine-tagged protein using HisTrap™ FF crude columns or kit, His GraviTrap™ columns, His MultiTrap products (discussed in Chapter 3), or when purifying a GST-tagged protein using GST MultiTrap products (discussed in Chapter 5). Use of any of these products eliminates the need to clarify the sample and will therefore speed up the purification procedure. This may be very important when purifying sensitive proteins, as a means to preserve their activity.

Major parameters to consider when preparing a sample for chromatographic purification include:

- Clarification (except for "crude" and GraviTrap columns as well as MultiTrap products; see above)
- Stabilization of target protein (protease inhibition, pH, ionic state, reducing agents, stabilizing additives, etc.)
- Suitable conditions for chromatographic purification to work (mainly absorption, optimizing binding of target protein and minimizing binding of contaminants)
- Available equipment
- Practicality and convenience (sample size, filtration/centrifugation equipment, etc.)

Protein stability

In the majority of cases, biological activity needs to be retained after purification. Retaining the activity of the target molecule is also an advantage when following the progress of the purification, because detection of the target molecule often relies on its biological activity. Denaturation of sample components often leads to precipitation or enhanced nonspecific adsorption, both of which will impair column function. Hence, there are many advantages to checking the stability limits of the sample and working within these limits during purification.

Proteins generally contain a high degree of tertiary structure, kept together by van der Waals' forces, ionic and hydrophobic interactions, and hydrogen bonding. Any conditions capable of destabilizing these forces may cause denaturation and/or precipitation. By contrast, peptides contain a low degree of tertiary structure. Their native state is dominated by secondary structures, stabilized mainly by hydrogen bonding. For this reason, peptides tolerate a much wider range of conditions than proteins. This basic difference in native structures is also reflected in that proteins are not easily renatured, while peptides often renature spontaneously. Protein quaternary structure and protein complexes may pose additional challenges to a successful protein purification. Protein complexes are often held together by weak interactions that require mild purification conditions, and perhaps removal of incomplete species of the complex. Some proteins require coenzymes or cofactors to be active, and membrane proteins may need lipids from their natural environment in the cell membrane to maintain their native structure.

It is advisable to perform stability tests before beginning to develop a purification protocol. The list below may be used as a basis for such testing:

- Test pH stability in steps of one pH unit between pH 2 and pH 9.
- Test salt stability with 0 to 2 M NaCl and 0 to 2 M $(NH_4)_2SO_4$ in steps of 0.5 M (include buffering agents as well).
- Test the temperature stability in 10°C steps from 4°C to 40°C. At a minimum, first test in the cold room and at ambient temperature (22°C).

- Test the stability and occurrence of proteolytic activity by leaving an aliquot of the sample at room temperature overnight. Centrifuge each sample, if possible, and measure activity and UV absorbance at 280 nm in the supernatant. Run an SDS-polyacrylamide gel to check the size of the target protein. Sometime taking a UV-VIS spectrum (190 to 800 nm) may be very useful (e.g., for cytochromes) because active structure may be required for native spectra.

Sample clarification

Centrifugation and filtration are standard laboratory techniques for sample clarification and are used routinely when handling small samples.

It is highly recommended to centrifuge and filter any sample immediately before chromatographic purification, unless purifying a histidine-tagged protein using HisTrap FF crude columns or kit, His GraviTrap columns, or His MultiTrap products (discussed in Chapter 3), or when purifying a GST-tagged protein using GST MultiTrap products (discussed in Chapter 5).

A clarified sample that is not used immediately may within minutes start to precipitate. In this situation, reclarification is recommended.

Keeping samples on ice until use is often recommended, even when purification is performed at room temperature.

Centrifugation

Centrifugation removes most particulate matter, such as cell debris. If the sample is still not clear after centrifugation, use filter paper or a 5 µm filter as a first step and one of the filters listed in Table 6 as a second step.

For small sample volumes or proteins that adsorb to filters, centrifuge at 10 000 x g for 15 min.

For cell lysates, centrifuge at 40 000 to 50 000 x g for 30 min (may be reduced to 10 to 15 min if speed is of the essence).

Serum samples can be filtered through glass wool after centrifugation to remove any remaining lipids.

Use the cooling function of the centrifuge and precool the rotor by storing it in the cold room (or by starting to cool the centrifuge well in advance with the rotor in place).

Filtration

Filtration removes particulate matter. Membrane filters that give the least amount of nonspecific binding of proteins are composed of cellulose acetate or polyvinylidene fluoride (PVDF). For sample preparation before chromatography, select a filter pore size in relation to the bead size of the chromatographic medium as shown in Table 6.

Table 6. Selecting filter pore sizes.

Nominal pore size of filter	Particle size of chromatographic medium
1 µm	90 µm and greater
0.45 µm	30 or 34 µm
0.22 µm	3, 10, 15 µm or when extra-clean samples or sterile filtration is required

Check the recovery of the target protein in a test run. Some proteins may adsorb nonspecifically to filter surfaces.

Filters become "saturated" — that is, they have a certain capacity. It may be necessary to check the capacity when setting up a protocol.

Desalting and buffer exchange

Desalting columns are suitable for many different sample volumes and will rapidly remove low-molecular-weight contaminants in a single step at the same time as transferring the sample into the correct buffer conditions. If desalting is the first chromatographic step, clarification will be needed. Centrifugation and/or filtration of the sample before desalting is recommended. Detailed procedures for buffer exchange and desalting are given in Chapter 9.

Dialysis and centrifugal ultrafiltration/concentration are also options for desalting and/or buffer exchange, but the speed of using a desalting column makes it an especially attractive option.

The need for changed condition can sometimes be met simply by dilution (to reduce ion strength), addition [to increase ammonium sulfate concentration for hydrophobic interaction chromatography (HIC)], or titration to adjust pH.

At laboratory scale, when samples are reasonably clean after filtration or centrifugation, the buffer exchange and desalting step can be omitted. For affinity chromatography or ion exchange chromatography, it may be sufficient to adjust the pH of the sample and, if necessary, adjust the ionic strength of the sample.

Rapidly process small or large sample volumes. Use before and/or between purification steps, if needed (remember that each extra step can reduce yield and that desalting also dilutes the sample).

Remove salts from proteins with molecular weight M_r > 5000.

Use 100 mM ammonium acetate or 100 mM ammonium hydrogen carbonate if volatile buffers are required.

Detection and quantitation

Detection and quantitation of the target protein are needed when optimizing purification protocols. For over-expressed proteins, the high concentration in itself can be used for detection of the target protein fraction in a chromatogram, but in such a case verification of the identify of the protein in the final preparation is needed. Specific detection of tagged proteins can often be accomplished by analyzing the presence of the tag by activity or immunoassay, or simply by the spectral properties of the tag. This may be especially important when multiple constructs with the same tag are prepared in high-throughput platforms. Specific detection of the target protein can be obtained by functional assays, immunodetection, and mass spectrometry. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is the key method for checking purity of proteins. The target protein band can often be identified using the apparent M_r obtained by including molecular weight markers in the analysis. Subsequent verification of protein identity should always be obtained. Optimizing purification protocols may require functional assays to assess the intactness of the target protein. Detection methods specific for histidine- and GST-tagged proteins are discussed in Chapters 3 and 5, respectively. In general:

- The relative yield of tagged protein can often be determined by measuring the absorbance at 280 nm (suitable for both histidine- and GST-tagged proteins), because the purity after a single purification step is high, that is, most of the eluted material may be considered to be the target protein. The extinction coefficient of the target protein will be needed. A good estimation may be obtained by theoretical calculation from the amino acid composition of the protein.
- The yield of protein may also be determined by standard chromogenic methods (e.g., Lowry, BCA™ protein assay, Bradford, etc.).

- Immunoassays can be used for quantitation if a suitable standard curve can be produced. In this case, it is not necessary to purify the tagged protein so long as a purified standard is available. Therefore, these techniques may be used for quantitation during protocol development. The immunoassay technique is also particularly suitable for screening large numbers of samples when a simple yes/no answer is required (e.g., when testing fractions from a chromatographic run).

Assessing protein expression

Suboptimal expression of the target protein can be addressed by various methods, based on the cause of the problem. If no target protein is detected in the extract, this may mean that the insert has been cloned in an incorrect reading frame. It is essential that the protein-coding DNA sequences are cloned in the proper translational reading frame in the vector. The best way to verify that the insert is in-frame is to sequence the cloning junctions.

If yield of the target protein is low, it may be because the culture conditions have not been optimized for its expression. Investigate the effect of cell strain, medium composition, incubation temperature, and induction conditions (if applicable). Exact conditions will vary for each tagged protein expressed.

With *E. coli* systems, analyze a small aliquot of an overnight culture by, for example, SDS-PAGE, and if available, use an activity assay.

Generally, a highly expressed protein will be visible by Coomassie™ blue staining when 5 to 10 µl of an induced culture whose A_{600} is ~1.0 is loaded on the gel. Nontransformed host *E. coli* cells and cells transformed with the parental vector should be run in parallel as negative and positive controls, respectively.

The presence of the tagged protein in a total cell extract and its absence from a clarified lysate may indicate the presence of inclusion bodies. Check for inclusion bodies using light microscopy. They are often visible as dense spots in the cells. Refer to Chapter 8 for information on handling inclusion bodies.

It is also worthwhile to check for expression by immunoblotting. Run an SDS-PAGE of induced cells and transfer the proteins to a nitrocellulose or PVDF membrane (such as Hybond™-C or Hybond-P). Detect tagged protein using anti-histidine or anti-GST antibody, as appropriate, or an antibody directed toward the specific target protein. Some tagged proteins may be masked on SDS-PAGE by a bacterial protein of approximately the same molecular weight. Immunoblotting can be used to identify tagged proteins in these cases.

If the target protein is present in the post-lysate pellet, consider methods to enrich it. Alternatively, choose to secrete the product or add a stabilizing tag.

If the target protein is adsorbed to cell debris, test extraction at varying ionic strengths and pH to dissociate it.

Occasionally, a high basal level of expression is observed, and this may pose problems of its own (e.g., this is a major concern if the expressed protein is toxic). The cause may be a leaky promoter. Different vector systems rely on different constitutive and induced promoters, thus the most straightforward means of addressing this problem is to try another system. It is also possible that the vector is simply not compatible with the expression host; trying another vector or host should alleviate this problem.

Various modifications to recombinant proteins can arise during growth, and these too may affect expression levels. These modifications include aggregation; misfolding and random disulfide bridges; deamidation of asparagine and glutamine; oxidation of methionine; proteolytic cleavage; and other modifications such as glycosylation, phosphorylation, and acylation. Discussion of these modifications is beyond the scope of this handbook, but a simple first approach to reducing or eliminating problems relating to them is, as above, to investigate the effect of cell strain, medium composition, incubation temperature, and induction conditions. Exact conditions will vary for each tagged protein expressed.

Analytical tools useful for determining if a recombinant protein is correctly expressed are summarized in Table 7.

Table 7. Analytical tools for assessing protein expression.

Analytical tool	Characteristic being assessed
SDS-PAGE and immunoblotting	Size
	Proteolytic cleavage
Native PAGE	Aggregation
Isoelectric focusing (IEF)	Heterogeneity
Tests for biological activity	Stability at different pH, ionic strengths, protein concentrations, detergent concentrations
N-terminal sequencing	Heterogeneous N-terminus
Mass spectrometry	Size, sequence hetereogeneities, post-translational heterogeneities, chemical modifications of amino acid residues
C-terminal sequencing (difficult method performed in specialized labs)	Truncated forms

Chapter 2
Manual and automated purification

Recombinant proteins are needed for research and industrial purposes in different qualities (e.g., with native structure or denatured) and quantities (from microgram to gram scales). One needs to choose a purification method that will yield protein of a quality and quantity that fits the intended use. The number of samples that must be purified is also an important consideration. It may be possible to save valuable time and protein samples by investing in a chromatography system.

Tagged recombinant proteins for simple purification

When a recombinant protein is fused to a peptide or protein tag, such as histidine or glutathione S-transferase (GST), the properties of the tag can be exploited for purification purposes. Affinity chromatography methods have been developed for each of the commonly used tags, and there is a good chance of a successful purification of a tagged protein in a single step.

Manual purification techniques

For small-scale purification of tagged proteins, a single affinity chromatography step with a simple elution by a step gradient is usually sufficient. Manual purification can be performed in batch or by using gravity-flow or spin columns, or 96-well plates.

When a tagged protein is purified by a batch method, the protein sample is added to a purification medium usually in a disposable plastic tube. The medium is then washed and the tagged protein is eluted. The batch method is suited to purification on a small scale.

A tagged protein can also be purified by simply passing the protein sample through a disposable column prepacked with an appropriate medium. There are columns especially designed for use by gravity flow, for example, for histidine-tagged proteins His GraviTrap. A 1-ml His GraviTrap column can purify approximately 40 mg of a histidine-tagged protein. In addition, there are HiTrap columns suitable for use with a syringe or peristaltic pump for both histidine- and GST-tagged proteins (HisTrap and GSTrap™ columns, respectively). In general the binding capacity for a histidine-tagged protein using a HisTrap column is at least 40 mg per ml of medium. HiTrap columns can also be connected to ÄKTAdesign™ chromatography systems (see the next section in this chapter). The different connections are easy to make because HiTrap columns come with all necessary connectors included.

When purification can be performed on a small scale or for expression screening, 96-well plates or prepacked spin columns are convenient. For both histidine- and GST-tagged proteins, prepacked 96-well plates (MultiTrap) are available. Samples are pipetted into the prepacked wells of the plate, with wash and elution by centrifugation or vacuum. Each well has a capacity to purify up to about 1 mg of histidine-tagged protein (His MultiTrap) and 0.5 mg of GST-tagged proteins (GST MultiTrap). Using these plates, 96 samples can be processed simultaneously. When many plates require processing, a robotic system can be used for plate handling.

Prepacked spin columns (SpinTrap) designed for use in a microcentrifuge can offer an alternative to screening using 96-well plates. His SpinTrap is such a spin column designed for the rapid purification and screening of histidine-tagged proteins. Each column has the capacity to purify approximately 750 µg of histidine-tagged protein. The GST SpinTrap Purification Module includes prepacked spin columns for purifying up to 400 µg of GST-tagged protein per column.

Automated purification using ÄKTAdesign chromatography systems

A chromatography system should be used when reproducible results are important and when manual purification becomes too time-consuming and inefficient. Manual purification can become inefficient when processes have to be repeated to obtain enough purified sample, when large sample volumes have to be handled or when there are many different samples to be purified. In addition, the quality and reproducibility of protein separations can be improved by using a chromatography system. Systems provide more control than manual purification because of the ability to automatically monitor the progress of the purification. Systems are robust and convenient to use. Not only can systems perform simple step-gradient elution, but they can also provide high-resolution separations using accurately controlled linear-gradient elution. They can work at the high flow rates of modern media. Following is a description of the use of ÄKTAdesign chromatography systems suited to purification of tagged proteins.

ÄKTAprime™ plus (Fig 5) is an economical and easy-to-learn system for the purification of tagged proteins. With push button control, it offers simple one-step purification of proteins. This system includes preprogrammed methods for the purification of histidine- and GST- tagged proteins. In fact, there are preprogrammed methods for the use of any HiTrap column. In addition, recovery of the recombinant protein is often better than when the same protein is purified manually. With optimized purification protocols and prepacked columns, yields and purity are highly consistent. Together with the appropriate columns, tagged proteins can be purified in a single chromatography step on ÄKTAprime plus from microgram to gram scale.

Fig 5. ÄKTAprime plus.

Fig 6. ÄKTAexplorer.

Purification of tagged proteins can also be performed on more advanced chromatography systems. ÄKTAexplorer™ (Fig 6) is a system where multiple samples (up to eight) of a tagged protein can be automatically purified in a single step. This is very convenient because manual work between samples is eliminated. Like ÄKTAprime plus, ÄKTAexplorer is a chromatography system that allows easy purification of proteins from microgram to gram scale.

Another advantage offered by ÄKTAexplorer is that multiple samples of tagged proteins can be purified automatically in multiple chromatography steps with the add-on ÄKTA™ 3D plus Kit. Using more than a single chromatography step is important when a single affinity step does not yield the purity required for a specific application or when a buffer-exchange or polishing step is needed after the affinity step. When using the kit together with ÄKTAexplorer 100, up to six samples of tagged proteins can be automatically purified in a single run, with protocols containing one or two steps. When a protocol with three steps is selected, up to four samples can be purified. Often affinity chromatography (AC) is the first step, and some protocols have a second purification step, gel filtration (GF), or ion exchange (IEX). For added convenience and reproducibility, the purification protocols use recommended prepacked columns. This system is capable of producing up to 50 mg of tagged protein of greater than 90% purity per sample. These proteins are useful in structural and functional studies or in drug target screening.

ÄKTAxpress™ (Fig 7) is recommended when higher automation is required. ÄKTAxpress is a modular system (from 1 to 12 modules controlled by one computer) for automated parallel purification of up to 48 samples of tagged proteins with purification protocols containing up to four steps. The purification protocols may begin with AC followed by other purification steps such as desalting, IEX, and GF. In addition, automatic on-column or off-column tag-removal steps can be integrated in the purification protocol. All modules can work on the same protocol, or each module can work independently. The purification protocols use prepacked columns and deliver purified samples of up to 50 mg of tagged protein of > 95% purity. These purified samples are suitable, for example, for use in structural studies.

Fig 7. Four modules of ÄKTAxpress system.

There are other ÄKTAdesign systems available that can also be used for the purification of tagged proteins. Standard ÄKTAdesign configurations are given in Fig 8.

More details about methods for purification are given in Chapter 3 and 4 for histidine-tagged proteins and Chapter 5 for GST-tagged proteins.

Table 8. Ways of working with standard ÄKTAdesign configurations.

	Standard ÄKTAdesign configurations						
Way of working	**ÄKTA prime plus**	**ÄKTA purifier™**	**ÄKTA explorer**	**ÄKTA xpress**	**ÄKTA pilot™**	**ÄKTA crossflow™**	**ÄKTA process™**
Manufacturing and production					•		•
UNICORN™ software		•	•	•	•	•	•
One-step simple purification	•			•			
Reproducible performance for routine purification	•	•	•	•	•	•	•
System control and data handling for regulatory requirements		•	•	•	•	•	•
Automatic method development and optimization		•	•		•	•	
Automatic buffer preparation		•	•				
Automatic pH scouting		•	•				
Automatic media or column scouting			•		•		
Automatic multistep purification			•	•			
Method development and scale-up			•		•	•	
Sanitary design cGMP					•	•	•
Scale-up, process development, and transfer to production					•	•	

ÄKTAprime plus

ÄKTApurifier

ÄKTAexplorer

ÄKTAxpress

ÄKTApilot

ÄKTAcrossflow

ÄKTAprocess

Fig 8. The standard ÄKTAdesign configurations.

Chapter 3
Purification of histidine-tagged recombinant proteins

Introduction

Histidine-tagged proteins have a high selective affinity for Ni^{2+} and several other metal ions that can be immobilized on chromatographic media using chelating ligands. Consequently, a protein containing a histidine tag will be selectively bound to metal-ion-charged media such as Ni Sepharose High Performance (HP) and Ni Sepharose 6 Fast Flow (FF) while other cellular proteins will not bind or bind weakly. This chromatographic technique is often termed immobilized metal ion affinity chromatography (IMAC). In general, the histidine-tagged protein is the strongest binder among all the proteins in a crude sample extract (from, for example, a bacterial lysate). Eukaryotic extracts often have slightly more proteins that can bind. Moreover, histidine tags are small and generally less disruptive than other tags to the properties of the proteins on which they are attached. Because of this, tag removal may not always be a priority.

Histidine-tagged protein expressed in *E. coli* can accumulate in two main forms, as biologically functional soluble proteins or as inclusion bodies. Inclusion bodies are insoluble aggregates of denatured or partly denatured protein that lack biological activity but often give a high yield of the recombinant protein. To restore biological function of proteins expressed as inclusion bodies, solubilization, refolding, and purification are necessary. This topic is discussed in more detail in Chapter 8.

Expression

General considerations for the expression of tagged proteins are discussed in Chapter 1, as are the factors that should be considered when selecting the vector and host.

Purification overview

Figure 9 gives an overview of a typical purification workflow for histidine-tagged proteins, including purification under denaturing conditions. On-column refolding and purification of histidine-tagged proteins are also discussed in Chapter 8.

For simple, one-step purification of histidine-tagged proteins, a range of products is available designed to meet specific purification needs. These products can be used for the purification of proteins containing polyhistidine tags of different lengths (four to 10 histidine residues). A tag that is six residues long $(histidine)_6$ is most common. Under the standard binding and elution conditions described in this handbook, a shorter tag, for example, $(histidine)_4$, will bind more weakly and a longer $(histidine)_{10}$ will bind more strongly as compared with $(histidine)_6$. This difference in binding strength can be used to advantage during purification. For example, because a longer tag binds more strongly, a higher concentration of imidazole can be included in the sample during loading (to prevent unwanted host cell proteins from binding) as well as be used during the washing step before elution. This can facilitate the removal of contaminants that may otherwise be copurified with a shorter tagged protein. For information on optimizing protein purification of histidine-tagged proteins, refer to Chapter 4.

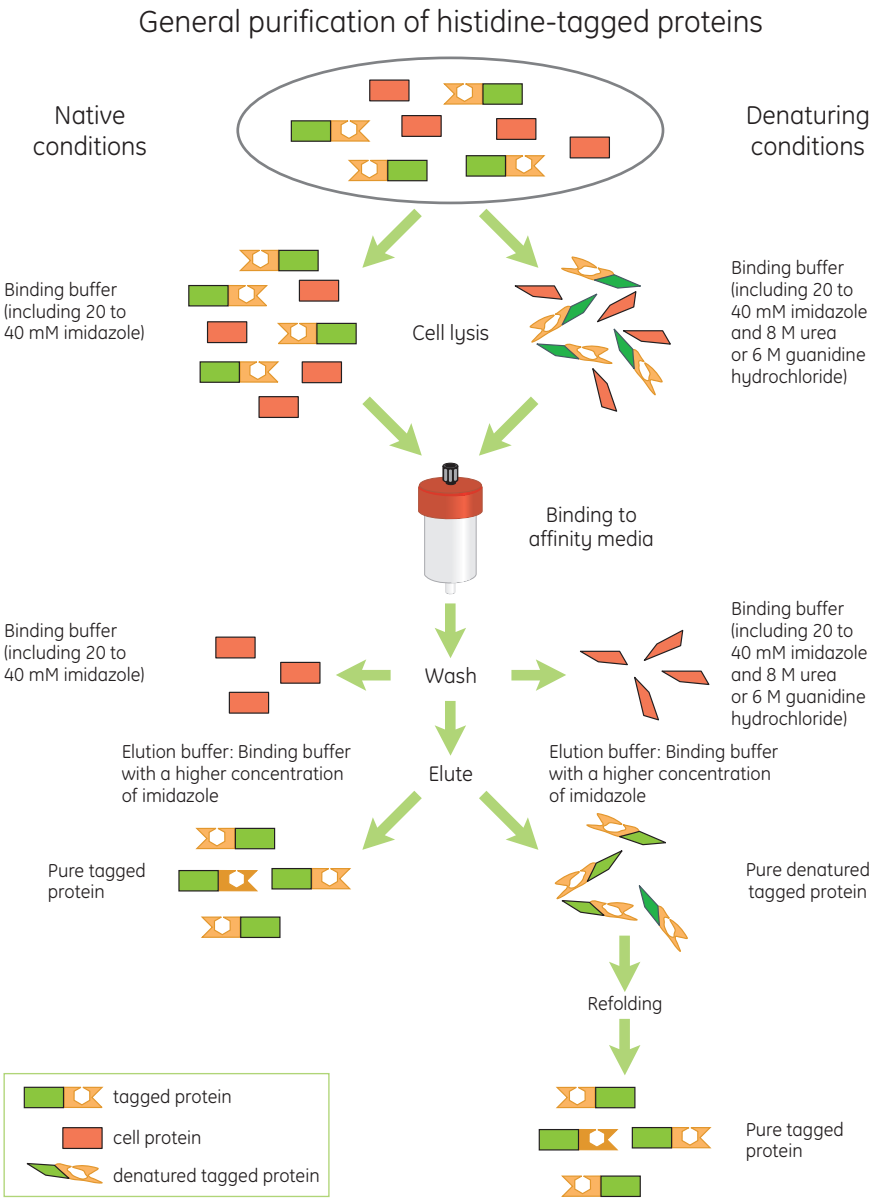

Fig 9. General purification workflow for histidine-tagged proteins (assumes use of Ni^{2+}-charged affinity media, but other metal-ion-charged media follow the same workflow).

General considerations

Types of media and formats

Media for purifying histidine-tagged proteins are available precharged with Ni^{2+} ions as well as uncharged. Uncharged media can be charged with different metal ions in order to adjust selectivity. Charged media from GE Healthcare include Ni Sepharose High Performance and Ni Sepharose 6 Fast Flow in lab packs and prepacked formats. Uncharged media include IMAC Sepharose High Performance and IMAC Sepharose 6 Fast Flow in lab packs and prepacked formats.

Ni Sepharose High Performance and Ni Sepharose 6 Fast Flow consist of highly cross-linked agarose beads with an immobilized chelating group. As the product names indicate, the media are precharged with Ni^{2+} ions. The media are compatible with all commonly used aqueous buffers, reducing agents,

denaturants such as 6 M guanidine-HCl (Gua-HCl) and 8 M urea, and a range of additives commonly used in protein purification. Refer to Appendix 1 for a list of characteristics of the media.

Different sizes and types of prepacked columns and 96-well filter plates together with easily packed Ni Sepharose High Performance and Ni Sepharose 6 Fast Flow bulk media (lab packs) provide fast, convenient alternatives to the traditional batch method of protein purification. 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.

We recommend always trying the precharged Ni Sepharose High Performance or Ni Sepharose 6 Fast Flow media first. If you determine that increased selectivity would be advantageous, next try applying other metal ions to one of the uncharged media. Test more than one metal ion to determine the one best suited for your separation. GE Healthcare offers several uncharged IMAC purification products for such purposes: convenient, prepacked 1-ml and 5-ml HiTrap IMAC HP and HiTrap IMAC FF and 20-ml HiPrep™ IMAC FF 16/10 columns, as well as IMAC Sepharose High Performance and IMAC Sepharose 6 Fast Flow bulk media. Refer to Appendix 1 for a list of characteristics of the media.

Monitor purification steps by one or more of the detection methods referred to later in this chapter. The choice of purification equipment should also be made according to the needs of the purification (see Chapter 2).

Metal ion

In general, Ni^{2+} is the preferred metal for purification of recombinant histidine-tagged proteins. Note, however, that in some cases it may be wise to test other metal ions, for example Zn^{2+} and Co^{2+}, as the strength of binding depends on the nature of the histidine-tagged protein as well as the metal ion. This topic is also discussed in Chapter 4.

Leakage of Ni^{2+} from Ni Sepharose Fast Flow and High Performance is low under all normal conditions. The leakage is lower than for other precharged IMAC media tested (see GE Healthcare Data File 11-0008-86). In addition, leakage of metal ions from IMAC Sepharose High Performance and IMAC Sepharose 6 Fast Flow is lower under normal conditions than is the case with other IMAC media tested. For very critical applications, leakage during purification can be reduced even further by performing a blank run before loading the sample (see purification procedures).

Working with nickel-containing products may produce an allergic reaction.

Buffers

Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.22 µm or 0.45 µm filter before use.

We recommend use of the His Buffer Kit (available separately) to eliminate time-consuming buffer preparation, thus promoting fast, reproducible, and convenient purification work. The kit contains phosphate buffer concentrates and highly pure 2 M imidazole stock solution optimized for rapid purification of histidine-tagged proteins.

We recommend binding at neutral to slightly alkaline pH (pH 7 to 8) in the presence of 0.5 to 1.0 M NaCl. Including salt in the buffers and samples eliminates ion-exchange effects but can also have a marginal effect on the retention of proteins.

Sodium phosphate buffers are often used. Tris-HCl can generally be used, but should be avoided in cases where the metal-protein affinity is weak, because it may reduce binding strength. Avoid chelating agents such as EDTA or citrate in buffers. Imidazole is usually used for elution of histidine-tagged proteins due to its efficiency at replacing the histidine tag by also interacting with the

metal ion. Low concentrations of imidazole should be used to wash out more weakly bound host cell proteins to increase the purity of the target protein. Use highly pure imidazole, which gives essentially no absorbance at 280 nm.

Membrane proteins must be purified in the present of a detergent in the sample and buffers. Notice that the NaCl concentration may have to be optimized to avoid precipitation. Proteins expressed as inclusion bodies can be solubilized in denaturants such as 8 M urea or 6 M Gua-HCl. The solubilized and denatured protein can then be purified in the presence of the denaturant. If on-column refolding is to be performed, an eluent with low concentration (or zero concentration) should be prepared. Refer to Chapter 8 for a discussion of working with inclusion bodies.

Samples containing urea can be analyzed directly by SDS-PAGE whereas samples containing Gua-HCl must be buffer-exchanged to a buffer with urea before SDS-PAGE, due to the high ionic strength of Gua-HCl solutions.

Imidazole

Imidazole competes with proteins for binding to Ni Sepharose and IMAC Sepharose. Equilibration buffer (binding and wash buffer) and sample should be complemented with a low concentration of imidazole to reduce nonspecific binding of host cell proteins. The initial low concentration of imidazole establishes a counter-ligand to the immobilized metal ion, which is important for controlled chromatography. At somewhat higher concentrations, imidazole may also decrease the binding of histidine-tagged proteins. The imidazole concentration in each step must therefore be optimized to ensure the best balance of high purity (low binding of host cell proteins) and high yield (strong binding of histidine-tagged target protein). The concentration of imidazole in the binding buffer and sample that will give optimal purification results is protein dependent, and is usually slightly higher (20 to 40 mM is recommended) for Ni Sepharose 6 Fast Flow and Ni Sepharose High Performance than for similar media on the market (see GE Healthcare Data File 11-0008-86 for a discussion of this topic). See Chapter 4 for a discussion on optimizing purification of histidine-tagged proteins by altering the imidazole concentration.

Use high-purity imidazole as this will give very low or no absorbance at 280 nm.

If imidazole needs to be removed from the protein, use a HiTrap Desalting, PD-10 Desalting, or HiPrep 26/10 Desalting column depending on the sample volume.

Alternative elution solutions

As alternatives to imidazole elution, histidine-tagged proteins can be eluted by other methods or combinations of methods; for example, lowering of pH within the range of 2.5 to 7.5 can be used. Below pH 4, metal ions will be stripped off the medium.

Note: It is not always possible to elute with lower pH when using a metal ion other than Ni^{2+}. This is protein and metal ion dependent.

EGTA and EDTA, which are strong chelating compounds, can also be used for elution, but they will strip the metal ions from the medium and thereby cause protein elution. The co-eluted metal ions will remain chelated in the protein solution, but are easily removed with a desalting column, such as HiTrap Desalting, PD-10 Desalting, or HiPrep 26/10 Desalting.

Note: The column needs to be recharged with metal ions before the next purification.

General procedure for sample preparation

For optimal conditions for growth, induction, and cell lysis of recombinant histidine-tagged proteins, please refer to established procedures. The following is a general procedure for sample preparation and cell lysis from bacterial cultures. Other established procedures may also work.

This procedure works well with the majority of the purification protocols included in this chapter. However, some modifications of the procedures are noted where relevant.

1 Harvest cells from the culture by centrifugation at 7000 to 8000 × g for 10 min or at 1000 to 1500 × g for 30 min at 4°C.

2. Discard the supernatant. Place the bacterial pellet on ice.

3. Dilute cell paste (bacterial pellet) by adding 5 to 10 ml of binding buffer for each gram of cell paste.

To prevent the binding of host cell proteins with exposed histidines, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).

4a. Enzymatic lysis: Add 0.2 mg/ml lysozyme, 20 µg/ml DNase, 1 mM $MgCl_2$, 1 mM Pefabloc™ SC or phenylmethylsulfonyl fluoride (PMSF) (final concentrations). Stir for 30 min at room temperature or 4°C, depending on the sensitivity of the target protein.

4b. Mechanical lysis: Disrupt cells by sonication on ice for approximately 10 min (in several short bursts), by homogenization with a French press (or other homogenizer), or by freezing/thawing at least five times.

Mechanical lysis time may have to be extended to obtain an optimized lysate for sample loading to avoid problems with back pressure. This is important when direct loading of unclarified, crude sample without any clarification is performed (using HisTrap FF crude columns). Different proteins have different sensitivity to cell lysis, and caution should be exercised to avoid heating and frothing of the sample. If the sonicated or homogenized unclarified cell lysate is frozen before use, precipitation and aggregation may increase. Additional sonication of the lysate can then prevent increased back-pressure problems when loading on the column.

5. Measure and adjust pH if needed.

Do not use strong bases or acids for pH adjustment, as this may increase the risk of precipitation.

The sample should be fully dissolved. To avoid column clogging, we recommend centrifugation and filtration through a 0.45 µm or 0.22 µm filter to remove cell debris or other particulate material. *Note:* this is **NOT** necessary when using HisTrap FF crude, His GraviTrap, His MultiTrap HP, or His MultiTrap FF.

If the sample is prepared in a buffer other than 20 mM phosphate buffer, 0.5 M NaCl, pH 7.4, adjust its NaCl concentration to 0.5 M and pH to 7 to 8. This can be achieved by addition of concentrated stock solutions, by dilution with the binding buffer, or by buffer exchange (on HiTrap Desalting, PD-10 Desalting, or HiPrep 26/10 Desalting column, depending on the sample volume). **IMPORTANT!** To minimize binding of host cell proteins, the sample should have the same concentration of imidazole as the binding buffer. The concentration of imidazole is protein dependent and should be determined empirically. We recommend starting with 20 to 40 mM imidazole.

If the recombinant histidine-tagged protein is expressed as inclusion bodies, they must be solubilized using 6 M Gua-HCl or 8 M urea, and the chosen denaturant must be present in all buffers during chromatography. Advice for working with inclusion bodies can be found in Chapter 8 and in the troubleshooting section later in this chapter.

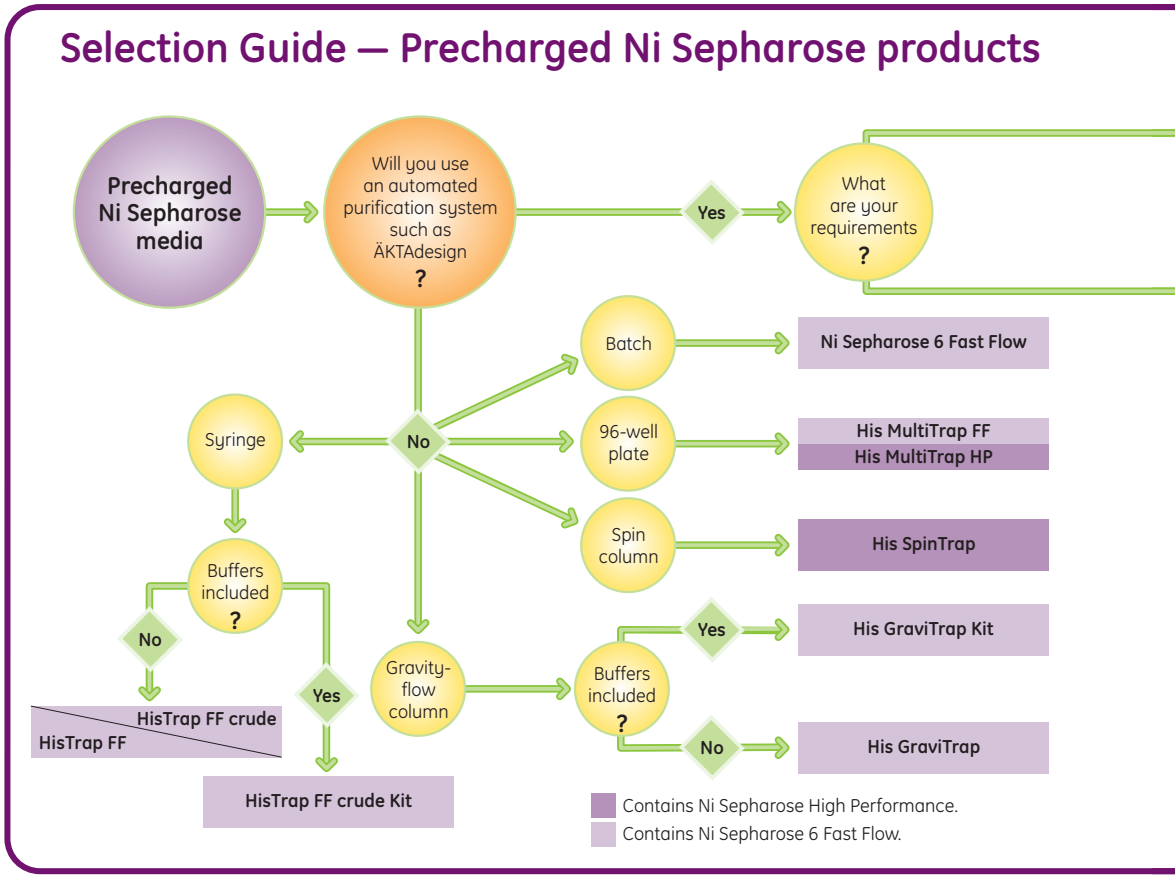

Fig 10. Selection guide for the precharged Ni Sepharose products.

Figure 10 provides a selection guide for the precharged Ni Sepharose products, and Table 9 describes these options in more detail. In general, Ni Sepharose High Performance is recommended when high resolution and high capacity are important, whereas Ni Sepharose 6 Fast Flow is recommended when scale-up is required. Similar information for the uncharged media follows later in this chapter, starting on page 72.

Table 9. Purification options for histidine-tagged proteins using precharged media.

Product	Format or column size	Approx. protein binding capacity	Description	High-throughput screening	Mini-preps	Batch/ gravity flow	Syringe	ÄKTAdesign system
Ni Sepharose High Performance	25 ml 100 ml	40 mg/ml	For high resolution and elution of a more concentrated sample (high-performance purification).	+	(+)	-	-	+

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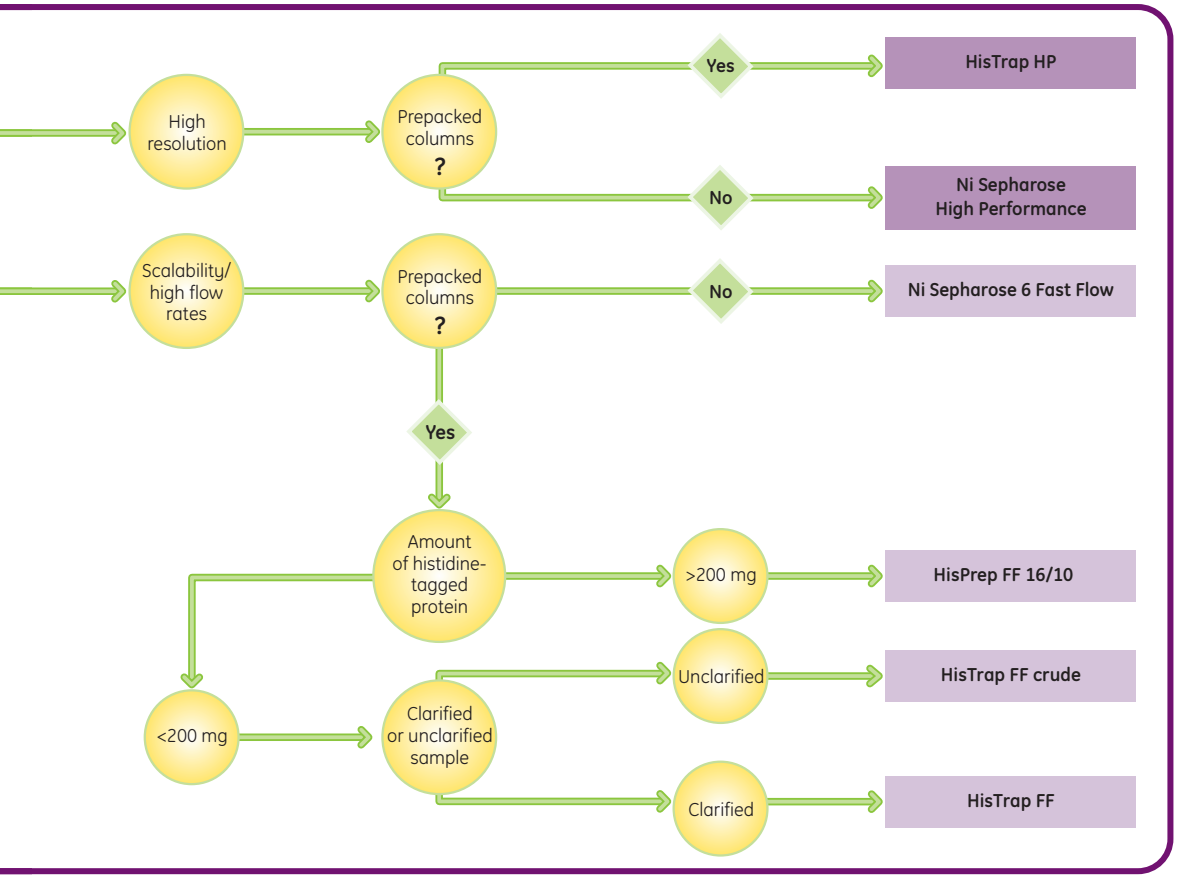


Table 9. Purification options for histidine-tagged proteins using precharged media (continued).

Product	Format or column size	Approx. protein binding capacity	Description	High-throughput screening	Mini-preps	Batch/ gravity flow	Syr-inge	ÄKTAdesign system
HisTrap HP	1 ml 5 ml	40 mg/ column 200 mg/ column	For use mainly with a peristaltic pump or chromatography system. For high resolution and elution of a more concentrated sample (high-performance purification).	-	-	-	(+)	+
His SpinTrap	100 µl	0.75 mg/ column	For simple minipreps of histidine-tagged proteins and rapid expression screening.	-	+	-	-	-
His MultiTrap HP	96-well filter plates	1 mg/ well	For high-throughput screening. Can use with robotics or manually by centrifugation or vacuum.	+	-	-	-	-

continues on following page

Table 9. Purification options for histidine-tagged proteins using precharged media (continued).

Product	Format or column size	Approx. protein binding capacity	Description	High-throughput screening	Mini-preps	Batch/gravity flow	Syringe	ÄKTAdesign system
Ni Sepharose 6 Fast Flow	5 ml 25 ml 100 ml 500 ml	40 mg/ml	Excellent for scale-up due to high capacity and high flow properties.	+	+	+	-	+
HisPrep™ FF 16/10	20 ml	800 mg/column	For use with a chromatography system. Scale-up purification.	-	-	-	-	+
HisTrap FF	1 ml 5 ml	40 mg/column 200 mg/column	For use with syringe, peristaltic pump, or chromatography system. Provides excellent flow properties. Scale-up purification.	-	-	-	+	+
HisTrap FF crude	1 ml 5 ml	40 mg/column 200 mg/column	For use with uncentrifuged, crude cell lysates. For use with syringe, peristaltic pump, or chromatography system.	-	-	-	+	+
HisTrap FF crude Kit	3 × 1 ml	40 mg/column	Kit includes 3 × 1 ml HisTrap FF crude columns, all necessary buffers, connectors, a syringe, and instructions.	-	-	-	+	-
His GraviTrap and His GraviTrap Kit	1 ml	40 mg/column	For use with gravity flow, allows direct purification of both clarified and unclarified cell lysates. Kit includes columns and His Buffer Kit.	-	-	+	-	-
His MultiTrap FF	96-well filter plates	0.8 mg/well	For high-throughput screening. Can use with robotics or manually by centrifugation or vacuum.	+	-	-	-	-
Companion product								
His Buffer Kit	1 kit	N/A	Premade buffers for manual purification of histidine-tagged proteins.	-	+	+	+	-

Contains Ni Sepharose 6 Fast Flow
Contains Ni Sepharose High Performance

Purification using Ni Sepharose High Performance

Ni Sepharose High Performance consists of highly cross-linked 6% agarose beads (34-µm diameter) to which a chelating group has been immobilized and subsequently charged with Ni^{2+} ions. The chelating group charged with Ni^{2+} provides very high binding capacity for histidine-tagged proteins, and the medium shows negligible leakage of the Ni^{2+} ion.

Ni Sepharose High Performance is compatible with all commonly used aqueous buffers, reducing agents, and denaturants such as 6 M Gua-HCl and 8 M urea, as well as a range of other additives (see Appendix 1). It is stable over a broad pH range. This high chemical and physical stability and broad compatibility maintains the biological activity and increases the yield of the purified product, at the same time as it greatly expands the range of suitable operating conditions, including procedures used to clean the medium.

The good flow rates and distinctly separated peaks containing concentrated material make Ni Sepharose High Performance the medium of choice for high-performance purifications. See Appendix 1 for the main characteristics of Ni Sepharose High Performance.

Ni Sepharose High Performance is supplied preswollen in 20% ethanol, in pack sizes of 25 and 100 ml, as well as in the convenient prepacked formats described later in this chapter.

Fig 11. Ni Sepharose High Performance precharged with Ni^{2+} for high-performance purification of histidine-tagged proteins.

Column packing

Refer to Appendix 4 for general guidelines for column packing.

Ideally, Sepharose High Performance media are packed in XK or Tricorn™ columns in a two-step procedure: Do not exceed 1.0 bar (0.1 MPa) in the first step and 3.5 bar (0.35 MPa) in the second step. If the packing equipment does not include a pressure gauge, use a packing flow rate of 5 ml/min (XK 16/20 column) or 2 ml/min (Tricorn 10/100 column) in the first step, and 9 ml/min (XK 16/20 column) or 3.6 ml/min (Tricorn 10/100 column) in the second step. If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate your pump can deliver. This should also give a well-packed bed.

1. Assemble the column (and packing reservoir if necessary).
2. Remove air from the end-piece and adapter by flushing with distilled water. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with water.
3. Resuspend the medium and pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.

4. If using a packing reservoir, immediately fill the remainder of the column and reservoir with water. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.
5. Open the bottom outlet of the column and set the pump to run at the desired flow rate.
6. Maintain packing flow rate for at least 3 bed volumes after a constant bed height is reached. Mark the bed height on the column.
7. Stop the pump and close the column outlet.
8. If using a packing reservoir, disconnect the reservoir and fit the adapter to the column.
9. With the adapter inlet disconnected, push the adapter down into the column until it reaches the mark. Allow the packing solution to flush the adapter inlet. Lock the adapter in position.
10. Connect the column to a pump or a chromatography system and start equilibration. Readjust the adapter if necessary.

    Note: For subsequent chromatography procedures, do not exceed 75% of the packing flow rate.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

Adjust the sample to the composition and pH of the binding buffer by: adding buffer, NaCl, imidazole, and additives from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange. To prevent the binding of host cell proteins with exposed histidine, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).

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

Buffer preparation

Binding buffer:	20 mM sodium phosphate, 0.5 M NaCl, 20 to 40 mM imidazole, pH 7.4 . (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)
Elution buffer:	20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4

Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.45-µm filter before use. Use high-purity imidazole, as this will give a very low or no absorbance at 280 nm.

The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. In the binding buffer, 20 to 40 mM imidazole is suitable for many proteins; 500 mM imidazole in the elution buffer ensures complete elution of the target protein.

As an alternative to elution with imidazole, lower the pH to approximately pH 4.5. (Metal ions will be stripped off the medium below pH 4.0).

Purification

1. If the column contains 20% ethanol, wash it with 5 column volumes of distilled water. Use a linear flow rate of 50 to 100 cm/h. Refer to Appendix 6 for flow rate calculations.
2. Equilibrate the column with 5 to 10 column volumes of binding buffer at a linear flow rate of 150 cm/h.
3. Apply the pretreated sample.
4. Wash with binding buffer until the absorbance reaches the baseline.
5. Elute with elution buffer using a step or linear gradient.
   For step elution, 5 column volumes of elution buffer are usually sufficient.
   For linear gradient elution, a shallow gradient, over 20 column volumes, may separate proteins with similar binding strengths.
6. After elution, regenerate the column by washing it with 5–10 column volumes of binding buffer. The column is now ready for a new purification.

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

Use the elution buffer as blank when measuring absorbance manually. If imidazole needs to be removed from the protein, use HiTrap Desalting, PD-10 Desalting, or HiPrep 26/10 Desalting column.

Ni Sepharose is compatible with reducing agents. However, we recommend removal of any weakly bound Ni^{2+} ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store Ni Sepharose High Performance with buffers that include reducing agents.

Leakage of Ni^{2+} from Ni Sepharose is low under all normal conditions. The leakage is lower than for other precharged IMAC media tested.
For very critical applications, leakage during purification can be even further diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer **without** reducing agents.

1. Wash the column with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding buffer.

Purification using Ni Sepharose 6 Fast Flow

Ni Sepharose 6 Fast Flow consists of 90-µm beads of highly cross-linked agarose, to which a chelating ligand has been immobilized and subsequently charged with Ni^{2+} ions. The ligand density of Ni Sepharose 6 Fast Flow ensures high binding capacity, and the medium shows negligible leakage of Ni^{2+} ions. The high flow rate property of the Sepharose 6 Fast Flow matrix makes it well-suited for scaling-up but also for gravity-flow purposes. In addition, the medium is compatible with a wide range of additives commonly used in the purification of histidine-tagged proteins. See Appendix 1 for the main characteristics of Ni Sepharose 6 Fast Flow.

Fig 12. Ni Sepharose 6 Fast Flow is designed for scaling up purification of histidine-tagged proteins but it works well also for gravity-flow purification.

Ni Sepharose 6 Fast Flow is useful for batch/gravity-flow purification of histidine-tagged proteins using Disposable PD-10 Columns. Ni Sepharose 6 Fast Flow prepacked in Disposable PD-10 Columns shows excellent performance in terms of fast purification time and total protein recovered during gravity-flow purification. See His GraviTrap on page 66 and also Data File 11-0008-86.

Ni Sepharose 6 Fast Flow is supplied preswollen in 20% ethanol, in pack sizes of 5, 25, 100, and 500 ml, as well as in convenient prepacked formats as described later in this chapter.

Column packing

Refer to Appendix 4 for general guidelines for column packing.

Ideally, Sepharose 6 Fast Flow media are packed in XK or Tricorn columns in a two-step procedure: Do not exceed 0.5 bar (0.05 MPa) in the first step and 1.5 bar (0.15 MPa) in the second step. If the packing equipment does not include a pressure gauge, use a packing flow rate of 2.5 ml/min (XK 16/20 column) or 0.9 ml/min (Tricorn 10/100 column) in the first step, and 8.7 ml/min (XK 16/20 column) or 4.7 ml/min (Tricorn 10/100 column) in the second step.

1. Assemble the column (and packing reservoir if necessary).
2. Remove air from the end-piece and adapter by flushing with distilled water. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with water.
3. Resuspend the medium and pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.
4. If using a packing reservoir, immediately fill the remainder of the column and reservoir with water. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.
5. Open the bottom outlet of the column and set the pump to run at the desired flow rate.
6. Maintain packing flow rate for at least 3 bed volumes after a constant bed height is reached. Mark the bed height on the column.
7. Stop the pump and close the column outlet.
8. If using a packing reservoir, disconnect the reservoir and fit the adapter to the column.
9. With the adapter inlet disconnected, push the adapter down into the column until it reaches the mark. Allow the packing solution to flush the adapter inlet. Lock the adapter in position.
10. Connect the column to a pump or a chromatography system and start equilibration. Readjust the adapter if necessary.

    Note: For subsequent chromatography procedures, do not exceed 75% of the packing flow rate.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

Adjust the sample to the composition and pH of the binding buffer by: adding buffer, NaCl, imidazole, and additives from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange. To prevent the binding of host cell proteins with exposed histidine, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).

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

Buffer preparation

Binding buffer:	20 mM sodium phosphate, 0.5 M NaCl, 20 to 40 mM imidazole, pH 7.4 . (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)
Elution buffer:	20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4

Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.45-µm filter before use. Use high-purity imidazole, as this will give a very low or no absorbance at 280 nm.

The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. In the binding buffer, 20 to 40 mM imidazole is suitable for many proteins; 500 mM imidazole in the elution buffer ensures complete elution of the target protein.

As an alternative to elution with imidazole, lower the pH to approximately pH 4.5. (Metal ions will be stripped off the medium below pH 4.0.)

Purification using a packed column

1. If the column contains 20% ethanol, wash it with 5 column volumes of distilled water. Use a linear flow rate of 50 to 100 cm/h.
2. Equilibrate the column with 5 to 10 column volumes of binding buffer at a linear flow rate of 150 cm/h.
3. Apply the pretreated sample.
4. Wash with binding buffer until the absorbance reaches the baseline.
5. Elute with elution buffer using a step or linear gradient.
   For step elution, 5 column volumes of elution buffer are usually sufficient.
   For linear gradient elution, a shallow gradient, over 20 column volumes, may separate proteins with similar binding strengths.
6. After elution, regenerate the column by washing it with 5–10 column volumes of binding buffer. The column is now ready for a new purification.

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

Use the elution buffer as blank when measuring absorbance manually. If imidazole needs to be removed from the protein, use HiTrap Desalting, a PD-10 Desalting, or HiPrep 26/10 Desalting column.

Ni Sepharose is compatible with reducing agents. However, we recommend removal of any weakly bound Ni^{2+} ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store Ni Sepharose 6 Fast Flow with buffers that include reducing agents.

Leakage of Ni^{2+} from Ni Sepharose is low under all normal conditions. The leakage is lower than for other precharged IMAC media tested.
For very critical applications, leakage during purification can be even further diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer **without** reducing agents.

1. Wash the column with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding buffer.

Purification using batch/gravity-flow

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

Adjust the sample to the composition and pH of the binding buffer by: adding buffer, NaCl, imidazole, and additives from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange. To prevent the binding of host cell proteins with exposed histidines, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).

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

Buffer preparation

Binding buffer:	20 mM sodium phosphate, 0.5 M NaCl, 20 to 40 mM imidazole, pH 7.4 . (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)
Elution buffer:	20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4

Water and chemicals used for buffer preparation should be of high purity. Use high-purity imidazole, as this will give a very low or no absorbance at 280 nm.

The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. In the binding buffer, 20 to 40 mM imidazole is suitable for many proteins; 500 mM imidazole in the elution buffer ensures complete elution of the target protein.

As an alternative to elution with imidazole, lower the pH to approximately pH 4.5. (Metal ions will be stripped off the medium below pH 4.0.)

Preparing the empty Disposable PD-10 Column

1. Wash the filter with 20% ethanol.
2. Rinse the filter with distilled water.
3. Insert the filter into the empty Disposable PD-10 Column. (Other empty gravity-flow columns can also be used.)

Medium preparation

1. Gently shake the bottle until the slurry is homogeneous.
2. Remove a sufficient amount of slurry from the bottle and transfer to a centrifuge tube.
3. Sediment the Ni Sepharose 6 Fast Flow by centrifugation at 500 × g for 5 min.
4. Discard the supernatant and replace with 5 ml of distilled water.
5. Gently shake the slurry for 3 min and resediment by centrifugation at 500 × g for 5 min.
6. Repeat steps 4 and 5 using binding buffer instead of distilled water.
7. Transfer the slurry to a measuring cylinder.
8. Add an appropriate volume of binding buffer to make a 50% slurry.

Purification using gravity flow

1. Add sample to the 50% slurry. Binding capacity of Ni Sepharose 6 Fast Flow is protein dependent and the average is 40 mg/ml. This means that 1 ml of the 50% slurry can bind approximately 20 mg of histidine-tagged protein.
2. Incubate sample and the Ni Sepharose 6 Fast Flow slurry on a shaker at low speed for 1 h.
3. Load sample/Ni Sepharose 6 Fast Flow mix onto the PD-10 column and collect the flowthrough.
4. Wash with 2 to 5 medium volumes of binding buffer and collect the flowthrough. For example, if 0.5 ml of Ni Sepharose 6 Fast Flow is used (1 ml of 50% slurry), wash with 1 to 2.5 ml of binding buffer.
5. Elute with 4 medium volumes of elution buffer and collect the eluted fractions in four separate tubes.
6. Measure absorbance at 280 nm using a spectrophotometer and confirm purity of the pooled fractions by SDS-PAGE. Use elution buffer as the blank.

Ni Sepharose is compatible with reducing agents. However, we recommend removal of any weakly bound Ni^{2+} ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store Ni Sepharose 6 Fast Flow with buffers that include reducing agents.

Leakage of Ni^{2+} from Ni Sepharose is low under all normal conditions. The leakage is lower than for other precharged IMAC media tested.
For very critical applications, leakage during purification can be even further diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer **without** reducing agents.

1. Wash the medium with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 medium volumes of elution buffer.
3. Equilibrate with 10 medium volumes of binding buffer.

This can be done by centrifugal washes of the suspended medium or, much more efficiently, by washing the medium on a sintered glass filter (medium grade G3 type).

High-throughput screening using His MultiTrap HP and His MultiTrap FF 96-well filter plates

His MultiTrap HP and His MultiTrap FF are prepacked, disposable 96-well filter plates for reproducible, high-throughput screening of histidine-tagged recombinant protein expression. Typical applications are expression screening of different constructs, screening for solubility of proteins, and optimization of the conditions for small-scale parallel purification. The plates are prepacked with precharged Ni Sepharose High Performance and Ni Sepharose 6 Fast Flow, respectively.

Each well of the prepacked His MultiTrap HP and His MultiTrap FF contains 500 µl of a 10% slurry of Ni Sepharose High Performance or Ni Sepharose 6 Fast Flow in storage solution (50 µl of medium in 20% ethanol) and has a capacity for purifying up to 1.0 mg and 0.8 mg of histidine-tagged protein, respectively. The plates are made of polypropylene and polyethylene.

Characteristics of the media and of His MultiTrap HP and His MultiTrap FF are listed in Appendix 1. The Ni^{2+}-charged media are compatible with all commonly used aqueous buffers, reducing agents, denaturants, such as 6 M Gua-HCl and 8 M urea, and a range of other additives.

Prepacked His MultiTrap HP and His MultiTrap FF plates provide well-to-well and plate-to-plate reproducibility in terms of yield and purity of eluted protein. Automated robotic systems can be used, as well as manual handling using centrifugation or vacuum pressure. The purification procedure can easily be scaled up because Ni Sepharose is available in both larger prepacked formats and as lab packs. Scaling up from His MultiTrap plates to a HisTrap 1-ml or 5-ml column while keeping the same conditions (e.g., Fast Flow or High Performance medium, imidazole concentration, etc.) provides highly consistent results and shortens the optimization time at scale-up.

Fig 13. His MultiTrap HP and His MultiTrap FF are prepacked 96-well filter plates for high-throughput expression screening of histidine-tagged proteins.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

- Lysis with commercial kits could give large cell debris particles that may interfere with drainage of the wells during purification. This problem can be solved by centrifugation or filtration of the sample before adding it to the wells.

- After thorough cell disruption, it is possible to apply unclarified lysate directly to the wells without pre-centrifugation and/or filtration of the sample.
  Apply the unclarified lysate to the wells directly after preparation, as the lysate may precipitate unless used immediately or frozen before use. New lysing of the sample can then prevent clogging of the wells when loading the plate.

- If the sample is too viscous, an extension of the duration of mechanical treatment of the sample to ensure a more complete lysis is recommended (keep the sample on ice to prevent overheating).

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 500 mM NaCl, 20 to 40 mM imidazole, pH 7.4. (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)

Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4.

To increase the purity, use as high a concentration of imidazole as possible in the sample and binding buffers without losing binding capacity. Refer to Chapter 4 for additional information on this topic.

Centrifugation procedure for high-throughput screening

Preparing the filter plate

1. Peel off the bottom seal from the 96-well filter plate. Be sure to hold the filter plate over a sink to accommodate any leakage of storage solution when removing the bottom seal.
2. Hold the filter plate upside down and gently shake it to dislodge any medium adhering to the top seal. Return the filter plate to an upright position.
3. Place the filter plate against the bench surface and peel off the top seal.
4. Position the filter plate on top of a collection plate.

   Note: Remember to change or empty the collection plate as necessary during the following steps.
5. Centrifuge the filter plate for 2 min at 500 × g to remove the ethanol storage solution from the medium.
6. Add 500 µl of deionized water to each well. Centrifuge the plate for 2 min at 500 × g.
7. Add 500 µl of binding buffer to each well to equilibrate the medium. Centrifuge for 2 min at 500 × g. Repeat once. The filter plate is now ready for use.

Blank run: Reducing agents may be used in sample and buffers. In such a case, perform a blank run by applying 500 µl of elution buffer/well before step 7. **No reducing agent should be used in buffer during blank runs**. Reequilibrate with binding buffer **including** reducing agent before sample application. Do not leave His MultiTrap plates with buffers including reducing agents when not in use.

Centrifugation procedure

Do not apply a force of more than 700 × g during centrifugation.

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

   Note: If the yield of protein is too low, increase the incubation time and/or gently agitate the filter plate to effect mixing.
2. Centrifuge the plate at 100 × g for 4 min or until all the wells are empty. Discard the flowthrough.
3. Add 500 µl of binding buffer per well to wash out any unbound sample. Centrifuge at 500 × g for 2 min. Repeat once or until all unbound sample is removed.
4. Add 200 µl of elution buffer per well and mix for 1 min.

   Note: The volume of elution buffer can be varied (50 to 100 µl per well), depending on the concentration of target protein required.
5. Change the collection plate and centrifuge at 500 × g for 2 min to collect the eluted protein. Repeat twice or until all the target protein has been eluted.

   Note: High-purity protein should yield an A_{280} reading of < 0.1. If necessary, change the collection plate between each elution to prevent unnecessary dilution of the target protein.

Vacuum procedure for high-throughput screening

If problems with foaming, reproducibility, or bubbles in the collection plate occur using vacuum, the centrifugation procedure should be considered. The distance between the filter plate and the collection plate is critical; adjust the distance if necessary.

Preparing the filter plate

1. Peel off the bottom seal from the 96-well filter plate. Be sure to hold the filter plate over a sink to accommodate any leakage of storage solution when removing the bottom seal.
2. Hold the filter plate upside down and gently shake it to dislodge any medium adhering to the top seal. Return the filter plate to an upright position.
3. Place the filter plate against the bench surface and peel off the top seal.
4. Position the filter plate on top of a collection plate.

   Note: Remember to change or empty the collection plate as necessary during the following steps.
5. Set the vacuum to -0.15 bar. Place the 96-well plate and collection plate on the vacuum manifold to remove the ethanol storage solution from the medium.
6. Add 500 µl of deionized water to each well. Apply vacuum to drain the water from the wells.
7. Add 500 µl of binding buffer to each well to equilibrate the medium. Remove the solution as in step 5. Repeat once. The filter plate is now ready for use.

Blank run: Reducing agents may be used in sample and buffers. In such a case, run a blank run by applying 500 µl of elution buffer/well before step 7. **No reducing agent should be used in buffer during blank runs.** Reequilibrate with binding buffer **including** reducing agent before sample application. Do not leave His MultiTrap plates with buffers including reducing agents when not in use.

Vacuum procedure

Do not apply a pressure in excess of -0.5 bar during vacuum operation.

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

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

   Note: If the yield of protein is too low, increase the incubation time and/or gently agitate the filter plate.
2. Remove the flowthrough by applying a vacuum of -0.15 bar until all the wells are empty. Slowly increase the vacuum to -0.30 bar and turn off the vacuum after approximately 5 sec. Discard the flowthrough.

Increasing the vacuum too quickly can result in foaming under the filter plate and subsequent cross-contamination of samples.

3. Add 500 µl of binding buffer per well to wash out any unbound sample. Apply a vacuum of -0.15 bar as in step 2. Repeat once or until all unbound sample is removed.
4. Add 200 µl of elution buffer per well and mix for 1 min.

   Note: The volume of elution buffer can be varied (50 to 100 µl per well), depending on the concentration of target protein required.
5. Change the collection plate and apply a vacuum of -0.15 bar to collect the eluted protein. Repeat twice or until all the target protein has been eluted.

   Note: High-purity protein should yield an A_{280} reading of < 0.1. If necessary, change the collection plate between each elution to prevent unnecessary dilution of the target protein.

Application example

Determining solubility effects of detergents in buffers during purification of membrane proteins using His MultiTrap FF

The 96-well plate format of His MultiTrap FF and His MultiTrap HP allows high-throughput screening and purification of histidine-tagged proteins. In this example, His MultiTrap FF was used to screen eight detergents for their effect on the solubility of six histidine-tagged membrane proteins. Results from purification screening of two proteins, GlpG protein (EM29) and cation transporter (EM43), are shown in a dot blot and SDS-PAGE in Figure 14. The results show that conditions to find the most appropriate detergent for the membrane proteins in the study can be readily optimized, with high reproducibility, using MultiTrap 96-well filter plate.

96-well filter plate:	His MultiTrap FF
Sample:	Six *E. coli* lysates containing histidine-tagged membrane proteins: probable transporter, ion transporter, putative transferase, regulatory protein, GlpG protein, and cation transporter; GlpG protein (EM29) and cation transporter (EM43) are shown here
Sample preparation:	Chemical and freeze/thaw lysis
Sample volume:	100 µl/well
Elution method:	Centrifugation
Elution volume:	3 × 50 µl/well
Lysis buffer:	20 mM sodium phosphate, pH 7.4, 100 mM sodium chloride, 20 mM imidazole, 0.5 mM TCEP, 5 U/ml Benzonase™ Nuclease, 1 mg/ml lysozyme, EDTA-free protease inhibitor cocktail, 1% to 2% detergent, and 1X BugBuster™ Protein Extraction Reagent, 25 U/ml Benzonase Nuclease, 1 kU/ml rLysozyme™ Solution, and 2X Complete Protease Inhibitor Cocktail Tablet solution
Binding buffer:	20 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 20 mM imidazole, 0.5 mM TCEP, 1–2% detergent
Wash buffer:	20 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 40 mM imidazole, 0.5 mM TCEP, 0.03% DDM, 1–2% detergent
Elution buffer:	20 mM sodium phosphate pH 7.4, 500 mM sodium chloride, 500 mM imidazole, 0.5 mM TCEP, 0.03% DDM, 1–2% detergent
Detergents:	1% Fos-Choline 12 (FC12), 1% undecyl maltoside (UDM), 1% dodecyl maltoside (DDM), 1% Cymal-5, 1% Cymal-6, 2% octyl glucoside (OG), 1% Triton™ X-100 (TX-100), 1% lauryl dimethylamine oxide (LDAO)
Data evaluation:	Dot-blot analysis on nitrocellulose membrane. Histidine-tagged proteins were detected using HisProbe™-HRP chemistry. SDS-PAGE with Coomassie staining

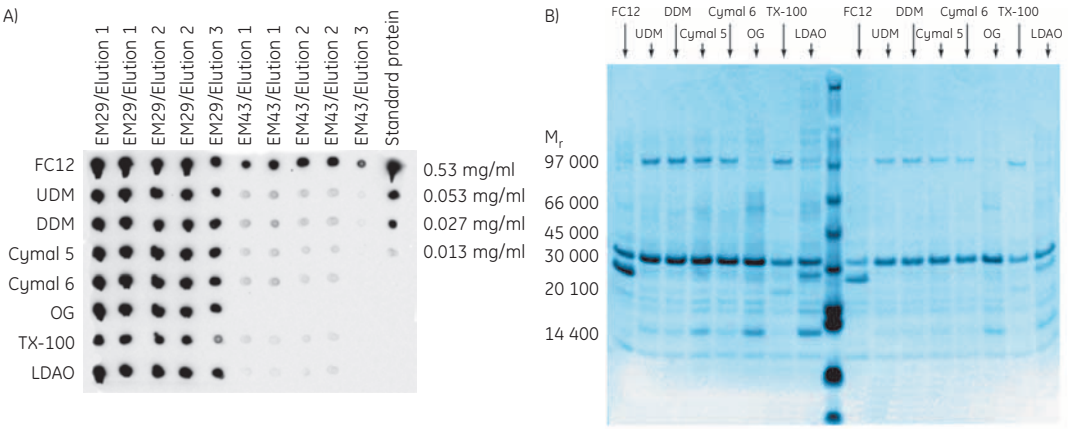


Fig 14. (A) Dot blot of membrane proteins EM29 and EM43 purified on His MultiTrap FF in the presence of different detergents. Repeats of eluates 1 and 2 shown in the dot blot are two independent extractions and purifications. (B) SDS-PAGE (Coomassie staining) of EM29 purifications (elutions 1 and 2 in the blot) with eight different detergents on His MultiTrap FF.

Minipreps using His SpinTrap

His SpinTrap is designed for efficient minipreps (small-scale purification) of histidine-tagged proteins directly from clarified or unclarified cell lysates. The columns may also be used for screening of large numbers of small-scale lysates, as well as optimization of purification conditions. The columns are prepacked with Ni Sepharose High Performance. Refer to Appendix 1 for the main characteristics of His SpinTrap.

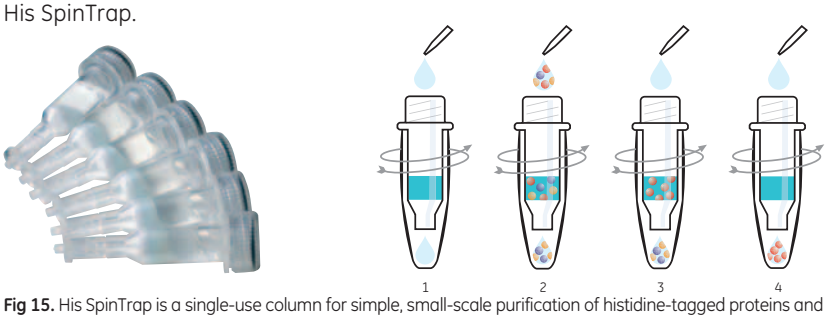


Fig 15. His SpinTrap is a single-use column for simple, small-scale purification of histidine-tagged proteins and rapid expression screening. Purifying histidine-tagged proteins with His SpinTrap is a simple, four-stage procedure that can be performed in 10 min using a microcentrifuge: (1) After placing the column in a 2-ml microcentrifuge tube, equilibrate by adding binding buffer and centrifuge; (2) add sample; (3) wash with binding buffer; (4) elute the target protein with elution buffer.

His SpinTrap columns are used together with a standard microcentrifuge. One purification run takes approximately 10 min. For optimal performance, use His SpinTrap together with buffers prepared using His Buffer Kit. Purification of unclarified samples on His SpinTrap columns minimizes loss of target protein caused by manual operations such as sample precentrifugation, transfer to centrifugation tubes, and collecting supernatant. In addition, loading unclarified sample directly to the His SpinTrap columns reduces sample preparation time, which minimizes degradation of sensitive target proteins. See Figures 15 and 16 for schematics showing the procedure.

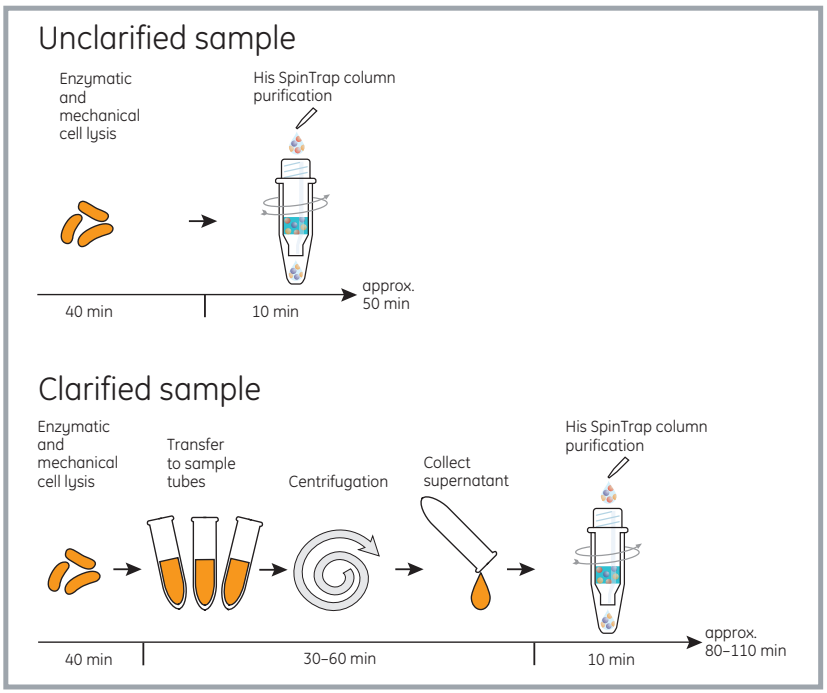


Fig 16. Total times for preparing and purifying unclarified samples are 30 to 60 min less than for clarified samples because the extra time needed to clarify the cell lysate by centrifugation is eliminated.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

The procedure below has been used successfully in our own laboratories for sample preparation prior to use of His SpinTrap, but other established procedures may also work. Use standard 2-ml microcentrifuge tubes.

1. Dilute the cell paste: Add 1 ml of binding buffer to resuspend cell paste obtained from 20 to 50 ml of cell culture (depending on expression level).

To prevent host cell proteins binding to exposed histidines, it is essential that the sample and binding buffers contain the same concentration of imidazole.

2a. Enzymatic lysis: Add 0.2 mg/ml lysozyme, 20 µg/ml DNase, 1 mM $MgCl_2$, and 1 mM Pefabloc SC or PMSF. Vortex the tubes gently and incubate at room temperature for 30 min.

Chemical lysis kits can also be used, but make sure that they do not contain any chelating agent.

2b. Mechanical lysis: Disrupt cells by repeated freeze/thaw, homogenization, or sonication.

You can also apply clarified sample to the column by spinning at full speed in a microcentrifuge for 10 min to remove insoluble material. Collect supernatant and purify on His SpinTrap.

Buffer preparation

Recommended buffers for native conditions can easily be prepared from His Buffer Kit.

Native conditions:

Binding buffer: 20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4

Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4

Denaturing conditions:

Binding buffer: 20 mM Tris-HCl, 8 M urea, 500 mM NaCl, 5 mM imidazole, pH 8.0 + 1 to 5 mM β-mercapto-ethanol

Elution buffer: 20 mM Tris-HCl, 8 M urea, 500 mM NaCl, 500 mM imidazole, pH 8.0 + 1 to 5 mM β-mercapto-ethanol

The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. Under native conditions, 20 to 40 mM imidazole in the binding buffer is suitable for many proteins; 500 mM imidazole in the elution buffer is most often sufficient to completely elute the target protein.

As an alternative to elution with imidazole, you can lower the pH to approximately pH 4.5. (Note that metal ions will be stripped off the medium below pH 4.0.)

Purification

Perform purifications on His SpinTrap using a standard microcentrifuge. Place the column in a 2-ml microcentrifuge tube to collect the liquid during centrifugation. Use a new 2-ml tube for every step (steps 1 to 6).

1. Invert and shake the column repeatedly to resuspend the medium. Loosen the top cap one-quarter of a turn and break off the bottom closure.
2. Place the column in a 2-ml microcentrifuge tube and centrifuge for 30 s at 70 to 100 × g (approx. 1000 rpm in an Eppendorf™ 5415R, 24-position fixed-angle rotor) to remove the storage liquid.
3. Remove and discard the top cap. Equilibrate the column by adding 600 µl of binding buffer. Centrifuge for 30 s at 70 to 100 × g.
4. Add up to 600 µl (total) of prepared sample. Centrifuge for 30 s at 70 to 100 × g.

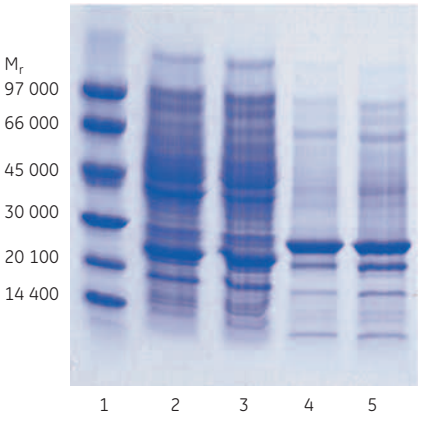
You can make several sample applications as long as you do not exceed the binding capacity of the column (see Appendix 1).

5. Wash with 600 µl of binding buffer. Centrifuge for 30 s at 70 to 100 × g.
6. Elute the target protein twice with 200 µl of elution buffer. Centrifuge for 30 s at 70 to 100 × g and collect the purified sample. The first 200 µl will contain the majority of the target protein.

Application example

Purification of unclarified sample using His SpinTrap

The performance of His SpinTrap columns in purifying a histidine-tagged protein from unclarified *E. coli* lysate was assessed. Histidine-tagged green fluorescent protein, GFP-$(His)_6$, in *E. coli* BL-21 lysate, was subjected to enzymatic lysis followed by sonication for 10 min, and the unclarified lysate was loaded directly on His SpinTrap. For comparison, half of the sample was also clarified by centrifugation before purification. Samples and binding buffer contained 60 mM imidazole. To ensure complete elution of GFP-$(His)_6$, which has a high affinity for Ni Sepharose High Performance, the elution buffer contained 800 mM imidazole rather than the more usual 500 mM. Purification time for the unclarified and clarified sample was 10 min. The final purity of eluates from unclarified and clarified samples was similar as confirmed by SDS-PAGE (Fig 17).



Column:	His SpinTrap
Equilibration:	600 µl binding buffer
Sample application:	600 µl unclarified or clarified *E. coli* BL-21 lysate containing 150 µg GFP-$(His)_6$
Wash:	600 µl binding buffer
Elution:	2 × 200 µl elution buffer
Binding buffer:	20 mM sodium phosphate, 500 mM NaCl, 60 mM imidazole, pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl, 800 mM imidazole, pH 7.4

Lanes
1. LMW markers
2. Unclarified sample, start material (diluted 1:10)
3. Clarified sample, start material (diluted 1:10)
4. Unclarified sample, eluted pool
5. Clarified sample, eluted pool

Fig 17. SDS-PAGE (ExcelGel™ SDS Gradient 8–18) under reducing conditions of unclarified and clarified *E. coli* lysate containing GFP-$(His)_6$. Similar purity and recovery were observed for both unclarified and clarified sample.

Purification using HisTrap HP and HisTrap FF

HisTrap HP and HisTrap FF are 1-ml and 5-ml HiTrap columns packed with Ni Sepharose High Performance or Ni Sepharose 6 Fast Flow, respectively. Sample application, washing, and elution can be performed using a syringe with a supplied adapter, a peristaltic pump, or a liquid chromatography system such as ÄKTAdesign (see Table 8 for equipment choices).

HisTrap HP and HisTrap FF columns are made of polypropylene, which is biocompatible and non-interactive with biomolecules. The top and bottom frits are manufactured from porous polyethylene. Columns are delivered with a stopper on the inlet and a snap-off end on the outlet. Every package includes all necessary components for connection of the columns to different types of equipment. For quick scale-up of purifications, two or three HisTrap columns (1 ml or 5 ml) can be connected in series (back pressure will be higher). Note that HisTrap HP and HisTrap FF columns cannot be opened or refilled.

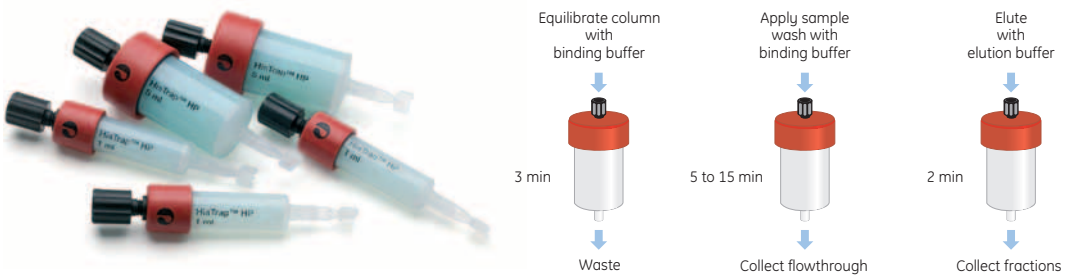


Fig 18. HisTrap HP and HisTrap FF 1-ml and 5-ml columns allow convenient and simple one-step purification of histidine-tagged proteins. HisTrap HP 1-ml and 5-ml columns shown here. The simple purification scheme is shown at right.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

Adjust the sample to the composition and pH of the binding buffer by: adding buffer, NaCl, imidazole, and additives from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange. To prevent the binding of host cell proteins with exposed histidines, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).

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

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 500 mM NaCl, 20 to 40 mM imidazole, pH 7.4. (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)

Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4.

Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.22 µm or a 0.45 µm filter before use. Use high-purity imidazole as this will give very low or no absorbance at 280 nm.

The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. Under native conditions, 20 to 40 mM imidazole in the binding buffer is suitable for many proteins; 500 mM imidazole in the elution buffer is most often sufficient to completely elute the target protein.

As an alternative to elution with imidazole, you can lower the pH to approximately pH 4.5. (Note that metal ions will be stripped off the medium below pH 4.0.)

Purification

1. Fill the syringe or pump tubing with distilled water. Remove the stopper and connect the column to the syringe (use the connector supplied), laboratory pump, or chromatography system "drop to drop" to avoid introducing air into the system.
2. Remove the snap-off end at the column outlet.
3. Wash out the ethanol with 3 to 5 column volumes of distilled water.
4. Equilibrate the column with at least 5 column volumes of binding buffer. Recommended flow rates are 1 ml/min (1-ml column) and 5 ml/min (5-ml column).
5. Apply the pretreated sample using a syringe fitted to the Luer connector or by pumping it onto the column. For best results, use a flow rate of 0.2 to 1 ml/min (1-ml column) and 0.5 to 5 ml/min (5-ml column) during sample application*.
6. Wash with binding buffer (generally at least 5 to 10 column volumes) until the absorbance reaches a steady baseline or no material remains in the effluent. Maintain a flow rate of 1 to 2 ml/min (1-ml column) and 5 to 10 ml/min (5-ml column) for washing.
7. Elute with elution buffer using a one-step or linear gradient. For step elution, 5 column volumes is usually sufficient. For linear gradient elution, 10 to 20 column volumes are usually sufficient. Maintain a flow rate of 1 to 2 ml/min (1-ml column) and 5 to 10 ml/min (5-ml column) for elution.
8. After elution, regenerate the column by washing it with 3 to 5 column volumes of binding buffer. The column is now ready for a new purification.

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

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

Ni Sepharose is compatible with reducing agents. However, we recommend removal of any weakly bound Ni^{2+} ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store HisTrap columns with buffers that include reducing agents.

Leakage of Ni^{2+} from Ni Sepharose is low under all normal conditions. The leakage is lower than for other precharged IMAC media tested.
For very critical applications, leakage during purification can be even further diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer **without** reducing agents.

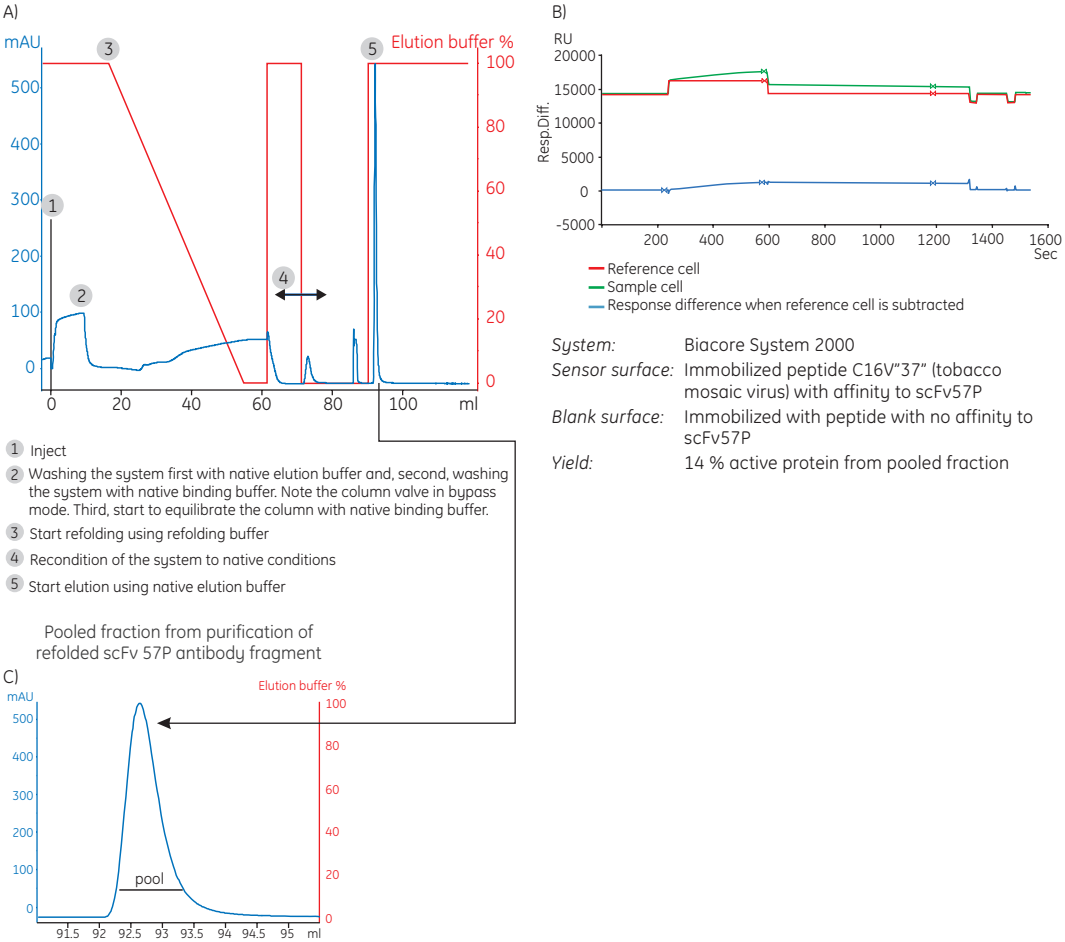
1. Wash the column with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding buffer.

Application examples

1. One-step on-column refolding and purification of a histidine-tagged protein from inclusion bodies using HisTrap HP

Histidine-tagged, solubilized single chain Fv antibody fragment Fab 57P, 10 ml (concentration 0.67 mg/ml) from *E. coli* inclusion bodies was refolded and purified using a 1-ml HisTrap HP column. The yield from refolding was 14% according to analysis using Biacore™ System 2000; see Figure 19B. The parameters of refolding could be optimized and the final method automated for both refolding and purification using HisTrap HP. Figure 19A-C shows the results from the refolding, purification, and analysis.

Sample:	Histidine-tagged, solubilized single chain Fv antibody fragment Fab 57, 10 ml (conc. 0.67 mg/ml) *E. coli* inclusion bodies
Column:	HisTrap HP 1 ml
Solubilizing buffer:	20 mM Tris-HCl, 6 M Gua-HCl, 1 mM DTE, 1 mM Na_2-EDTA, 0.1 mM Pefabloc, pH 7.5
Denatured binding buffer:	20 mM Tris-HCl, 5 mM imidazole, 0.5 M NaCl, 8 M urea, 1 mM DTE, 0.1 mM Pefabloc, pH 7.5
Refolding buffer:	20 mM Tris-HCl, 5 mM imidazole, 0.5 M NaCl, 0.5 M arginine-HCl, 1 mM reduced glutathione (GSH), 1 mM oxidized glutathione (GSSG), pH 7.5
Native binding buffer:	20 mM Tris-HCl, 10 mM imidazole, 0.5 M NaCl, pH 7.5
Native elution buffer:	20 mM Tris-HCl, 500 mM imidazole, 0.5 M NaCl, pH 7.5
Flow rate:	1 ml/min
System:	ÄKTAexplorer 10



System:	Biacore System 2000
Sensor surface:	Immobilized peptide C16V"37" (tobacco mosaic virus) with affinity to scFv57P
Blank surface:	Immobilized with peptide with no affinity to scFv57P
Yield:	14 % active protein from pooled fraction

Fig 19 (A). On-column folding and purification. (B) Sensogram of the interaction between immobilized peptide and refolded protein. (C) Enlarged figure of pooled fraction from purification of refolded scFv 57P antibody fragment.

2. Two-step purification of a high-molecular-weight histidine-tagged protein using HisTrap HP

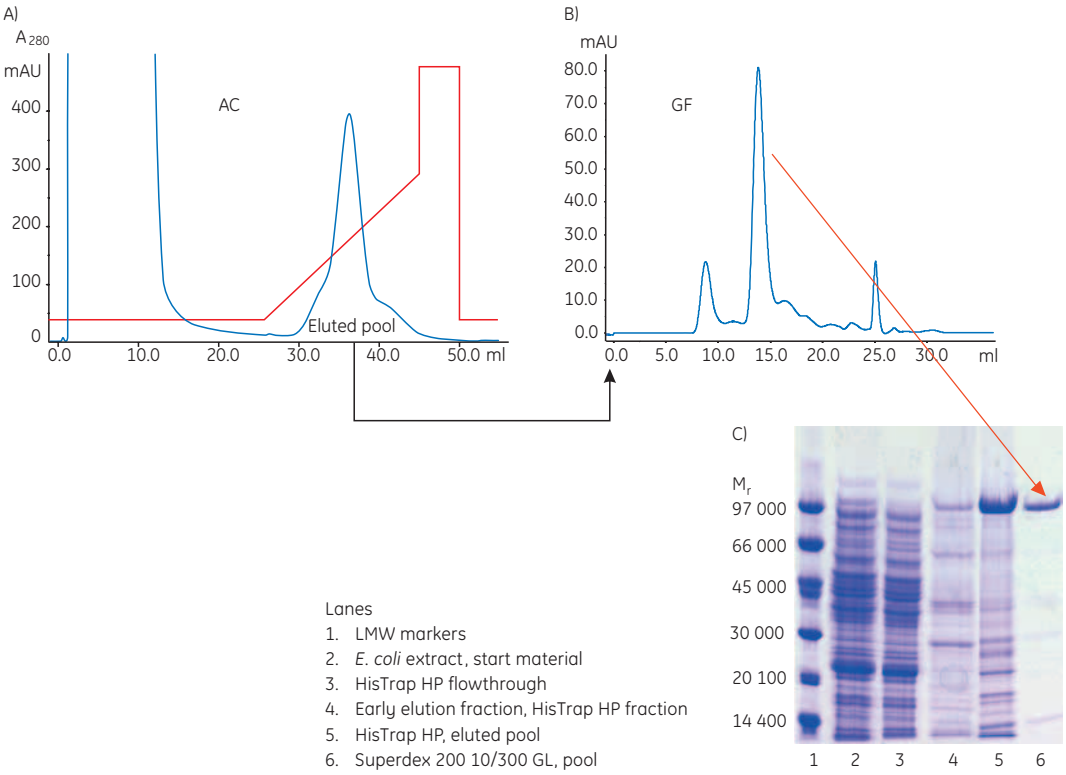
The high-molecular-weight protein histidine-tagged mannanase Man 26A from *Cellulomonas fimi* (M_r 100 000) was purified in its enzymatically active form using a 1-ml HisTrap HP column (Fig 20A). A second purification step using gel filtration with Superdex™ 200 was added to obtain a purity of 95% (Figs 20B and 20C, respectively).

A. Affinity chromatography (AC)

Sample:	10 ml *E. coli* extract with low-level expression of a histidine-tagged mannanase, Man 26A, from *Cellulomonas fimi* (M_r ~ 100 000)
Column:	HisTrap HP 1 ml
Binding buffer:	20 mM sodium phosphate, 30 mM imidazole, 500 mM NaCl, pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.4
Gradient:	25 ml linear gradient 30–300 mM imidazole
Flow rate:	1 ml/min
System:	ÄKTAexplorer 100

B. Gel filtration (GF)

Sample:	0.5 ml concentrated sample from HisTrap HP 1-ml column
Column:	Superdex 200 10/300 GL
Buffer:	PBS, pH 7.5
Flow rate:	0.5 ml/min
System:	ÄKTAexplorer 100



Lanes

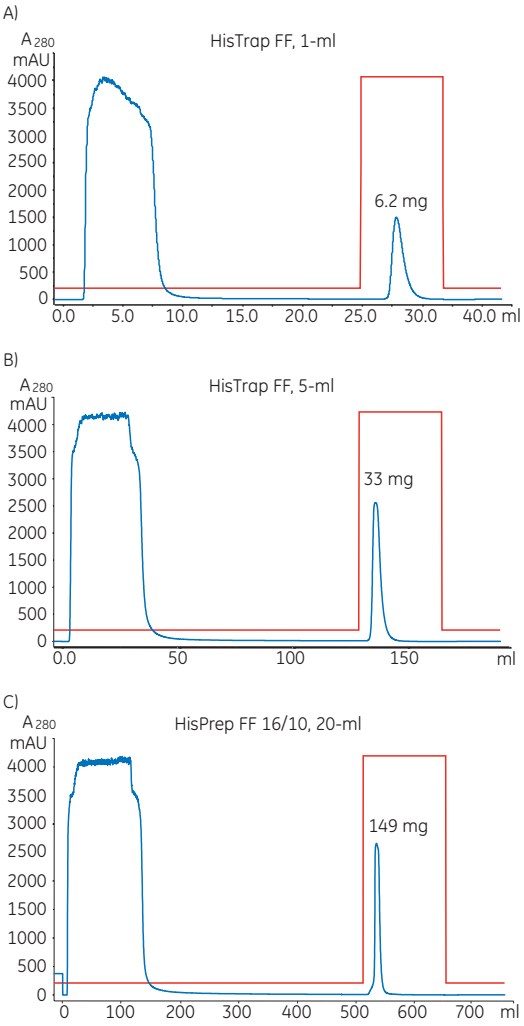
1. LMW markers
2. *E. coli* extract, start material
3. HisTrap HP flowthrough
4. Early elution fraction, HisTrap HP fraction
5. HisTrap HP, eluted pool
6. Superdex 200 10/300 GL, pool

Fig 20. (A) First purification step, affinity chromatography, using HisTrap HP 1-ml column. (B) Second purification step with gel filtration using Superdex 200 30/100 GL. (C) SDS-PAGE.

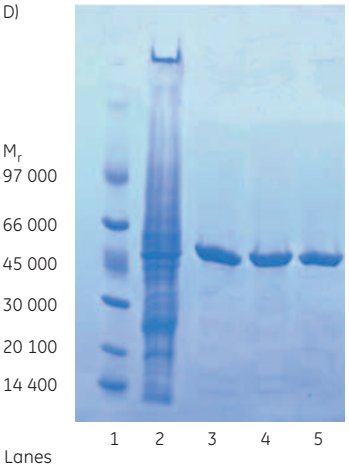
3. Scaling up purification from HisTrap FF to pilot scale

Histidine-tagged maltose binding protein MBP-$(His)_6$ was purified from an *E. coli* extract. Samples containing 8, 40, and 160 mg, respectively, were loaded on a 1-ml HisTrap FF column, a 5-ml HisTrap FF column, and a 20-ml HisPrep FF 16/10 column, all of which were run at the same linear flow rate. The results show that scaling up the column dimension while running at the same linear flow rate provides highly consistent results (Fig 21A–C). Pooled fractions were analyzed by SDS-PAGE and showed almost identical results in terms of purity and recovery (Fig 21D).

To go from laboratory to pilot scale, higher sample load is necessary. Scale-up was conducted with a high load (88% of the binding capacity) of MBP-$(His)_6$. The high sample load required optimization of the binding and washing buffer to avoid loss of MBP-$(His)_6$ during the washing step. An imidazole concentration of 5 mM was found to give the best recovery and purity results. Two separate runs were conducted using HisPrep FF 16/10 columns to show the reproducibility of the purification. The protocol was then scaled up 10-fold. Pooled fractions analyzed by SDS-PAGE gave almost identical results in terms of recovery and purity between the different runs and different scales to indicate a successful process scale-up (Figure 22B).

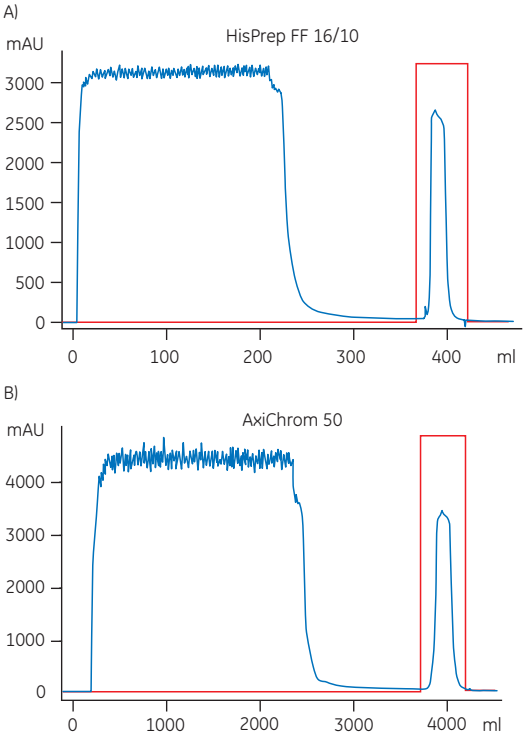


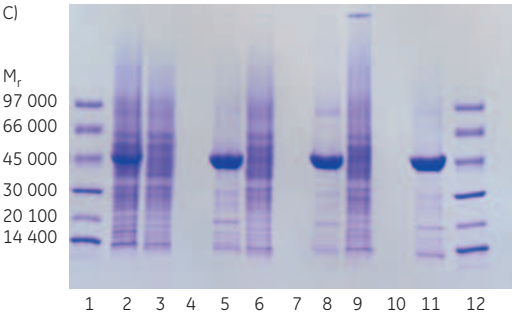
Columns: HisTrap FF 1 ml, HisTrap FF 5 ml, HisPrep FF 16/10 (20 ml)
Sample: Histidine-tagged Maltose Binding Protein, MBP-$(His)_6$, in *E. coli* extract
Binding buffer: 20 mM sodium phosphate, 25 mM imidazole, 500 mM NaCl, pH 7.4
Elution buffer: 20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.4
Flow rates: HisTrap FF 1 ml: 1 ml/min
HisTrap FF 5 ml: 5 ml/min
HisPrep FF 16/10: 5 ml/min



Lanes
1. LMW markers
2. Start material
3. Eluted pool, HisTrap FF 1 ml
4. Eluted pool, HisTrap FF 5 ml
5. Eluted pool, HisPrep FF 16/10 (20 ml)

Fig 21. Scale-up from (A) HisTrap FF 1 ml via (B) HisTrap FF 5 ml to (C) a HisPrep 16/10 (20 ml) prepacked column. The samples loaded contained approximately 8, 40, and 160 mg of MBP-$(His)_6$, respectively. Recovery in milligrams is shown in each chromatogram. (D) SDS-PAGE (ExcelGel SDS Gradient 8–18) under nonreducing conditions confirms that scaling up from the 1-ml to the 20-ml column does not significantly affect the purification result.





Lanes

1. LMW markers
2. Sample loaded, *E. coli* extract
3. Flowthrough HisPrep FF 16/10 run 1
4. Wash HisPrep FF 16/10 run 1
5. Eluted pool HisPrep FF 16/10 run 1
6. Flowthrough HisPrep FF 16/10 run 2
7. Wash HisPrep FF 16/10 run 2
8. Eluted pool HisPrep FF 16/10 run 2
9. Flowthrough AxiChrom 50 run
10. Wash AxiChrom 50 run
11. Eluted pool AxiChrom 50 run
12. LMW markers

Fig 22. Scale-up from (A) HisPrep FF 16/10 (20 ml) to (B) AxiChrom™ 50 (210 ml) column. ÄKTAexplorer 100 was used for the purification runs on HisPrep FF 16/10 columns and ÄKTApilot was used for AxiChrom 50 purification. All systems were controlled by UNICORN software. Note that only the chromatogram for run 1 on HisPrep FF 16/10 is presented. (C) SDS-PAGE of various fractions from both purifications.

Purification using HisTrap FF with ÄKTAprime plus

These procedures use a HisTrap FF 1-ml column but also can be used with a HisTrap HP 1-ml column.

Buffer preparation

Binding buffer (port A1): 20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4.

Elution buffer (port B): 20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4.

Use high-purity water and chemicals and filter all buffers through a 0.45 µm filter before use. Prepare at least 500 ml of eluent.

20 to 40 mM imidazole should be included in the binding buffer to reduce nonspecific binding of non-histidine-tagged proteins. The concentration of imidazole is protein dependent, and if the protein of interest elutes or does not bind at a certain imidazole concentration, then reduce the concentration.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

Adjust the sample to the composition and pH of the binding buffer by: adding buffer, NaCl, imidazole, and additives from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange. To prevent the binding of host cell proteins with exposed histidines, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).

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

System preparation

This example uses ÄKTAprime plus. Once the system is prepared, the remaining steps (under Selecting Application Template and starting the method) will be performed automatically.

1. Place each inlet tubing from port A (8-port valve) in the binding buffer and the tubing from port B (2-port valve) in the elution buffer.
2. Place the three brown waste tubings in waste.
3. Connect the column between port 1 on the injection valve (7-port valve) and the UV flow cell.
4. Fill the fraction collector rack with 18-mm tubes and position the white plate on the fractionation arm against the first tube.

For step elution, the number of tubes to insert in the fraction collector will vary with the sample volume. Fill the fraction collector with 20 tubes + one tube/ml sample. For example, if the sample volume is 10 ml, fill the fraction collector with 20 + 10 = 30 tubes. However, note that the maximum capacity of the fraction collector is 95 tubes, limiting the sample volume to 75 ml.

For gradient elution, use a minimum of 40 tubes.

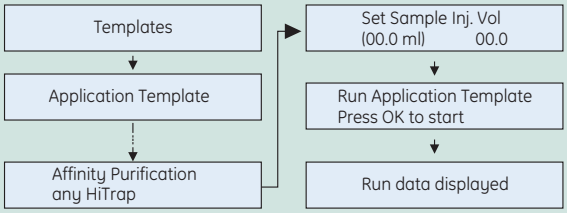
5. Connect a sample loop large enough for your sample between port 2 and 6 on the injection valve. Use a syringe to manually fill the loop.

   Note: If a Superloop™ is needed, additional information is supplied in the instructions for Superloop.

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

Selecting Application Template and starting the method for step elution

1. Check the communication to PrimeView™. At the lower right corner of the screen the text **Controlled By:** prime should be displayed.
2. Use the arrow and OK buttons to move in the menu tree until you find **Affinity purification any HiTrap.**



3. Enter the sample volume and press **OK** to start the template.

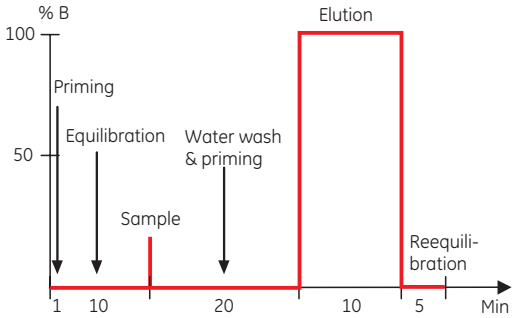
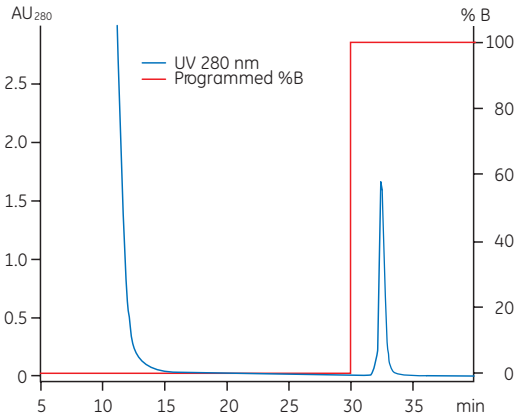


Fig 23. Theoretical gradient in **Affinity Purification any HiTrap Application Template.** Total separation time = 47 min + sample application time.

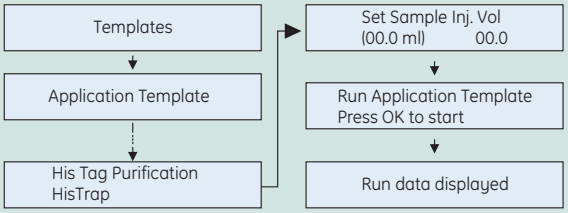


Sample: Clarified homogenate of *E. coli* expressing histidine-tagged protein
Column: HisTrap HP 1 ml
Binding buffer (port A1): 20 mM phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4
Elution buffer (port B): 20 mM phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4

Fig 24. Typical result from step elution of a histidine-tagged protein.

Selecting Application Template and starting the method for gradient elution

1. Check the communication to PrimeView. At the lower right corner of the screen the text **Controlled By:** prime should be displayed.
2. Use the arrow and OK buttons to move in the menu tree until you find **His Tag Purification HisTrap.**



3. Enter the sample volume and press **OK** to start the template.

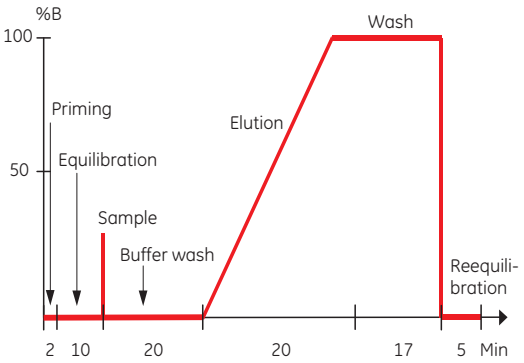
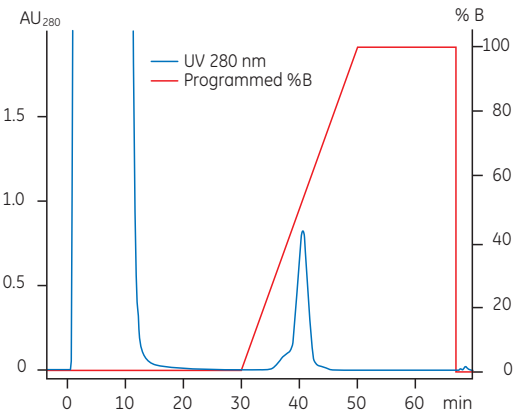


Fig 25. Theoretical gradient in **His Tag Purification HisTrap Application Template.** Total separation time = 74 min + sample application time.



Sample:	Clarified homogenate of *E. coli* expressing histidine-tagged protein
Column:	HisTrap HP 1 ml
Binding buffer (port A1):	20 mM phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4
Elution buffer (port B):	20 mM phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4

Fig 26. Typical result from gradient elution of a histidine-tagged protein.

Purification from unclarified cell lysate using HisTrap FF crude

HisTrap FF crude is a ready-to-use column, prepacked with precharged Ni Sepharose 6 Fast Flow, for purification of histidine-tagged recombinant proteins. After thorough cell disruption, it is possible to load the unclarified, crude cell lysate directly on the column without centrifugation and filtration of the sample.

Direct loading of unclarified cell lysates decreases the total purification time and may increase the possibility of purifying sensitive target proteins without losing their activity.

Fig 27. HisTrap FF crude columns allow simple, one-step purification of histidine-tagged proteins without sample pretreatment such as centrifugation and filtration.

HisTrap FF crude columns are made of polypropylene that is biocompatible and does not interact with biomolecules. Columns are delivered with a stopper on the inlet and a snap-off end on the outlet. The columns have porous polyethylene top and bottom frits with a pore size optimized for loading of unclarified cell lysates directly on the column without causing back-pressure problems or leakage of the Ni Sepharose 6 Fast Flow beads. Columns can be operated with a syringe and the supplied Luer connector, a peristaltic pump, or a chromatography system such as ÄKTAdesign. Note that HisTrap FF crude columns cannot be opened or refilled.

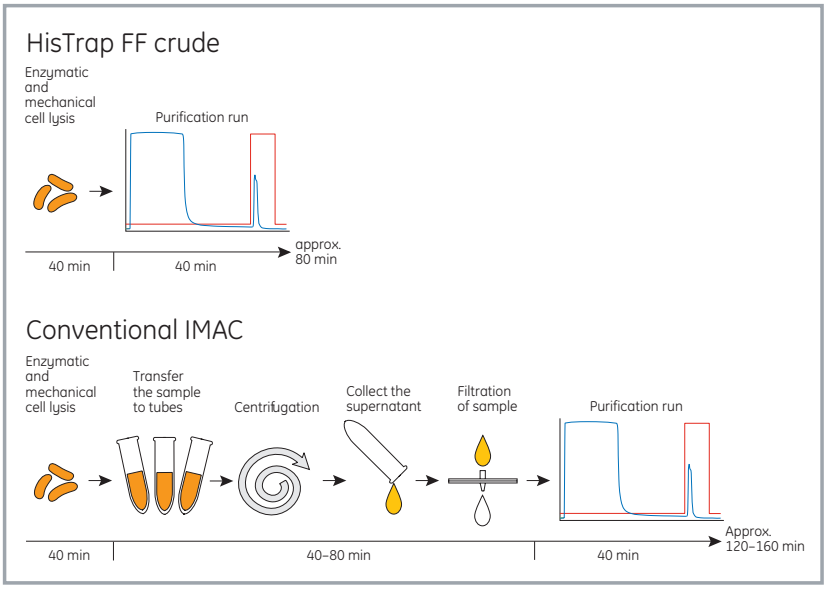


Fig 28. HisTrap FF crude columns save time during purification of histidine-tagged proteins compared with conventional IMAC.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

For direct loading of an unclarified sample, it is critical to obtain good cell lysis in order to avoid problems with back pressure.

The protocol below has been used successfully in our own laboratories, but other established procedures may also work.

1. Dilution of cell paste: Add 5 to 10 ml of binding buffer for each gram of cell paste. To prevent the binding of host cell proteins with exposed histidines, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).
2. Enzymatic lysis: 0.2 mg/ml lysozyme, 20 µg/ml DNase, 1 mM $MgCl_2$, 1 mM Pefabloc SC or PMSF (final concentrations). Stir for 30 min at room temperature or 4°C depending on the sensitivity of the target protein.
3. Mechanical lysis*: Sonication on ice, approximately 10 min
   or
   Homogenization with a French press or other homogenizer
   or
   Freeze/thaw, repeated at least five times.

   * Mechanical lysis time may have to be extended compared with standard protocols to secure an optimized lysate for sample loading (to prevent clogging of the column and back pressure problems). Different proteins have different sensitivity to cell lysis, and caution has to be taken to avoid frothing and overheating of the sample.
4. Adjust the pH of the lysate: Do not use strong bases or acids for pH adjustment (precipitation risk). Apply the unclarified lysate on the column directly after preparation.

If the sonicated or homogenized unclarified cell lysate is frozen before use, precipitation and aggregation may increase. New sonication of the lysate can then prevent increased back-pressure problems when loading on the column.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 500 mM NaCl, 20 to 40 mM imidazole, pH 7.4. (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)

Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4.

The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. Under native conditions, 20 to 40 mM imidazole in the binding buffer is suitable for many proteins; 500 mM imidazole in the elution buffer is most often sufficient to completely elute the target protein. Use high-purity imidazole as this will give very low or no absorbance at 280 nm.

As an alternative to elution with imidazole, you can lower the pH to approximately pH 4.5. (Note that metal ions will be stripped off the medium below pH 4.0.)

Purification

1. Fill the pump tubing or syringe with distilled water. Remove the stopper and connect the column to the chromatography system tubing, syringe (use the provided Luer connector), or laboratory pump "drop to drop" to avoid introducing air into the system.
2. Remove the snap-off end at the column outlet.
3. Wash out the ethanol with 3 to 5 column volumes of distilled water.

4. Equilibrate the column with at least 5 column volumes of binding buffer. Recommended flow rates are 1 ml/min or 5 ml/min for the 1-ml and 5-ml columns, respectively.
5. Apply the unclarified lysate with a pump or syringe. Typical loading volumes of unclarified lysate (highly dependent on specific sample, sample pretreatment, and temperature at sample loading):
   - HisTrap FF crude 1 ml: Up to 100 ml
   - HisTrap FF crude 5 ml: Up to 500 ml

   Note that the protein binding capacity may also limit the amount of sample that can be applied.

Continuous stirring of the sample during sample loading is recommended to prevent sedimentation. Sample loading at 4°C may increase the viscosity of the sample. An adverse effect of increased sample viscosity is that maximum back pressure for the column is reached at a lower sample volume loading on the column. Large volumes may increase back pressure, making the use of a syringe more difficult.

6. Wash with binding buffer until the absorbance reaches a steady baseline (generally at least 10 to 15 column volumes).
7. Elute with elution buffer using a step gradient or a linear gradient. For step elution, 5 column volumes of elution buffer is usually sufficient. A shallow gradient, for example, a linear gradient over 20 column volumes or more, can separate proteins with similar binding strengths.
8. After elution, regenerate the column by washing it with 5 to 10 column volumes of binding buffer. The column is now ready for a new purification.

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

Unclarified lysates may cause increased air bubble formation during purification. An attached flow restrictor in the chromatography system can prevent this if it causes problems. If a flow restrictor is attached, it is important to change the pressure limit to 0.5 MPa (5 bar) on the ÄKTAdesign system (where the column and the flow restrictor give a pressure of 0.3 MPa and 0.2 MPa, respectively).

Ni Sepharose is compatible with reducing agents. However, we recommend removal of any weakly bound Ni^{2+} ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store HisTrap FF crude columns with buffers that include reducing agents.

Leakage of Ni^{2+} from Ni Sepharose is low under all normal conditions. The leakage is lower than for other precharged IMAC media tested.
For very critical applications, leakage during purification can be even further diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer **without** reducing agents.

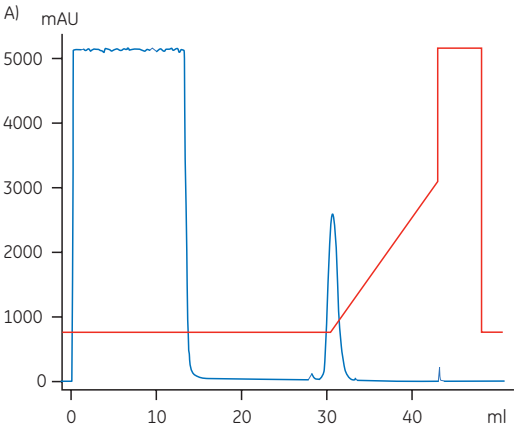
1. Wash the column with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding buffer.

Application examples

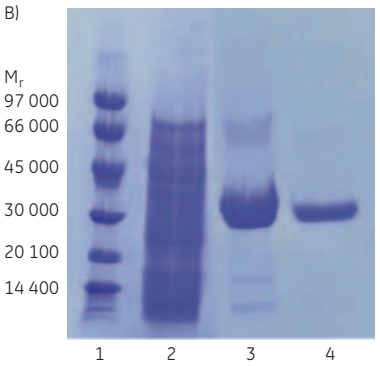
1. Purification of histidine-tagged protein expressed at low levels in *Pichia pastoris* using HisTrap FF crude

HisTrap FF crude columns are well-suited for purification of histidine-tagged proteins expressed at low levels from hosts such as *Pichia pastoris*. Using HisTrap FF crude columns, highly pure protein can be obtained directly from unclarified lysates of *P. pastoris*.

Figure 29 shows the purification of a histidine-tagged *Saccharomyces cerevisiae* hydrolase expressed at low levels in *P. pastoris*. **Unclarified** sample was loaded directly onto a HisTrap FF crude 5-ml column without any centrifugation or filtration of the sample. Purity of the protein from the unclarified sample was high as determined by SDS-PAGE.



Column:	HisTrap FF crude 5 ml
Sample:	Unclarified lysate of YNR064c (*Saccharomyces cerevisiae* hydrolase) expressed in *P. pastoris*
Sample volume:	130 ml
Binding buffer:	20 mM sodium phosphate, 500 mM NaCl, 75 mM imidazole, pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, 3 mM KCl, pH 7.4
Flow rate:	5 ml/min
System:	ÄKTAexplorer 100



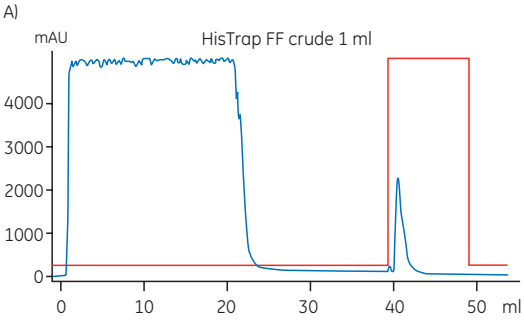
Lanes
1. LMW markers
2. Start material
3. Eluted pool (diluted 1:2)
4. Eluted pool (diluted 1:4)

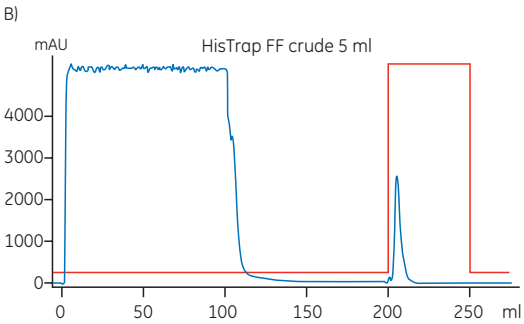
Fig 29. (A) Purification of an unclarified sample of histidine-tagged *Saccharomyces cerevisiae* hydrolase expressed in *P. pastoris* on HisTrap FF crude 5 ml. (B) SDS-PAGE under nonreducing conditions (ExcelGel SDS Gradient 8–18) shows the high purity obtained of the low-level expression protein.

2. Scale-up from 1-ml to 5-ml HisTrap FF crude

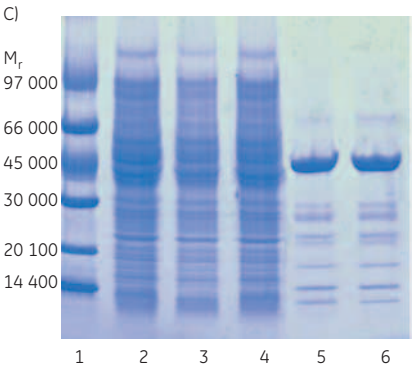
An experiment was performed to scale up from 1-ml to 5-ml HisTrap FF crude columns. The sample was unclarified *E. coli* extract containing MBP-$(His)_6$, which had been prepared by enzymatic lysis in combination with homogenization prior to loading on the column. The samples contained approximately 8 and 40 mg of MBP-$(His)_6$ for the 1-ml and 5-ml columns, respectively.

SDS-PAGE shows that the purity and recovery (mg protein/ml medium) of the histidine-tagged protein purified on the two columns was almost identical.





Column: HisTrap FF crude columns, 1 ml and 5 ml
Sample: Unclarified *E. coli* BL21 lysate containing MBP-$(His)_6$
Binding buffer: 20 mM sodium phosphate, 500 mM NaCl, 25 mM imidazole, pH 7.4
Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4
Flow rate: HisTrap FF crude 1 ml: 1 ml/min
HisTrap FF crude 5 ml: 5 ml/min
System: ÄKTAexplorer 100



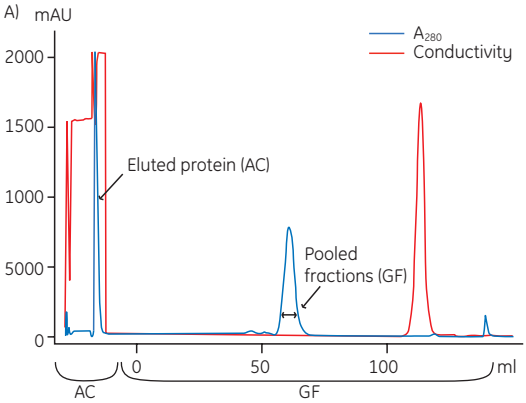
Lanes
1. LMW markers
2. Start material (diluted 1:10)
3. Flowthrough, 1-ml column (diluted 1:10)
4. Flowthrough, 5-ml column (diluted 1:10)
5. Eluted pool 1-ml column (diluted 1:5)
6. Eluted pool 5-ml column (diluted 1:5)

Fig 30. Scale-up from (A) 1-ml to (B) 5-ml HisTrap FF crude columns. Recovery of protein was 6.3 and 35.2 mg for the 1-ml and 5-ml columns, respectively. (C) SDS-PAGE under nonreducing conditions (ExcelGel SDS Gradient 8–18) confirms that scaling up from the 1-ml to the 5-ml column does not significantly affect the purification result.

3. Automated, multi-step purification using HisTrap FF crude

HisTrap FF crude columns can be run on ÄKTAdesign systems such as ÄKTAxpress for high-throughput purification of histidine-tagged proteins. ÄKTAxpress enables automated, parallel purification of histidine-tagged proteins with the capacity to run a number of different multi-step protocols. A method wizard supplied with the UNICORN control software makes it easy to create methods for different purification protocols. Figure 31 shows an automated two-step purification of an unclarified lysate of *E. coli* containing MBP-$(His)_6$. The first step in the purification protocol was affinity chromatography (AC), using HisTrap FF crude 1-ml column. The eluted peak from the affinity step was automatically collected in a loop and reinjected onto a HiLoad™ 16/60 Superdex 75 pg gel filtration column in the second step of the purification. Purity of the protein in fractions from the gel filtration step was confirmed by SDS-PAGE (Fig 31B).

The results show that HisTrap FF crude together with ÄKTAxpress facilitates and enables significant time savings in the purification of histidine-tagged proteins without compromising sample purity.

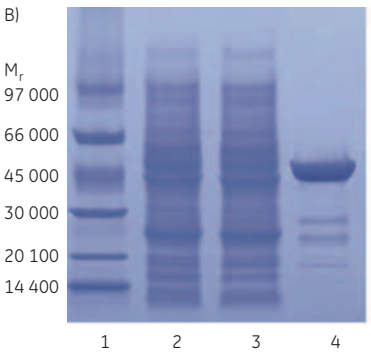


Affinity chromatography (AC) conditions

Column:	HisTrap FF crude 1 ml
Sample:	Unclarified *E. coli* BL21 lysate containing MBP-$(His)_6$
Binding buffer:	20 mM sodium phosphate, 500 mM NaCl, 25 mM imidazole, pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4
Flow rate:	1 ml/min
System:	ÄKTAxpress

Gel filtration (GF) conditions

Column:	HiLoad 16/60 Superdex 75 pg
Buffer:	10 mM sodium phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4
Flow rate:	1.5 ml/min
System:	ÄKTAxpress



Lanes
1. LMW markers
2. Start material (diluted 1:20)
3. Flowthrough, HisTrap FF crude 1-ml (diluted 1:10)
4. Pool from HiLoad 16/60 Superdex 75 pg (diluted 1:2)

Fig 31. (A) Automated two-step purification of MBP-$(His)_6$ from an unclarified lysate of *E. coli* using HisTrap FF crude and HiLoad 16/60 Superdex 75 pg on ÄKTAxpress. (B) SDS-PAGE was performed under nonreducing conditions on ExcelGel SDS Gradient 8–18.

Purification using a syringe and HisTrap FF crude Kit

HisTrap FF crude Kit is designed for rapid and convenient purification of histidine-tagged proteins using premade buffers and a syringe. Histidine-tagged proteins can be purified directly from unclarified cell lysates. This saves time because the pretreatment of the sample is minimized and may increase the activity of the target protein.

The kit contains three ready-to-use 1-ml HisTrap FF crude columns (containing Ni Sepharose 6 Fast Flow), buffer concentrates, a 5-ml syringe, and connectors. The kit provides a sufficient volume of buffer concentrates to perform 10 to 12 purifications when operated with a syringe. The special design of the column, together with Ni Sepharose 6 Fast Flow, provides fast, easy, and reproducible separations in a convenient format. Note that HisTrap FF crude columns cannot be opened or refilled.

Direct loading of unclarified cell lysates decreases the total purification time and may increase the possibility of purifying sensitive target proteins without losing their activity.

Fig 32. HisTrap FF crude Kit provides convenient and simple purification of histidine-tagged proteins.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

For direct loading of an unclarified sample, it is critical to obtain good cell lysis in order to avoid problems with back pressure.

The protocol below has been used successfully in our own laboratories, but other established procedures may also work.

1. Dilution of cell paste: Add 5 to 10 ml of binding buffer for each gram of cell paste. To prevent the binding of host cell proteins with exposed histidines, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).
2. Enzymatic lysis: 0.2 mg/ml lysozyme, 20 µg/ml DNase, 1 mM $MgCl_2$, 1 mM Pefabloc SC or PMSF (final concentrations). Stir for 30 min at room temperature or 4°C depending on the sensitivity of the target protein.
3. Mechanical lysis*: Sonication on ice, approximately 10 min
   or
   Homogenization with a French press or other homogenizer
   or
   Freeze/thaw, repeated at least five times

   * Mechanical lysis time may have to be extended compared with standard protocols to secure an optimized lysate for sample loading (to prevent clogging of the column and back pressure problems). Different proteins have different sensitivity to cell lysis, and caution has to be taken to avoid frothing and overheating of the sample.
4. Adjust the pH of the lysate: Do not use strong bases or acids for pH adjustment (precipitation risk). Apply the unclarified lysate on the column directly after preparation.

If the sonicated or homogenized unclarified cell lysate is frozen before use, precipitation and aggregation may increase. New sonication of the lysate can then prevent increased back-pressure problems when loading on the column.

Buffer preparation

Binding buffer:	Mix 3 ml of Phosphate buffer 8× stock solution (included in kit) with 0.24 ml of 2 M imidazole (included in kit) and add water to 24 ml. Check pH and adjust to pH 7.4 to 7.6 if necessary. This buffer now contains 20 mM phosphate, 500 mM NaCl, and 20 mM imidazole.
Elution buffer:	Mix 1 ml of Phosphate buffer 8× stock solution (included in kit) with 2 ml of 2 M imidazole (included in kit) and add distilled water to 8 ml. Check pH and adjust to pH 7.4 to 7.6 if necessary. This buffer now contains 20 mM phosphate, 500 mM NaCl, and 500 mM imidazole.

The high salt concentration in the buffer stock solution may cause salt crystals to form at low temperature. These crystals will dissolve at room temperature. We therefore recommend that the buffer stock solutions be allowed to reach room temperature before use. The formation of salt crystals that dissolve at room temperature does not affect the performance of the product.

Table 10 provides the mixing table for 50 ml of buffer. To obtain the imidazole concentration indicated in the first column, mix Phosphate buffer 8 × stock solution, 2 M imidazole, and distilled water according to the table. Check pH and adjust to pH 7.4 to 7.6 if necessary. These buffers will contain 20 mM phosphate, 500 mM NaCl, and the concentrations of imidazole indicated. For one purification, 24 ml of the binding buffer and 8 ml of each elution buffer are sufficient.

Table 10. Mixing table for 50 ml of buffer.

Imidazole concentration in buffer mM	Phosphate buffer 8x stock solution pH 7.4 ml	2 M Imidazole pH 7.4 ml	Deionized water ml
0	6.25	0	to 50 ml
10	6.25	0.25	to 50 ml
20	6.25	0.50	to 50 ml
30	6.25	0.75	to 50 ml
40	6.25	1.00	to 50 ml
50	6.25	1.25	to 50 ml
60	6.25	1.50	to 50 ml
70	6.25	1.75	to 50 ml
80	6.25	2.00	to 50 ml
90	6.25	2.25	to 50 ml
100	6.25	2.50	to 50 ml
150	6.25	3.75	to 50 ml
200	6.25	5.00	to 50 ml
250	6.25	6.25	to 50 ml
300	6.25	7.50	to 50 ml
400	6.25	10.00	to 50 ml
500	6.25	12.50	to 50 ml

Ni Sepharose is compatible with reducing agents. However, we recommend removal of any weakly bound Ni^{2+} ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store HisTrap FF crude columns with buffers that include reducing agents.

Leakage of Ni^{2+} from Ni Sepharose is low under all normal conditions. The leakage is lower than for other precharged IMAC media tested.
For very critical applications, leakage during purification can be even further diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer **without** reducing agents.

1. Wash the column with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding buffer.

Basic protein purification

When high yield is more important than optimal purity, use the following procedure. When optimal purity is required, use the optimized procedure (next page) instead.

1. Using the buffer concentrate provided, prepare 24 ml of binding buffer.
2. Using the buffer concentrate provided, prepare 8 ml of elution buffer.
3. Fill the syringe with distilled water. Remove the stopper and connect the column to the syringe with the provided Luer connector "drop to drop" to avoid introducing air into the column. (If air becomes trapped in the column, wash it with distilled water until the air disappears.)
4. Remove the snap-off end. Wash the column with 5 ml of distilled water.
5. Using the syringe, equilibrate the column with 5 to 10 ml of binding buffer.
6. Apply the unclarified lysate with the syringe. Collect the flowthrough fraction. A pump (e.g., Peristaltic Pump P-1) is convenient for large volumes (more than 15 ml) using a maximum flow rate of 3 ml/min.

Sample loading at 4°C may increase the viscosity of the sample. An adverse effect of increased sample viscosity is that maximum back pressure for the column is reached at a lower sample volume loading on the column. Large volumes may increase back pressure, making the use of a syringe more difficult. Typical loading volumes of unclarified lysate (highly dependent on specific sample, sample pretreatment, and temperature at sample loading): Up to 100 ml.

7. Wash with 10 ml of binding buffer. Collect the wash fraction.
8. Elute with 5 ml of elution buffer. Avoid dilution of the eluate by collecting it in 1-ml fractions.
9. Check the different fractions for the purified protein (e.g., by SDS-PAGE and/or Western blotting). The purified protein is most likely found in the second and third milliliter of the elution step.

For A_{280} measurement, use the elution buffer as a blank. If imidazole needs to be removed, use HiTrap Desalting, HiPrep 26/10 Desalting, or PD-10 Desalting Columns.

10. After elution, regenerate the column by washing it with 10 ml of binding buffer. The column is now ready for a new purification.

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this and on cleaning and storage, refer to Appendix 1.

Optimized protein purification

When optimal purity is needed, the following general procedure for stepwise gradient elution should be used.
The next time the same protein is to be purified, the number of steps can be reduced to those described under "Basic protein purification" with the optimal imidazole concentrations selected here.

1. Prepare binding buffer and five steps of elution buffer ranging from 40 mM to 500 mM imidazole. Check pH of each after mixing and adjust to pH 7.4 to 7.6 if necessary. See buffer mixing table, page 62.
2. Fill the syringe with distilled water. Remove the stopper and connect the column to the syringe with the provided Luer connector "drop to drop" to avoid introducing air into the column. (If air becomes trapped in the column, wash it with distilled water until the air disappears.)
3. Remove the snap-off end. Wash the column with 5 ml of distilled water.
4. Using the syringe, equilibrate the column with 5 to 10 ml of binding buffer.
5. Apply the unclarified lysate with the syringe. Collect the flowthrough fraction. A pump (e.g., Peristaltic Pump P-1) is convenient for large volumes (more than 15 ml) using a maximum flow rate of 3 ml/min.

Sample loading at 4°C may increase the viscosity of the sample. An adverse effect of increased sample viscosity is that maximum back pressure for the column is reached at a lower sample volume loading on the column. Large volumes may increase back pressure, making the use of a syringe more difficult. Typical loading volumes of unclarified lysate (highly dependent on specific sample, sample pretreatment, and temperature at sample loading): Up to 100 ml.

6. Wash with 10 ml of binding buffer. Collect the wash fraction.
7. Start elution with 5 ml of the first elution buffer containing 40 mM imidazole. Avoid dilution by collecting the eluate in 1-ml fractions.
8. Proceed with the next imidazole concentration. (For example, elute with 5 ml of elution buffer containing 60 mM imidazole.) Collect the eluate in 1-ml fractions as above.
9. Proceed with the buffers of increasing imidazole concentration, as described in steps 6 and 7. The purified protein is most likely found in the second and third fraction of one of the elution steps.
10. Check the different fractions for the purified protein (e.g., by SDS-PAGE and/or Western blotting).

For A_{280} measurements, use the elution buffers as blanks. If imidazole is to be removed, use HiTrap Desalting, HiPrep 26/10 Desalting, or PD-10 Desalting Columns.

11. After elution, reequilibrate the column with 10 ml of binding buffer. The column is now ready for a new purification.

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

The results of the above purification provide information about the optimal binding and elution buffers. The optimal elution buffer is the one that eluted the histidine-tagged protein. The optimal binding (wash) buffer is the one from 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 of the purified protein. Use these buffers for the next purification of an identical protein.

The concentration of imidazole needed to prevent nonspecific binding of host cell proteins (without any elution of histidine-tagged protein) is generally more important to determine than the concentration needed for elution. A concentration of 500 mM can be used for elution in most cases.

Application example

Purification using HisTrap FF crude Kit

HisTrap FF crude Kit includes three 1-ml HisTrap FF crude columns, ready-made binding and elution buffer concentrates, connectors, a syringe, and instructions. The kit allows purification in a matter of minutes starting from an unclarified cell lysate using a simple, four-step procedure:

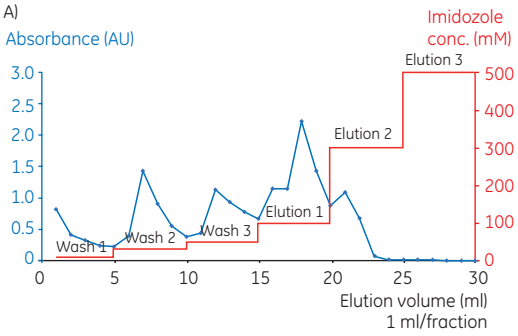
1. Lyse cells containing histidine-tagged protein.

2. Prepare buffers by mixing and diluting the concentrates.

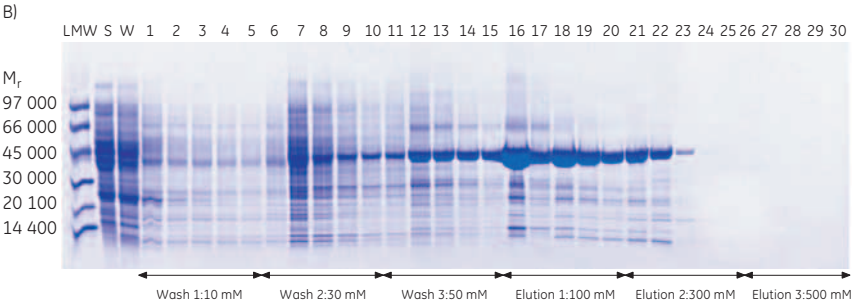
3. Use the syringe to load unclarified sample to the column, wash, and elute the target protein.

4. Check purity by SDS-PAGE.

As Figure 33 shows, MBP-$(His)_6$ is effectively purified on the HisTrap FF crude 1-ml column using a syringe and the buffers included in HisTrap FF crude Kit.



Column: HisTrap FF crude 1 ml
Sample: MBP-$(His)_6$, M_r 43 000
Flow rate: Approx. 1 ml/min
Buffers: Included in the HisTrap FF crude Kit, prepared according to the instructions
Fraction size: 1 ml
Equipment: Manual purification using a syringe



Lanes
LMW LMW markers
S Sample, unclarified, diluted 1:10
W Wash 5 ml, 10 mM imidazole

Lanes
Fraction 1–5 Wash 1, 10 mM imidazole in buffer
Fraction 6–10 Wash 2, 30 mM imidazole in buffer
Fraction 11–15 Wash 3, 50 mM imidazole in buffer
Fraction 16–20 Elution 1, 100 mM imidazole in buffer
Fraction 21–25 Elution 2, 300 mM imidazole in buffer
Fraction 26–30 Elution 3, 500 mM imidazole in buffer

Fig 33. (A) Purification of MBP-$(His)_6$ using HisTrap FF crude Kit. (B) Native SDS-PAGE (ExcelGel 8–18) of 1-ml fractions from the purification.

Gravity-flow purification using His GraviTrap and His GraviTrap Kit

His GraviTrap columns are designed for fast and simple purification of histidine-tagged proteins using gravity flow. Both clarified and unclarified sample can be applied to the column. The column is prepacked with Ni Sepharose 6 Fast Flow. Special column frits protect the medium from running dry during purification. A typical purification run on His GraviTrap is performed in approximately 30 min (depending on sample volume and viscosity of the solutions).

Fig 34. His GraviTrap connected to LabMate PD-10 Buffer Reservoir for convenient equilibration, sample application, and wash.

His GraviTrap columns are delivered in a package that can be converted into a column stand to simplify purification. LabMate™ PD-10 Buffer Reservoir can be connected to the columns for convenient handling of sample volumes above 10 ml. For optimal performance, use His GraviTrap with buffers prepared from His Buffer Kit.

The benefits of His GraviTrap and His Buffer Kit are combined in His GraviTrap Kit, which contains two packs of His GraviTrap and one pack of His Buffer Kit. His GraviTrap Kit contains columns and buffers for 20 purifications.

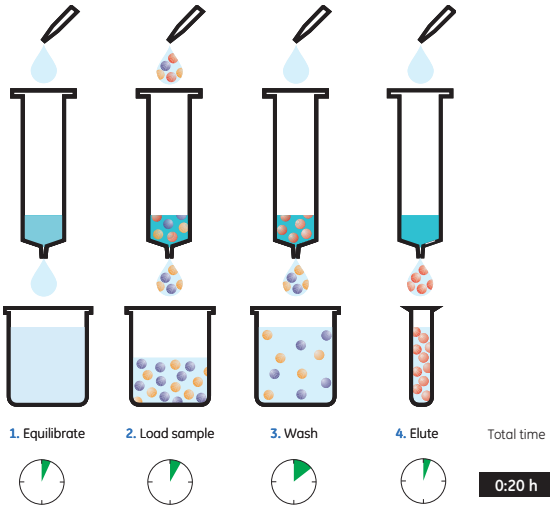


Fig 35. Purifying histidine-tagged proteins with His GraviTrap is a simple and quick four-stage procedure.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

For direct loading of an unclarified sample, it is critical to obtain good cell lysis in order to avoid problems with back pressure.

The protocol below has been used successfully in our own laboratories, but other established procedures may also work.

1. Dilution of cell paste: Add 5 to 10 ml of binding buffer for each gram of cell paste. To prevent the binding of host cell proteins with exposed histidines, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).
2. Enzymatic lysis: 0.2 mg/ml lysozyme, 20 µg/ml DNase, 1 mM $MgCl_2$, 1 mM Pefabloc SC or PMSF (final concentrations). Stir for 30 min at room temperature or 4°C depending on the sensitivity of the target protein.
3. Mechanical lysis*: Sonication on ice, approximately 10 min
   or
   Homogenization with a French press or other homogenizer
   or
   Freeze/thaw, repeated at least five times

   * Mechanical lysis time may have to be extended compared with standard protocols to secure an optimized lysate for sample loading (to prevent clogging of the column and back pressure problems). Different proteins have different sensitivity to cell lysis, and caution has to be taken to avoid frothing and overheating of the sample.
4. Adjust the pH of the lysate: Do not use strong bases or acids for pH adjustment (precipitation risk). Apply the unclarified lysate on the column directly after preparation.

If the sonicated or homogenized unclarified cell lysate is frozen before use, precipitation and aggregation may increase. New sonication of the lysate can then prevent increased back-pressure problems when loading on the column.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4.

Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4

The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. In the binding buffer, 20 to 40 mM imidazole is suitable for many proteins; 500 mM imidazole in the elution buffer ensures complete elution of the target protein.

As an alternative to elution with imidazole, lower the pH to approximately pH 4.5. (Metal ions will be stripped off the medium below pH 4.0.)

Purification

1. Cut off the bottom tip, remove the top cap, pour off excess liquid, and place the column in the Workmate column stand. If needed, mount LabMate (funnel) on top of the column.
2. Equilibrate the column with 10 ml of binding buffer. The frits protect the column from running dry during the run.
3. Add 0.5 to 35 ml of the prepared sample.

The protein binding capacity of the column is high (approx. 40 mg histidine-tagged protein/column); however, the value is protein dependent.

4. Wash with 10 ml of binding buffer.
5. Apply 3 ml of elution buffer and collect the eluate. Under denaturing conditions, elute twice with 3 ml of elution buffer.

If you use buffers containing denaturing agents or viscous solutions, perform the purification at room temperature.

Ni Sepharose is compatible with reducing agents. However, we recommend removal of any weakly bound Ni^{2+} ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store His GraviTrap columns with buffers that include reducing agents.

Leakage of Ni^{2+} from Ni Sepharose is low under all normal conditions. The leakage is lower than for other precharged IMAC media tested.
For very critical applications, leakage during purification can be even further diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer **without** reducing agents.

1. Wash the column with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding buffer.

Application example

Rapid purification of a high-molecular-weight histidine-tagged protein using His GraviTrap

His GraviTrap, prepacked with Ni Sepharose 6 Fast Flow, allows quick and simple purification of histidine-tagged proteins without the need for a pump or purification system. A single column allows purification of approximately 40 mg of protein in as little as 20 to 25 minutes. Large volumes of clarified or unclarified samples can easily be applied, and the purified protein can be eluted in a small volume, resulting in a highly concentrated target protein.

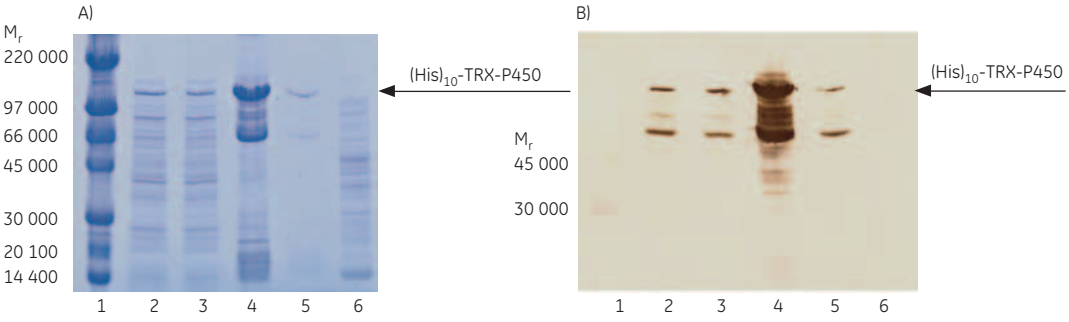
In this example, 20 ml of a clarified *E. coli* JM109 lysate containing $(His)_{10}$-TRX-P450 (M_r ~ 130 000) was purified in just 25 minutes and analyzed by SDS-PAGE and Western blot (Fig 36A–B). SDS-PAGE analysis shows three major protein bands in the eluted fractions. Western blot analysis and N-terminal sequencing (data not shown) confirm that each of the three bands in the eluates contains a histidine tag. The low-molecular-weight bands are truncated forms of the histidine-tagged target protein.

Method:

Equilibration:	10 ml binding buffer (including 40 mM imidazole)
Sample application:	20 ml sample (including 40 mM imidazole)
Wash:	2 × 10 ml binding buffer (including 40 mM imidazole)
Elution:	2 × 3 ml elution buffer

Western blot:

Electrophoresis and transfer:	PhastSystem™ and PhastGel™ Gradient 10–15
Membrane:	Hybond ECL
Primary antibody:	Anti-His antibody (mouse)
Secondary antibody:	Anti-mouse IgG, HRP-linked
Detection:	Colorimetric, DAB-enhanced liquid substrate



Lanes

1. High-Range Rainbow™ Molecular Weight Markers
2. Start material (diluted 1:20)
3. Flowthrough (diluted 1:20)
4. Eluate 1
5. Eluate 2
6. Negative control (JM109 nontransformed)

Fig 36. (A) SDS-PAGE and (B) Western blot of purified $(His)_{10}$-TRX-P450 using His GraviTrap column.

Scale-up purification using HisPrep FF 16/10

HisPrep FF 16/10 columns are specially designed 20-ml HiPrep columns, ready to use for easy, one-step preparative purification of histidine-tagged proteins. Prepacked with Ni Sepharose 6 Fast Flow, the columns exhibit high binding capacity and excellent flow properties. For easy scale-up, columns can be connected in series (back pressure will increase).

Fig 37. HisPrep FF 16/10 column for convenient scale-up purification of histidine-tagged proteins.

The column is made of polypropylene, which is biocompatible and noninteractive with biomolecules. Purifications can be easily achieved using a chromatography system such as ÄKTAdesign or other chromatography systems (connectors are included in each package for easy connections). Refer to Table 8 for a selection guide to purification equipment and to Appendix 1 for a list of HisPrep FF 16/10 column parameters. Note that HisPrep FF 16/10 columns cannot be opened or refilled.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

- Adjust the sample to the composition and pH of the binding buffer by: adding buffer, NaCl, imidazole, and additives from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange. To prevent the binding of host cell proteins with exposed histidines, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).
- Pass the sample through a 0.22 µm or a 0.45 µm filter and/or centrifuge it immediately before applying it to the column. If the sample is too viscous, to prevent it from clogging the column dilute it with binding buffer, increase lysis treatment (sonication, homogenization), or add DNase/RNase to reduce the size of nucleic acid fragments.

Buffer preparation

Binding buffer:	20 mM sodium phosphate, 500 mM NaCl, 20 to 40 mM imidazole, pH 7.4. (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4.

- The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. In the binding buffer, 20 to 40 mM imidazole is suitable for many proteins; 500 mM imidazole in the elution buffer ensures complete elution of the target protein.
- As an alternative to elution with imidazole, lower the pH to approximately pH 4.5. (Metal ions will be stripped off the medium below pH 4.0.)

Purification

For first-time use, it is important to set an appropriate pressure limit on the system and equilibrate the column by running 100 ml of binding buffer through it.

1. Apply the centrifuged and/or filtered sample (in binding buffer) to the column at a flow rate of 1 to 10 ml/min (30 to 300 cm/h).
2. Wash the column with 5 to 10 column volumes of binding buffer at 2 to 10 ml/min (60 to 300 cm/h).
3. Elute the bound protein with 5 to 10 column volumes of elution buffer at a flow rate of 2 to 10 ml/min (60 to 300 cm/h).
4. After elution, regenerate the column by washing it with approximately 100 ml of binding buffer. The column is now ready for a new purification.

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

Ni Sepharose is compatible with reducing agents. However, we recommend removal of any weakly bound Ni^{2+} ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store HisPrep columns with buffers that include reducing agents.

Leakage of Ni^{2+} from Ni Sepharose is low under all normal conditions. The leakage is lower than for other precharged IMAC media tested.
For very critical applications, leakage during purification can be even further diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer **without** reducing agents.

1. Wash the column with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding buffer.

Application example

Refer to Application example 3 on page 50.

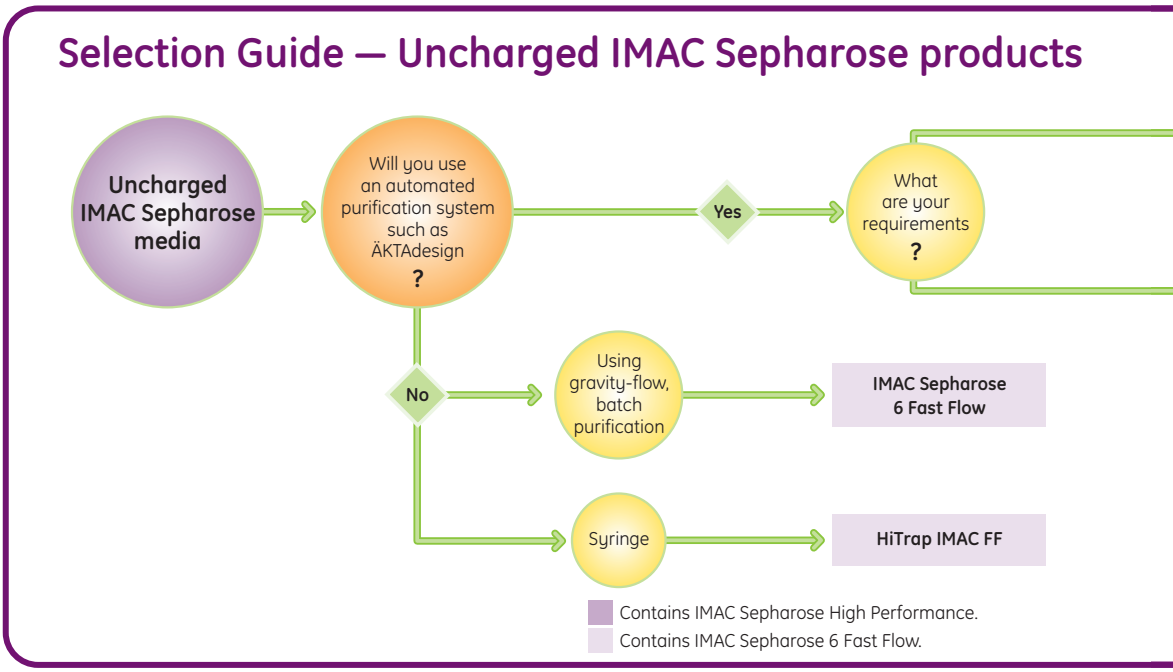


Fig 38. Selection guide for uncharged IMAC Sepharose products.

Figure 38 provides a selection guide for the uncharged IMAC Sepharose products, and Table 11 describes these options in more detail. In general, IMAC Sepharose High Performance is recommended when high resolution and high capacity are important, whereas IMAC Sepharose 6 Fast Flow is recommended when scale-up is required.

Table 11. Purification options for histidine-tagged proteins using uncharged IMAC Sepharose products.

Product	Format or column size	Approx. protein binding capacity	Description	High-throughput screening	Mini-preps	Batch/ gravity flow	Syringe	ÄKTAdesign system
IMAC Sepharose High Performance	25 ml 100 ml	40 mg/ml (Ni^{2+})	For high resolution and elution of a more concentrated sample (high-performance purification).	+	(+)	-	-	+
HiTrap IMAC HP	1 ml 5 ml	40 mg/ column (Ni^{2+}) 200 mg/ column (Ni^{2+})	For use mainly with a peristaltic pump or chromatography system. For high resolution and elution of a more concentrated sample (high-performance purification).	-	-	-	(+)	+
IMAC Sepharose 6 Fast Flow	25 ml 100 ml	40 mg/ml (Ni^{2+})	Excellent for scale-up due to high capacity and high flow properties.	+	+	+	-	+

continues on following page

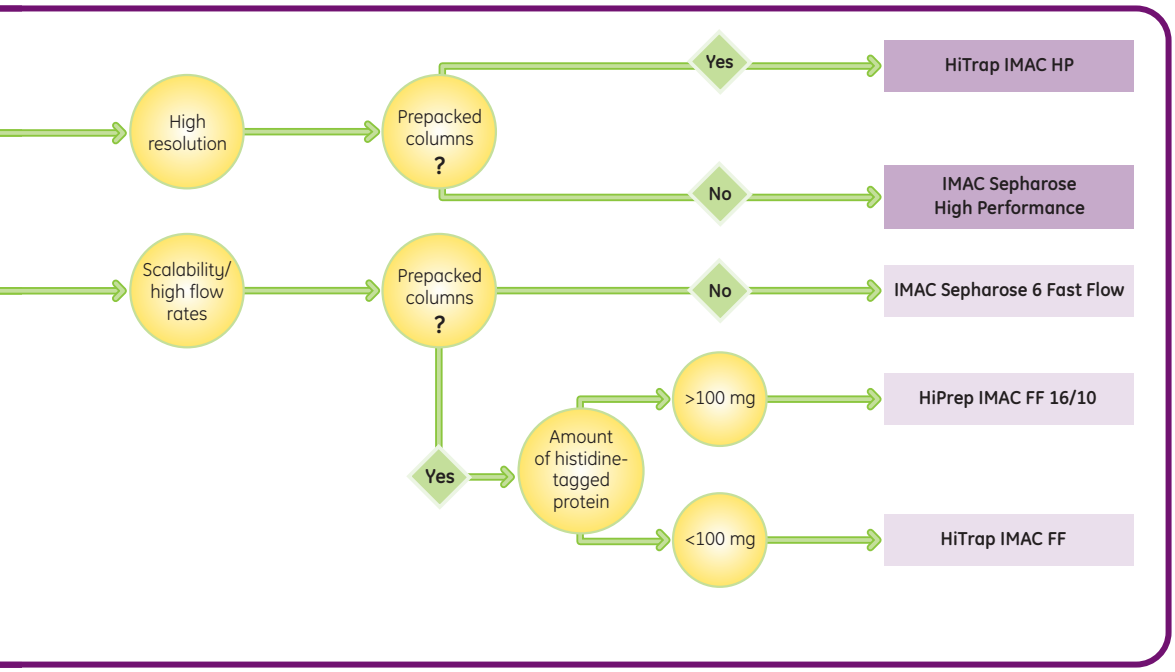


Table 11. Purification options for histidine-tagged proteins using uncharged IMAC Sepharose products (continued).

Product	Format or column size	Approx. protein binding capacity	Description	High-throughput screening	Mini-preps	Batch/ gravity flow	Syringe	ÄKTAdesign system
HiPrep IMAC FF 16/10	20 ml	800 mg/ column (Ni^{2+})	For use with a chromatography system. Scale-up purification.	-	-	-	-	+
HiTrap IMAC FF	1 ml	40 mg/ column (Ni^{2+})	For use with syringe, peristaltic pump, or chromatography system. Provides excellent flow properties. Scale-up.	-	-	-	+	+
	5 ml	200 mg/ column (Ni^{2+})						

Contains IMAC Sepharose High Performance

Contains IMAC Sepharose 6 Fast Flow

Purification using IMAC Sepharose High Performance

IMAC Sepharose High Performance is an uncharged medium consisting of 34-µm beads of highly cross-linked 6% agarose to which a chelating group has been covalently coupled. This chelating group will be charged with suitable metal ions by the user, allowing the medium to selectively retain target proteins. The small bead size allows high chromatographic resolution with distinctly separated peaks containing concentrated material. The medium is highly compatible with a range of additives and is well suited to high-performance purifications that produce concentrated products in the eluate. Refer to Appendix 1 for a list of the characteristics of IMAC Sepharose High Performance. IMAC Sepharose High Performance is supplied preswollen in 20% ethanol.

Fig 39. IMAC Sepharose High Performance is supplied free of metal ions, enabling it to be used across a range of applications for purifying histidine-tagged as well as native proteins. It is available in 25 ml and 100 ml lab packs as well as prepacked HiTrap IMAC HP 1-ml and 5-ml columns.

Packing a column

Refer to Appendix 4 for general guidelines for column packing.

Ideally, IMAC Sepharose High Performance media are packed in XK or Tricorn columns in a two-step procedure: Do not exceed 1.0 bar (0.1 MPa) in the first step and 3.5 bar (0.35 MPa) in the second step. If the packing equipment does not include a pressure gauge, use a packing flow rate of 5 ml/min (XK 16/20 column) or 2 ml/min (Tricorn 10/100 column) in the first step, and 9 ml/min (XK 16/20 column) or 3.6 ml/min (Tricorn 10/100 column) in the second step. If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate your pump can deliver in order to achieve a well-packed bed.

1. Assemble the column (and packing reservoir if necessary).
2. Remove air from the end-piece and adapter by flushing with distilled water. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with water.
3. Resuspend the medium and pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.
4. If using a packing reservoir, immediately fill the remainder of the column and reservoir with water. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.
5. Open the bottom outlet of the column and set the pump to run at the desired flow rate.

6. Maintain packing flow rate for at least 3 bed volumes after a constant bed height is reached. Mark the bed height on the column.
7. Stop the pump and close the column outlet.
8. If using a packing reservoir, disconnect the reservoir and fit the adapter to the column.
9. With the adapter inlet disconnected, push the adapter down into the column until it reaches the mark. Allow the packing solution to flush the adapter inlet. Lock the adapter in position.
10. Connect the column to a pump or a chromatography system and start equilibration. Readjust the adapter if necessary.

    Note: For subsequent chromatography procedures, do not exceed 75% of the packing flow rate.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

Adjust the sample to the composition and pH of the binding buffer by: adding buffer, NaCl, imidazole, and additives from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange. To prevent the binding of host cell proteins with exposed histidine, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).

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

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 0.5 to 1.0 M NaCl, 20 to 40 mM imidazole, pH 7.4. (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)

Elution buffer: 20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4.

Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.22 µm or a 0.45 µm filter before use. High-purity imidazole will give low to no absorbance at 280 nm.

The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. Under native conditions, 20 to 40 mM imidazole in the binding buffer is suitable for many proteins; 500 mM imidazole in the elution buffer is most often sufficient to completely elute the target protein.

Charging the medium with metal ion

1. Prepare a 0.1 M solution of the desired metal ion (e.g., Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}, or Ni^{2+}) in distilled water. Salts of chlorides, sulfates, etc., can be used (e.g., 0.1 M $CuSO_4$ or 0.1 M $NiSO_4$).

Solutions of Zn^{2+} ions should have a pH of approximately 5.5 or lower to avoid solubility problems that arise at pH 6 or higher. Fe^{3+} ions should be immobilized at low pH (approximately pH 3.0) to avoid formation of insoluble ferric compounds.

2. Wash the column with at least 2 column volumes of distilled water.
3. Apply at least 0.2 column volumes of the metal ion solution to the column.
4. Wash the column with at least 5 column volumes of distilled water to remove excess metal ions.

Washing with buffer before applying the metal ion solution may cause unwanted precipitation.

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

IMAC Sepharose High Performance is compatible with reducing agents. However, we recommend removal of any weakly bound metal ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store IMAC Sepharose High Performance with buffers that include reducing agents.

Leakage of metal ions is low under all normal conditions. For critical applications, leakage during purification can be diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer **without** reducing agents.

1. Wash the column with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding buffer.

Purification

1. If necessary, wash out the 20% ethanol with 5 column volumes of distilled water. Use a linear flow rate of 50 to 100 cm/h. Refer to Appendix 6 for flow rate calculations.
2. Equilibrate the column with 5 to 10 column volumes of binding buffer at a linear flow rate of 150 cm/h.
3. Apply the pretreated sample.
4. Wash the column with binding buffer until the absorbance reaches the baseline.
5. Elute with elution buffer using a step or linear gradient.
   For step elution, 5 column volumes of elution buffer are usually sufficient.
   For linear gradient elution, a shallow gradient, over 20 column volumes, may separate proteins with similar binding strengths.
6. After elution, regenerate the column by washing it with 5 to 10 column volumes of binding buffer. The column is now ready for a new purification.

Use the elution buffer as blank when measuring absorbance manually. If imidazole needs to be removed from the protein, use HiTrap Desalting, a PD-10 Desalting, or HiPrep 26/10 Desalting column.

Purification using IMAC Sepharose 6 Fast Flow

IMAC Sepharose 6 Fast Flow consists of 90-µm beads of highly cross-linked agarose to which a chelating group has been covalently coupled. This chelating group will be charged with suitable metal ions by the user, allowing the medium to selectively retain target proteins.

IMAC Sepharose 6 Fast Flow displays a high protein binding capacity. The binding capacity is protein dependent and metal-ion dependent. The medium is easy to pack and use, and its high flow properties make it excellent for scaling up. Refer to Appendix 1 for a list of the characteristics of this medium. IMAC Sepharose 6 Fast Flow is supplied preswollen in 20% ethanol.

Fig 40. IMAC Sepharose 6 Fast Flow provides high flow properties to enable scaled-up purification of histidine-tagged and native proteins. It provides numerous possibilities for optimizing purifications at both laboratory and process scale.

Packing a column

Refer to Appendix 4 for general guidelines for column packing.

Ideally, IMAC Sepharose 6 Fast Flow media are packed in XK or Tricorn columns in a two-step procedure: Do not exceed 0.5 bar (0.05 MPa) in the first step and 1.5 bar (0.15 MPa) in the second step. If the packing equipment does not include a pressure gauge, use a packing flow rate of 2.5 ml/min (XK 16/20 column) or 0.9 ml/min (Tricorn 10/100 column) in the first step, and 8.7 ml/min (XK 16/20 column) or 4.7 ml/min (Tricorn 10/100 column) in the second step.

1. Assemble the column (and packing reservoir if necessary).
2. Remove air from the end-piece and adapter by flushing with distilled water. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with water.
3. Resuspend the medium and pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.
4. If using a packing reservoir, immediately fill the remainder of the column and reservoir with water. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.
5. Open the bottom outlet of the column and set the pump to run at the desired flow rate.

6. Maintain packing flow rate for at least 3 bed volumes after a constant bed height is reached. Mark the bed height on the column.
7. Stop the pump and close the column outlet.
8. If using a packing reservoir, disconnect the reservoir and fit the adapter to the column.
9. With the adapter inlet disconnected, push the adapter down into the column until it reaches the mark. Allow the packing solution to flush the adapter inlet. Lock the adapter in position.
10. Connect the column to a pump or a chromatography system and start equilibration. Readjust the adapter if necessary.

    Note: For subsequent chromatography procedures, do not exceed 75% of the packing flow rate.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

Adjust the sample to the composition and pH of the binding buffer by: adding buffer, NaCl, imidazole, and additives from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange. To prevent the binding of host cell proteins with exposed histidine, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).

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

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 0.5 to 1.0 M NaCl, 20 to 40 mM imidazole, pH 7.4. (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)

Elution buffer: 20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4.

Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.22 µm or a 0.45 µm filter before use. High-purity imidazole will give low to no absorbance at 280 nm.

The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. Under native conditions, 20 to 40 mM imidazole in the binding buffer is suitable for many proteins; 500 mM imidazole in the elution buffer is most often sufficient to completely elute the target protein.

Charging the medium with metal ion

1. Prepare a 0.1 M solution of the desired metal ion (e.g., Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}, or Ni^{2+}) in distilled water. Salts of chlorides, sulfates, etc., can be used (e.g., 0.1 M $CuSO_4$ or 0.1 M $NiSO_4$).

Solutions of Zn^{2+} ions should have a pH of approximately 5.5 or lower to avoid solubility problems that arise at pH 6 or higher. Fe^{3+} ions should be immobilized at low pH (approximately pH 3.0) to avoid formation of insoluble ferric compounds.

2. Wash the column with at least 2 column volumes of distilled water.
3. Apply at least 0.2 column volumes of the metal ion solution to the column.
4. Wash the column with at least 5 column volumes of distilled water to remove excess metal ions.

Washing with buffer before applying the metal ion solution may cause unwanted precipitation.

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

IMAC Sepharose 6 Fast Flow is compatible with reducing agents. However, we recommend removal of any weakly bound metal ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store IMAC Sepharose 6 Fast Flow with buffers that include reducing agents.

Leakage of metal ions is low under all normal conditions. For critical applications, leakage during purification can be diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer **without** reducing agents.

1. Wash the column with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding buffer.

Purification

1. If necessary, wash out the 20% ethanol with 5 column volumes of distilled water. Use a linear flow rate of 50 to 100 cm/h. Refer to Appendix 6 for flow rate calculations.
2. Equilibrate the column with 5 to 10 column volumes of binding buffer at a linear flow rate of 150 cm/h.
3. Apply the pretreated sample.
4. Wash the column with binding buffer until the absorbance reaches the baseline.
5. Elute with elution buffer using a step or linear gradient.
   For step elution, 5 column volumes of elution buffer are usually sufficient.
   For linear gradient elution, a shallow gradient, over 20 column volumes, may separate proteins with similar binding strengths.
6. After elution, regenerate the column by washing it with 5 to 10 column volumes of binding buffer. The column is now ready for a new purification.

Use the elution buffer as blank when measuring absorbance manually. If imidazole needs to be removed from the protein, use HiTrap Desalting, a PD-10 Desalting, or HiPrep 26/10 Desalting column.

Purification using HiTrap IMAC HP and HiTrap IMAC FF columns

HiTrap IMAC HP and HiTrap IMAC FF are 1-ml and 5-ml columns prepacked with IMAC Sepharose High Performance or IMAC Sepharose 6 Fast Flow, respectively. Sample application, washing, and elution can be performed using a syringe with a supplied adapter, a peristaltic pump, or a liquid chromatography system such as ÄKTAdesign (see Table 8 for equipment choices).

HiTrap IMAC HP and HiTrap IMAC FF columns are made of polypropylene, which is biocompatible and noninteractive with biomolecules. The top and bottom frits are manufactured from porous polyethylene. Columns are delivered with a stopper on the inlet and a snap-off end on the outlet. Each package includes all necessary components for connecting the columns to different types of equipment. For quick scale-up of purifications, two or three HiTrap columns (1 ml or 5 ml) can be connected in series (back pressure will be higher). Note that HiTrap IMAC columns cannot be opened or refilled.

Fig 41. HiTrap IMAC HP 1-ml columns charged with Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}, respectively.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

Adjust the sample to the composition and pH of the binding buffer by: adding buffer, NaCl, imidazole, and additives from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange. To prevent the binding of host cell proteins with exposed histidines, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).

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

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 500 mM NaCl, 20 to 40 mM imidazole, pH 7.4. (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)

Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4.

Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.22 µm or a 0.45 µm filter before use. High-purity imidazole will give low to no absorbance at 280 nm.

The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. Under native conditions, 20 to 40 mM imidazole in the binding buffer is suitable for many proteins; 500 mM imidazole in the elution buffer is most often sufficient to completely elute the target protein.

Charging the column with metal ion

1. Prepare a 0.1 M solution of the desired metal ion (e. g., Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}, or Ni^{2+}) in distilled water. Salts of chlorides, sulfates, etc., can be used (e.g., 0.1 M $CuSO_4$ or 0.1 M $NiSO_4$).

Solutions of Zn^{2+} ions should have a pH of approximately 5.5 or lower to avoid solubility problems that arise at pH 6 or higher. Fe^{3+} ions should be immobilized at low pH (approximately pH 3.0) to avoid formation of insoluble ferric compounds.

2. Fill the pump tubing or syringe with distilled water. Remove the stopper and connect the column to the chromatography system tubing, syringe (use the connector supplied), or laboratory pump "drop to drop" to avoid introducing air into the system.
3. Remove the snap-off end at the column outlet.
4. Wash out the ethanol with 5 ml (HiTrap IMAC HP or FF 1-ml columns) or 15 ml (HiTrap IMAC HP or FF 5-ml columns) of distilled water. Do not use buffer to wash the column at this stage; a buffer wash may cause the metal ion to precipitate during step 5.
5. Charge the water-washed column by loading at least 0.5 ml (HiTrap IMAC HP or FF 1-ml columns) or 2.5 ml (HiTrap IMAC HP or FF 5-ml columns) of 0.1 M metal ion/salt solution.
6. Repeat the water wash described in step 4.
7. Equilibrate the column with at least 5 column volumes of binding buffer. Recommended flow rates are 1 ml/min or 5 ml/min for the 1-ml and 5-ml columns, respectively.

Washing with buffer before applying the metal ion solution may cause unwanted precipitation.

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

IMAC Sepharose is compatible with reducing agents. However, we recommend removal of any weakly bound metal ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store HiTrap IMAC columns with buffers that include reducing agents.

For critical applications, leakage of metal ions during purification can be diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer **without** reducing agents.

1. Wash the column with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding buffer.

Purification

1. Apply the pretreated sample using a syringe fitted to the connector or by pumping it onto the column. For best results, use a flow rate of 0.2 to 1 ml/min (1-ml column) and 0.5 to 5 ml/min (5-ml column) during sample application*.
2. Wash with binding buffer (generally at least 10 to 15 column volumes) until the absorbance reaches a steady baseline.
3. Elute with elution buffer using a one-step or linear gradient. For step elution, 5 column volumes usually suffice. A linear gradient over 20 column volumes or more may separate proteins with similar binding strengths.
4. After elution, regenerate the column by washing it with 5 to 10 column volumes of binding buffer. The column is now ready for a new purification.

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

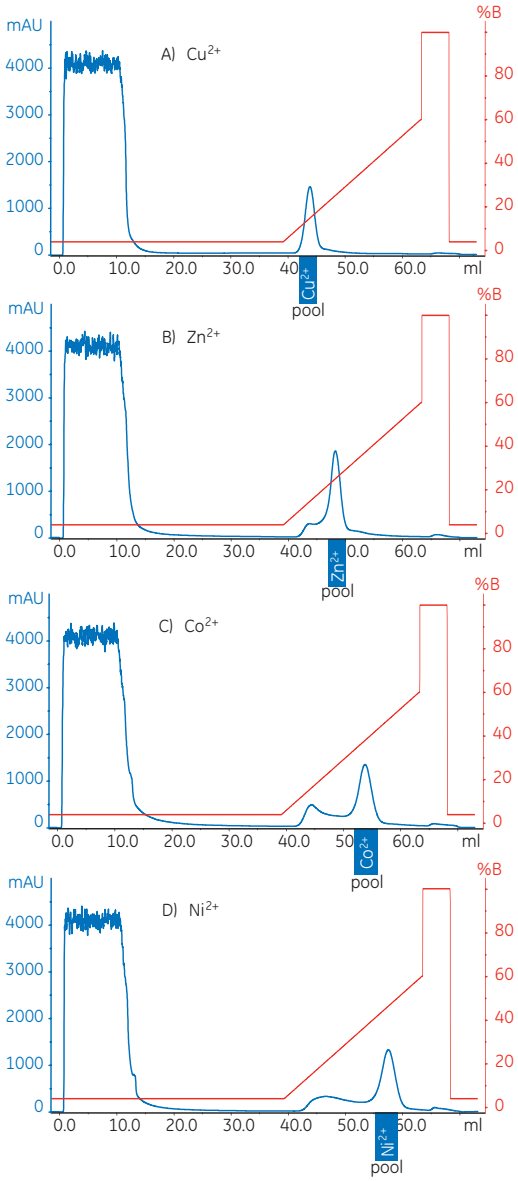
The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

Application example

Screening for optimized purity using different metal ions

YNR064c (M_r 33 700) is a $(histidine)_6$-tagged protein expressed in *Pichia pastoris*. It was purified using HiTrap IMAC HP 1-ml columns charged separately with Cu^{2+}, Zn^{2+}, Co^{2+}, or Ni^{2+}; conditions were otherwise the same for the four purifications. See Figures 42A-E for the resulting chromatograms and SDS-PAGE analysis of pooled fractions.

The results show that for this $(histidine)_6$-tagged target protein, the highest purity was achieved with Ni^{2+} or Cu^{2+}, although Cu^{2+}, at the conditions used, apparently gave a small loss of target protein (Figure 42E, lane 4).



Column: HiTrap IMAC HP 1 ml, charged with: A) Cu^{2+} B) Zn^{2+} C) Co^{2+} D) Ni^{2+} Conditions were otherwise the same for the four purifications

Sample: Histidine-tagged YNR064c (M_r 33 700) in clarified *Pichia pastoris* extract including 20 mM imidazole

Sample volume: 10 ml

Binding buffer: 20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4

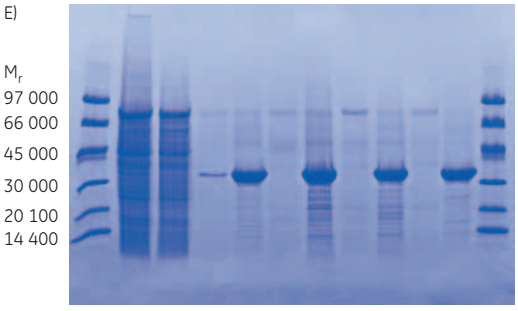
Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4

Flow rate: 1 ml/min

Gradient: 4–60% elution buffer (20–300 mM imidazole) in 25 ml 100% elution buffer (500 mM imidazole) in 5 ml

Detection: Absorbance, 280 nm

System: ÄKTAexplorer 10



Lanes

1. LMW markers
2. Start material, diluted 1:10
3. Flowthrough, diluted 1:10, Cu^{2+}
4. Wash, Cu^{2+}
5. Eluted pool, Cu^{2+}
6. Wash, Zn^{2+}
7. Eluted pool, Zn^{2+}
8. Wash, Co^{2+}
9. Eluted pool, Co^{2+}
10. Wash, Ni^{2+}
11. Eluted pool, Ni^{2+}
12. LMW markers

Fig 42. Purification of $(histidine)_6$-tagged YNR064c expressed in *Pichia pastoris* on four different HiTrap IMAC HP 1-ml columns charged separately with metal ions (A) Cu^{2+}, (B) Zn^{2+}, (C) Co^{2+}, or (D) Ni^{2+}. Pools selected after SDS-PAGE of individual 1-ml fractions (not shown) are indicated. (E) SDS-PAGE analysis: reducing conditions on ExcelGel SDS Gradient 8–18; Coomassie staining.

Preparative purification using HiPrep IMAC FF 16/10 column

HiPrep IMAC FF 16/10 is a ready-to-use 20-ml column, prepacked with uncharged IMAC Sepharose 6 Fast Flow. The column is well-suited for preparative purification of histidine-tagged recombinant proteins and untagged, naturally occurring proteins. HiPrep IMAC FF 16/10 provides fast, simple, and easy separations in a convenient format, and the IMAC Sepharose 6 Fast Flow medium is well-suited for scaling up.

The column is made of polypropylene, which is biocompatible and noninteractive with biomolecules. Separations can be easily achieved using a chromatography system such as ÄKTAdesign. Refer to Table 8 for a selection guide to purification equipment and to Appendix 1 for a list of HiPrep IMAC FF 16/10 column parameters.

IMAC Sepharose 6 Fast Flow is also available as prepacked 1-ml and 5-ml HiTrap IMAC FF columns and as a bulk medium in lab packs (25 and 100 ml) for packing columns.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

Adjust the sample to the composition and pH of the binding buffer by: adding buffer, NaCl, imidazole, and additives from concentrated stock solutions; by diluting the sample with binding buffer; or by buffer exchange. To prevent the binding of host cell proteins with exposed histidines, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Chapter 4).

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

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 500 mM NaCl, 20 to 40 mM imidazole, pH 7.4. (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)

Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4.

The optimal concentration of imidazole needed in the sample and buffer to obtain the best purity and yield differs from protein to protein. Under native conditions, 20 to 40 mM imidazole in the binding buffer is suitable for many proteins; 500 mM imidazole in the elution buffer is most often sufficient to completely elute the target protein.

Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.22 µm or a 0.45 µm filter before use. High-purity imidazole will give low to no absorbance at 280 nm.

Charging the column with metal ion

1. Prepare a 0.1 M solution of the desired metal ion (e. g., Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}, or Ni^{2+}) in distilled water. Salts of chlorides, sulfates, etc., can be used (e.g., 0.1 M $CuSO_4$ or 0.1 M $NiSO_4$).

Solutions of Zn^{2+} ions should have a pH of approximately 5.5 or lower to avoid solubility problems that arise at pH 6 or higher. Fe^{3+} ions should be immobilized at low pH (approximately pH 3.0) to avoid formation of insoluble ferric compounds.

2. Wash the column with at least 2 column volumes of distilled water.
3. Apply at least 0.2 column volumes of the metal ion solution to the column.
4. Wash the column with at least 5 column volumes of distilled water to remove excess metal ions.

Washing with buffer before applying the metal ion solution may cause unwanted precipitation.

Purification

1. Apply the centrifuged and/or filtered sample (in binding buffer) to the column at a flow rate of 1 to 10 ml/min (30 to 300 cm/h).
2. Wash the column with 5 to 10 column volumes of binding buffer at 2 to 10 ml/min (60 to 300 cm/h).
3. Elute the bound protein with 5 to 10 column volumes of elution buffer at a flow rate of 2 to10 ml/min (60 to 300 cm/h).
4. After elution, regenerate the column by washing it with 5 to 10 column volumes of binding buffer. The column is now ready for a new purification.

The column does not need to be stripped and recharged between each purification if the same protein is going to be purified. Reuse of any purification column depends on the nature of the sample and should only be performed with identical tagged proteins to prevent cross-contamination. For more information on this topic and on cleaning and storage, refer to Appendix 1.

IMAC Sepharose is compatible with reducing agents. However, we recommend removal of any weakly bound metal ions before applying buffer/sample that includes reducing agents. This can be accomplished by performing a blank run without reducing agents (see below). Do not store HiPrep IMAC FF 16/10 columns with buffers that include reducing agents.

For critical applications, leakage of metal ions during purification can be diminished by performing a blank run (as described below) before loading sample.

Blank run:

Use binding buffer and elution buffer without reducing agents.

1. Wash the column with 5 column volumes of distilled water (to remove the 20% ethanol).
2. Wash with 5 column volumes of elution buffer.
3. Equilibrate with 10 column volumes of binding buffer.

Application example

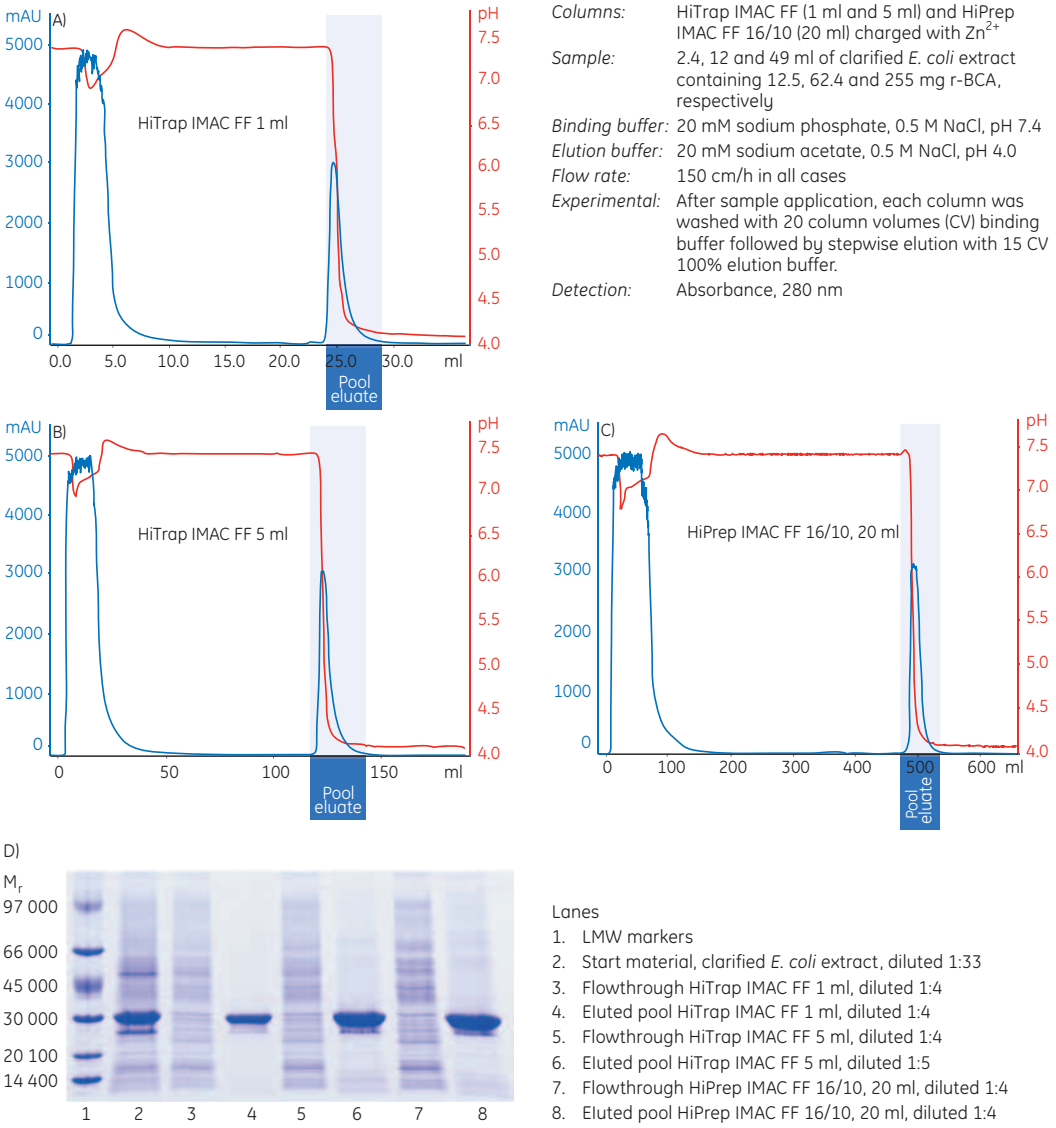
Optimization and scale-up of untagged protein

The IMAC technique can also be used to purify proteins that are not histidine-tagged. This application presents one such example. Experiments were performed to optimize the method and assess the efficiency of scaling up the capture step in the purification of recombinant bovine carbonic anhydrase II (r-BCA, M_r 30 000), a protein that naturally contains exposed histidine residues.

Three metal ions (Cu^{2+}, Ni^{2+}, and Zn^{2+}) and two elution methods (imidazole and pH) were tested to establish optimal conditions for purifying r-BCA in process-scale applications. HiTrap IMAC FF 1-ml columns were used to establish the conditions. High purity was obtained with all three metal ions tested; binding strength decreased in the order $Zn^{2+} = Ni^{2+} > Cu^{2+}$ (data not shown). Zn^{2+} is the preferred metal ion for process-scale purification because of its low toxicity, making it the appropriate choice for the scale-up experiments. Results also showed excellent recovery and purity in both elution methods (data not shown). Because pH elution is less expensive, it was chosen for the scale-up experiments.

In the scale-up studies, yields were very good (> 90%) with both HiTrap IMAC FF 5-ml and HiPrep IMAC FF 16/10, 20-ml columns (Fig 43). The loading was 74% of maximum binding capacity. No significant change in recovery and purity was seen between the different scales (Table 12). The recovery of the enzymatic activity was determined using an esterase activity assay and was found to be approximately 90% in all cases.

Metal ion leakage from the medium, an important concern in industrial applications, was also investigated in this study. Total leakage of Zn^{2+} was found to be very low, less than 3% in the HiPrep IMAC FF 16/10 scale. It should be noted that r-BCA needs one zinc ion in the active site for its enzymatic activity. A simple desalting step (HiPrep 26/10 Desalting) after purification removes all metal ions (except the one anchored to the active site of the protein).



Columns: HiTrap IMAC FF (1 ml and 5 ml) and HiPrep IMAC FF 16/10 (20 ml) charged with Zn^{2+}

Sample: 2.4, 12 and 49 ml of clarified *E. coli* extract containing 12.5, 62.4 and 255 mg r-BCA, respectively

Binding buffer: 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4

Elution buffer: 20 mM sodium acetate, 0.5 M NaCl, pH 4.0

Flow rate: 150 cm/h in all cases

Experimental: After sample application, each column was washed with 20 column volumes (CV) binding buffer followed by stepwise elution with 15 CV 100% elution buffer.

Detection: Absorbance, 280 nm

Lanes

1. LMW markers
2. Start material, clarified *E. coli* extract, diluted 1:33
3. Flowthrough HiTrap IMAC FF 1 ml, diluted 1:4
4. Eluted pool HiTrap IMAC FF 1 ml, diluted 1:4
5. Flowthrough HiTrap IMAC FF 5 ml, diluted 1:4
6. Eluted pool HiTrap IMAC FF 5 ml, diluted 1:5
7. Flowthrough HiPrep IMAC FF 16/10, 20 ml, diluted 1:4
8. Eluted pool HiPrep IMAC FF 16/10, 20 ml, diluted 1:4

Fig 43. Chromatograms showing scale-up of purification from (A) HiTrap IMAC FF 1-ml column to (B) HiTrap IMAC FF 5-ml column and (C) HiPrep IMAC FF 16/10 20-ml column. Sample was 2.4, 12, and 49 ml of clarified extract of *E. coli* containing 12.5, 62.4, and 255 mg of r-BCA, respectively. The load was approximately 74% of maximum binding capacity. (D) Nonreduced SDS-PAGE analysis on ExcelGel Gradient 8–18 of the main fractions from the scale-up experiments. The gel was stained with a 1% solution of PhastGel Blue R (Coomassie).

Table 12. Data and results from the scale-up purification of r-BCA on IMAC Sepharose 6 Fast Flow. Comparisons of r-BCA yields and recoveries for the different runs show scalability of the application.

Column	Fraction	Amount applied (mg)	Amount eluted (mg)	Recovery of protein	Recovery of r-BCA activity
HiTrap IMAC FF 1 ml	Clarified *E. coli* extract	12.5	-	-	-
	Eluted pool	-	11.7	94%	93%
HiTrap IMAC FF 5 ml	Clarified *E. coli* extract	62.4	-	-	-
	Eluted pool	-	56.1	90%	84%
HiPrep IMAC FF 16/10 (20 ml)	Clarified *E. coli* extract	255	-	-	-
	Eluted pool	-	235	92%	90%

Detection of histidine-tagged proteins

Table 13 reviews the methods available for detection of histidine-tagged proteins. These methods can be selected according to the experimental situation. For example, SDS-PAGE analysis, performed frequently during expression 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.

Table 13. Detection methods for histidine-tagged proteins.

Generic detection method (detects the tag)	Comments
ELISA assay using anti-His antibody	Highly specific, detects only histidine-tagged protein.
Western blot analysis using anti-His antibody and ECL detection systems	Highly specific, detects only histidine-tagged 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 Advance™.
Detection methods specific for the target protein	
SDS-PAGE with Coomassie or silver staining	Provides information on size and % purity. Detects tagged protein and contaminants.
Functional assays	Useful to assess if the purified histidine-tagged protein is active. Not always available. May require development and optimization.

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% β-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 to 10 µl of supernatant from crude extracts, cell lysates or purified fractions as appropriate.
2. Vortex briefly and heat for 5 min at 90°C 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 14).

Table 14. Separation size range for different percentages of acrylamide in the SDS-PAGE gel.

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

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

Western blot analysis

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

Anti-His Antibody

Blocking/Incubation buffer: 5% (w/v) nonfat dry milk and 0.1% (v/v) Tween 20 in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3)

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

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 GE Healthcare 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 histidine-tagged 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, ECL Plus, or ECL Advance 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 GE Healthcare.

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 to 200 ml of blocking/incubation buffer.
3. Incubate for 1 to 16 h at ambient temperature with gentle shaking.
4. Decant and discard the buffer.

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

Incubation of membrane blot with primary antibody

1. Prepare an appropriate dilution of anti-His antibody with blocking/incubation buffer, for example, 5 to 10 µl of antibody to 50 ml of buffer. Refer to GE Healthcare 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 h at ambient temperature with gentle shaking.
4. Decant and discard the antibody-buffer.
5. Rinse twice with 20 to 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 to 30 ml of blocking or wash buffer for 10 to 60 min at ambient temperature with gentle shaking.
8. Discard the wash and repeat.

Incubation of membrane blot with secondary antibody

1. Dilute an appropriate anti-mouse secondary antibody with blocking/incubation buffer according to the manufacturer's recommendation. Refer to GE Healthcare 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 h at ambient temperature with gentle shaking.
4. Decant and discard the antibody-buffer.
5. Rinse twice with 20 to 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 to 30 ml of blocking or wash buffer for 10 to 60 min 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.

Refer to GE Healthcare Application Note 18-1139-13 and product brochure 14-0003-87 for further information on optimization of antibody concentration for Western blotting.

ECL, ECL Plus, and ECL Advance 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.

Anti-His antibody from GE Healthcare is a monoclonal preparation and has been tested for its lack of nonspecific 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 tagged protein sample. Such antibodies can be removed by cross-absorbing the antibody with an *E. coli* sonicate to remove anti-*E. coli* antibodies. This *E. coli* should not contain a histidine-tag-encoding plasmid.

Tag removal by enzymatic cleavage

In most cases, functional tests can be performed using the intact histidine-tagged protein. If removal of the tag is necessary, then procedures similar to GST tag removal can be followed, that is, specific recognition sites are incorporated to allow subsequent enzymatic cleavage. The precise protocols required for cleavage and purification will depend on the original vectors and the properties of the specific enzymes used for cleavage.

rTEV protease (Invitrogen) has a $(histidine)_6$-tag and recognizes the amino acid sequence Glu-Asn-Leu-Tyr-Phe-Gln↓Gly. Glu, Tyr, Gln and Gly are needed for cleavage between the Gln and Gly residues (↓). N-terminal $(histidine)_6$-tags can be removed. The advantage of this enzymatic cleavage is that the protein of interest can be repurified using the same Ni Sepharose medium or prepacked column. The $(histidine)_6$-tag and the $(histidine)_6$-tag rTEV protease will both bind to the column, and the protein of interest can be collected in the flowthrough.

The amount of enzyme, temperature, and length of incubation required for complete digestion vary according to the specific tagged protein produced. Determine optimal conditions in preliminary experiments.
Remove samples at various time points and analyze by SDS-PAGE to estimate the yield, purity, and extent of digestion. Approximate molecular weights for SDS-PAGE analysis:

rTEV protease	M_r 29 000
Carboxypeptidase A*	M_r 94 000

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

There is no PreScission Protease recognition site available for use with histidine-tagged proteins.

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 gel filtration (usually no need for optimization), ion exchange, or hydrophobic interaction chromatography will need to be developed (see Appendix 9).

Application example

Automatic histidine tag removal using ÄKTAxpress

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

Four-step protocol: $(histidine)_6$-tagged protein cleaved with AcTEV protease

The example in Figure 44 shows purification results for a $(histidine)_6$-tagged protein, APC234 (M_r 32 500), expressed in *E. coli*. The M_r of the cleaved product is 30 000. After harvest, cell lysis was performed by sonication. The samples were clarified by centrifugation prior to sample loading.

Affinity chromatography (AC), desalting (DS), ion exchange (IEX), and gel filtration (GF) were all performed on ÄKTAxpress using columns as indicated in the figure. The purity of each sample was analyzed by SDS-PAGE (Coomassie staining). The reduced samples were applied on an SDS-polyacrylamide gel. Approximately 7.5 µg of protein was loaded per lane.

Columns:	AC: HisTrap HP, 5 ml DS: HiPrep 26/10 Desalting IEX: RESOURCE™ Q, 6 ml GF: HiLoad 16/60 Superdex 75 pg
Sample:	APC234, M_r 32 000 (cleaved product, M_r 30 000)
Cleavage conditions:	200 units of AcTEV™ protease/mg protein, 8 h incubation time at room temperature
AC binding buffer:	50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.5
AC cleavage buffer:	50 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole, pH 7.5
AC elution buffer:	50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.5
DS and IEX binding buffer:	50 mM Tris-HCl, pH 8.0
IEX elution buffer:	50 mM Tris-HCl, 1 M NaCl, pH 8.0
GF buffer:	50 mM Tris-HCl, 150 mM NaCl, pH 7.5

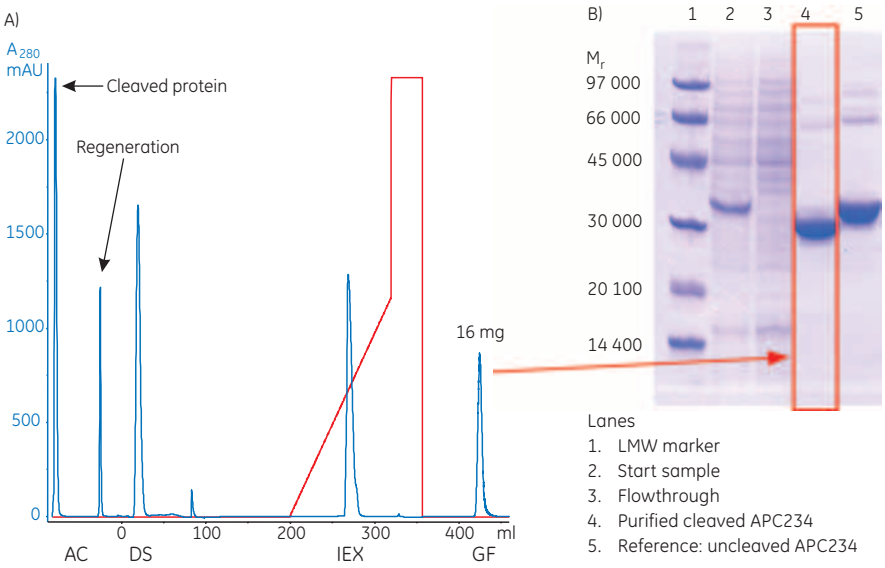


Fig 44. (A) Four-step protocol for purification of $(histidine)_6$-tagged protein cleaved with AcTEV protease. (B) Analysis of the cleaved purified protein using SDS-PAGE. The gel was stained with Coomassie.

Troubleshooting

The troubleshooting guide below addresses problems common to the majority of purification products discussed in this chapter, as well as problems specific to a particular method. In the latter case, the relevant product is indicated.

Problem	Possible cause	Solution
Column or plate wells have clogged OR Liquid not completely removed during centrifugation (His SpinTrap) OR Flow rate is too slow (His GraviTrap)	Cell debris is present.	Centrifuge and/or pass the sample through a 0.22 or 0.45 µm filter. Clean the media according to Appendix 1. If cleaning-in-place is unsuccessful, replace the media/prepacked column. Optimize sample pretreatment before the next sample loading. Try using HisTrap FF crude columns.
	The sample is too viscous due to too high a concentration of material or the presence of large nucleic acid molecules (may be evidenced by increased back pressure).	Increase dilution of the cell paste before lysis, or dilute after lysis. Increase time for lysis until the viscosity is reduced, and/or add an additional dose of DNase and Mg^{2+} (DNase I to 5 µg/ml, Mg^{2+} to 1 mM), and incubate on ice for 10 to 15 min. Increase the efficiency of the mechanical cell disruption (e.g., increase sonication time). Keep the sample on ice to avoid frothing and overheating as this may denature the target protein. Over-sonication can also lead to copurification of host proteins with the target protein. Freeze/thaw of the unclarified lysate may increase precipitation and aggregation. Sonication of the thawed lysate can prevent increased back pressure problems when loading on the column. If the purification has been performed at 4°C, move to room temperature if possible. Draw the lysate through a syringe needle several times. Decrease the flow rate during sample loading.
	Protein is difficult to dissolve or precipitates during purification.	First, screen for suitable conditions for solubility; vary pH, ionic strength, protein concentration, detergent, other additives that may affect solubility of the protein. If the protein cannot be kept in solution by these means, consider using more harsh conditions, such as 8 M urea, 6 M Gua-HCl, or SDS (or other harsh detergent). Add detergents, reducing agents or other additives to the sample [2% Triton X-100, 2% Tween 20, 2% Nonidet™ P-40, 2% cholate, 1% CHAPS, 1.5 M NaCl, 50% glycerol, 20 mM β-mercaptoethanol, 1 to 3 mM DTT or DTE (up to 5 mM is possible but depends on the sample and the sample volume), 5 mM TCEP, 10 mM reduced glutathione, 8 M urea, or 6 M Gua-HCl] and mix gently for 30 min to solubilize the tagged protein.

continues on following page

Problem	Possible cause	Solution
	Protein is difficult to dissolve or precipitates during purification.	Note that Triton X-100 and NP-40 (but not Tween) have a high absorbance at 280 nm. Furthermore, detergents cannot be easily removed by buffer exchange. Inclusion bodies: the protein can usually be solubilized (and unfolded; refolding needed to obtain active protein) from inclusion bodies using common denaturants such as 4 to 6 M Gua-HCl, 4 to 8 M urea, or strong detergents. Mix gently for 30 min or more to aid solubilization of the tagged protein. Purify in the presence of the denaturant. If possible, decrease the NaCl concentration in the elution buffer adjust ion strength or pH of sample.
No or low yield of histidine-tagged protein in the purified fractions	Elution conditions are too mild (histidine-tagged protein still bound).	Elute with increasing imidazole concentration or decreasing pH to determine the optimal elution conditions.
	Protein has precipitated in the column or wells.	For the next experiment, decrease amount of sample, or decrease protein concentration by eluting with linear imidazole gradient instead of imidazole steps. Try detergents or changed NaCl concentration, or elute under denaturing (unfolding) conditions (use 4 to 8 M urea or 4 to 6 M Gua-HCl).
	Nonspecific hydrophobic or other interactions are occurring.	Add a nonionic detergent to the elution buffer (e.g., 0.2% Triton X-100) or increase the NaCl concentration.
The histidine-tagged protein is found in the flowthrough	The concentration of imidazole in the sample and/or binding buffer is incorrect.	Alter the imidazole concentration—it may be too high.
	The histidine tag may be insufficiently exposed.	Perform purification of unfolded protein in urea or Gua-HCl as for inclusion bodies. To minimize dilution of the sample, solid urea or Gua-HCl can be added.
	Buffer/sample composition is incorrect.	Check pH and composition of sample and binding buffer. Ensure that chelating or strong reducing agents are not present in the sample at too high concentration, and that the concentration of imidazole is not too high.
	Histidine tag has been lost.	Check sequence of the construct on Western blot or extract using anti-His antibody.
	Incubation time is too short.	Decrease the flow rate or increase the incubation time of the sample in the wells/batch or use a lower centrifugation speed/vacuum.
Histidine-tagged protein is not completely eluted.		Elute with a larger volume of elution buffer and/or increase the concentration of imidazole.

continues on following page

Problem	Possible cause	Solution
Histidine-tagged protein found in the pellet (SDS-PAGE of samples collected during the preparation of the bacterial lysate may indicate that most of histidine-tagged protein is located in the centrifugation pellet)	Sonication may be insufficient.	Cell disruption may be checked by microscopic examination or monitored by measuring the release of nucleic acids at A_{260}. Addition of lysozyme (up to 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 and overheating as this may denature the target protein. Over-sonication can also lead to copurification of host proteins with the target protein.
	Protein was adsorbed to cell debris during extraction and lost upon clarification	Change extraction condition (pH, ionic strength, try detergent solubilization).
	The protein may be insoluble (inclusion bodies).	The protein can usually be solubilized (and unfolded) from inclusion bodies using common denaturants such as 4 to 6 M Gua-HCl, 4 to 8 M urea, or strong detergents. Prepare buffers containing 20 mM sodium phosphate, 8 M urea, or 6 M Gua-HCl, and suitable imidazole concentrations, pH 7.4 to 7.6. Buffers with urea should also include 500 mM NaCl. Use these buffers for sample preparation, as binding buffer and as elution buffer. For sample preparation and binding buffer, use 5 to 40 mM imidazole or the concentration selected during optimization trials (including urea or Gua-HCl). To minimize dilution of the sample, solid urea or Gua-HCl can be added.
The eluted protein is not pure (multiple bands on SDS-PAGE)	Proteases have partially degraded the tagged protein.	Add protease inhibitors (use EDTA with caution).
	Contaminants have high affinity for the metal ion.	Elute with a stepwise or linear imidazole gradient to determine optimal imidazole concentrations to use for binding and for wash; add imidazole to the sample in the same concentration as the binding buffer. Wash before elution with binding buffer containing as high concentration of imidazole as possible, without causing elution of the tagged protein. A shallow imidazole gradient (20 column volumes or more), may separate proteins with similar binding strengths. If optimized conditions do not remove contaminants, further purification steps may be necessary.
	Contaminants are associated with tagged protein, e.g., chaperonins attached to the target protein.	Add detergent and/or reducing agents before sonicating cells. Increase detergent levels (e.g., up to 2% Triton X-100 or 2% Tween 20), or add glycerol (up to 50%) to the wash buffer to disrupt nonspecific interactions. Consider increasing the imidazole concentration or changing the metal ion used for purification.
	Unbound material has been insufficiently removed by the washing step.	Repeat the wash step after sample application to obtain optimal yield.

continues on following page

Problem	Possible cause	Solution
Histidine-tagged protein is eluted during sample loading/ wash	Contaminants may have a high affinity for certain metal ions.	Charge the column using another metal ion.
	Buffer/sample composition is not optimal.	Check pH and composition of sample and binding buffer. Ensure that chelating or strong reducing agents are not present in the sample at a too high concentration, and that the concentration of imidazole is not too high.
	Histidine tag is partially obstructed.	Purify under denaturing conditions (use 4 to 8 M urea or 4 to 6 M Gua-HCl).
	Capacity is exceeded.	For applicable formats (i.e., prepacked HisTrap columns), join two or three columns together or change to a larger column.
Unwanted air bubbles have formed		Unclarified lysates may cause increased air bubble formation during purification. An attached flow restrictor in the chromatography system can prevent this. If a flow restrictor is attached, it is important to change the pressure limit to 0.5 MPa (5 bar) on the ÄKTAdesign system (where the column and the flow restrictor give a pressure of 0.3 MPa and 0.2 MPa, respectively).
MultiTrap: Leakage of solution after removing foils		Add 500 µl of deionized water twice before adding binding buffer to the wells. Remove the solution between the additions with either centrifugation or vacuum.
MultiTrap: Problem with reproducibility and/or foam in collection plate when using vacuum		Increase/decrease the vacuum. Add more wash steps before eluting the protein. Change to centrifugation.

Chapter 4
Optimizing purification of histidine-tagged proteins

Introduction

Three methods for optimizing purification of histidine-tagged proteins are discussed in this chapter:

- Optimizing using imidazole
- Optimizing using different metal ions
- Optimizing using multistep purifications

For general purification of histidine-tagged proteins, including typical workflow, descriptions of available media and product formats, procedures, and troubleshooting hints, refer to Chapter 3.

Optimizing using imidazole

The presence of surface-exposed histidine residues or other complex-forming amino acids can lead to nonspecific binding of untagged host cell proteins to purification media. These untagged proteins elute with the target protein and must subsequently be removed. Because the binding affinity of these contaminants is often lower than that of the tagged recombinant proteins, separating the contaminants from the protein of interest may be possible by optimizing the separation conditions.

The several examples below show how changes in imidazole concentration affect the purity of the histidine-tagged target protein.

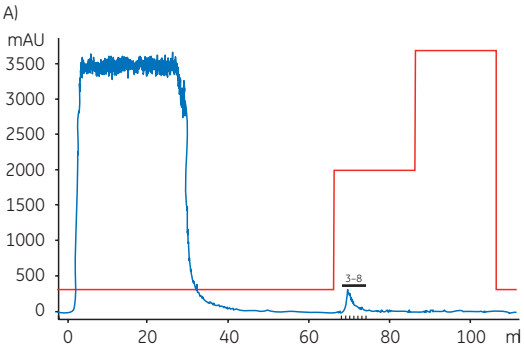
1. Employing imidazole as a competitive agent

In chromatographic runs that include imidazole as elution agent, the column should be preequilibrated with a low concentration of imidazole. If this step is omitted, uncontrollable effects may occur once the imidazole is introduced during the run.

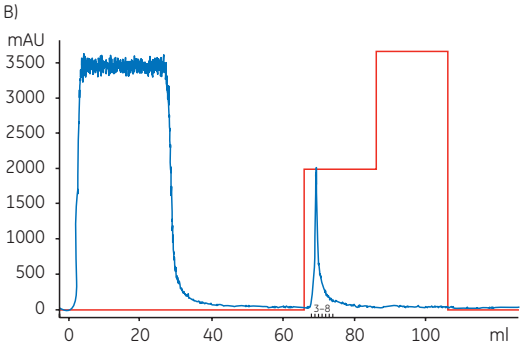
One way to reduce the binding of contaminant proteins during purification is to employ imidazole as a competitive agent (see Fig 45). In the experiment shown, histidine-tagged protein kinase G [$(His)_6$-PknG] from *Mycobacterium bovis* was purified using a concentration of 45 mM imidazole in the sample and binding buffer during final purification. The medium used in the experiment was Ni Sepharose High Performance (see Chapter 3).

To achieve a higher protein concentration, the protein was eluted in a two-step gradient (Fig 45A). To demonstrate the advantageous effect of imidazole, an additional purification was performed under the same conditions except that imidazole was omitted from the sample and binding buffer (Fig 45B). It is important to note that omission of imidazole is not generally recommended; this example is provided solely to demonstrate the negative effect of its absence on the purity of the eluted target protein.

SDS-PAGE of the pooled elution fractions indicated a large improvement in purity of the desired protein when 45 mM imidazole was included in the sample and binding buffer (Fig 45C). The yield of the target protein was maintained in the sample with 45 mM imidazole present. Note that the concentration of imidazole is protein dependent and thus must be determined case by case.



Column: Ni Sepharose High Performance, 2 ml in XK 16/20
Sample: Histidine-tagged PknG in 26 ml *E. coli* M15 extract
Binding buffer: 20 mM Tris, pH 8.0, 0.5 M NaCl, 1 % Triton X-100, 10% glycerol, 10 mM β-mercaptoethanol, 45 mM imidazole
Elution buffer: 20 mM Tris, pH 8.0, 0.5 M NaCl, 1% Triton X-100, 10% glycerol, 10 mM β-mercaptoethanol, 500 mM imidazole
Gradient: step 50% elution buffer, 20 CV; 100% elution buffer 20 CV
Flow rate: 1 ml/min
System: ÄKTApurifier 10



Column: Ni Sepharose High Performance, 2 ml in XK 16/20
Sample: Histidine-tagged PknG in 26 ml *E. coli* M15 extract
Binding buffer: 20 mM Tris, pH 8.0, 0.5 M NaCl, 1% Triton X-100, 10% glycerol, 10 mM β-mercaptoethanol
Elution buffer: 20 mM Tris, pH 8.0, 0.5 M NaCl, 1% Triton X-100, 10% glycerol, 10 mM β-mercaptoethanol, 500 mM imidazole
Gradient: step 50% elution buffer, 20 CV; 100% elution buffer 20 CV
Flow rate: 1 ml/min
System: ÄKTApurifier 10

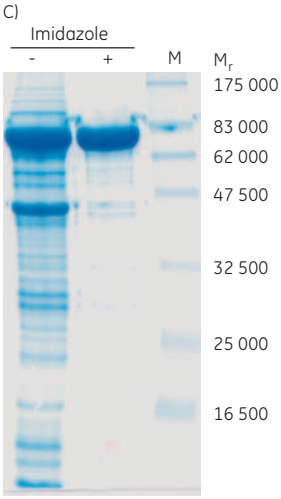


Fig 45. Purification of $(His)_6$-PknG with (A) and without (B) 45 mM imidazole in the sample and binding buffer. For each chromatogram, the lysate of 2 l of *E. coli* culture (sample volume 26 ml; filtered through a 0.45-µm syringe filter) was loaded on a 2-ml Ni Sepharose High Performance column (XK 16/20 column) using ÄKTApurifier. The kinase was eluted in a two-step gradient with 50% and 100% of elution buffer. (C) SDS-PAGE (12% gel) of $(His)_6$-PknG fractions showing eluates without (-) and with (+) 45 mM imidazole in the binding buffer.

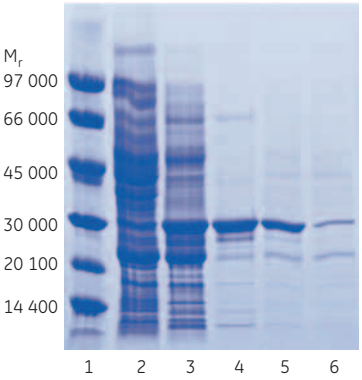
2. Determining optimal imidazole concentration using His SpinTrap

The imidazole concentration during binding and washing is an important factor affecting the final purity and yield of the target protein. His SpinTrap is a convenient and fast tool for determination of optimal imidazole concentration. Optimization is important for both purity and yield of the target protein. This was demonstrated by a series of experiments where a histidine-tagged protein, APB7-$(His)_6$ (M_r 28 000), was purified on His SpinTrap using 5, 50, 100, or 200 mM imidazole in samples and binding buffers. The elution buffer contained 500 mM imidazole.

An imidazole concentration of 5 mM resulted in low purity of the eluted sample (Fig 46, lane 3), while an increase to 50 mM imidazole prevented binding of most contaminants and improved purity (Fig 46, lane 4). Including 100 mM imidazole in the sample and binding buffer lowered yield while purity improved marginally (Fig 46, lane 5). The lower yield can be explained by leakage of target protein due to the high imidazole concentration during binding and washing. Further increase to 200 mM imidazole reduced yield even more (Fig 46, lane 6).

This example shows that higher imidazole concentrations during binding improve the purity, whereas too high concentration decreases the yield. The optimal imidazole concentration during binding is protein dependent. For many proteins, 20 to 40 mM imidazole is the best choice.

Column:	His SpinTrap
Equilibration:	600 µl binding buffer
Sample application:	600 µl clarified *E. coli* BL-21 lysate containing 400 µg APB7-$(His)_6$
Wash:	600 µl binding buffer
Elution:	2 × 200 µl elution buffer
Binding buffer:	20 mM sodium phosphate, 500 mM NaCl, 5–200 mM imidazole, pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4



Lanes
1. LMW markers
2. Start material (diluted 1:10)
3. Eluted pool, 5 mM imidazole during binding (diluted 1:2)
4. Eluted pool, 50 mM imidazole during binding (diluted 1:2)
5. Eluted pool, 100 mM imidazole during binding (diluted 1:2)
6. Eluted pool, 200 mM imidazole during binding (diluted 1:2)

Fig 46. SDS-PAGE under reducing conditions (ExcelGel SDS Gradient 8–18) of histidine-tagged APB7 protein. The imidazole concentration during binding affects the final purity and yield (compare lanes 3, 4, 5, and 6).

Optimizing using different metal ions

The strength of binding between a protein and a metal ion is affected by several factors, including the structure and characteristics of the target protein, the presence and properties of the protein affinity tag, the properties of the metal ion, and the pH and composition of the binding buffer. As a result, Ni^{2+}, the metal ion considered to have the strongest affinity to histidine-tagged proteins, may not always be the best choice for a given application. Under some circumstances, therefore, other transition metal ions, such as Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, and Zn^{2+}, may be better suited.

When the binding characteristics of a target protein are unknown, we recommend testing more than one metal ion to determine the one best suited for your separation. GE Healthcare offers several uncharged IMAC purification products for such purposes: convenient, prepacked 1-ml and 5-ml HiTrap IMAC HP and HiTrap IMAC FF and 20-ml HiPrep IMAC FF 16/10 columns, as well as IMAC Sepharose High Performance and IMAC Sepharose 6 Fast Flow bulk media. These products are described in Chapter 3, which also includes procedures for their use.

The following guidelines may assist in devising preliminary experiments to determine the metal ion most suitable for a given separation:

- Ni^{2+} is generally used for histidine-tagged recombinant proteins.
- Co^{2+} is also used for purification of histidine-tagged proteins, especially when a somewhat weaker binding of target proteins is preferred.
- Cu^{2+} and Zn^{2+} are frequently used for purification of untagged proteins. Cu^{2+} gives relatively strong binding to a range of proteins; some proteins will only bind to Cu^{2+}. Zn^{2+} ions often bind more weakly, a characteristic that is often exploited to achieve selective elution of the target protein. Both Cu^{2+} and Zn^{2+} can be used for histidine-tagged proteins and for process-scale separations.
- Fe^{3+} and Ca^{2+} are used more rarely than other metal ions. Take extra precautions when working with Fe^{3+}, as it reduces easily in neutral solutions, forming compounds that can be hard to dissolve. When working with Fe^{3+} at low pH, approximately 3, immobilize the ions to avoid precipitation of unwanted compounds. We also advise stripping immobilized Fe^{3+} ions after each run and recharging the column as required. Strongly bound Fe^{3+} ions and ferric compounds can be removed by leaving the medium in 50 mM EDTA overnight.

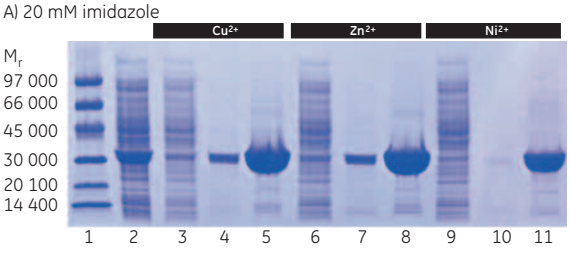
Below we present two examples showing how the selection of the most suitable metal ion and experimental conditions (including imidazole concentration) affects the purification of a given target protein.

1. Comparison study using Cu^{2+}, Zn^{2+}, and Ni^{2+} on HiTrap IMAC FF

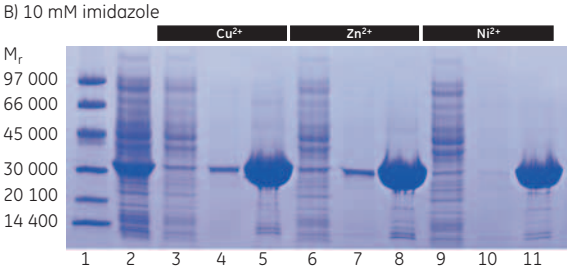
In this study, APB7, a $(histidine)_6$-tagged protein (M_r 28 000) expressed in *E. coli* BL-21, was purified on HiTrap IMAC FF 1-ml columns (prepacked with IMAC Sepharose 6 Fast Flow) and charged separately with Cu^{2+}, Zn^{2+}, and Ni^{2+}.

Screening experiments were performed to determine the optimal imidazole concentration for each ion [see Figure 47 (A to C)]. The results of each of these three purifications indicate:

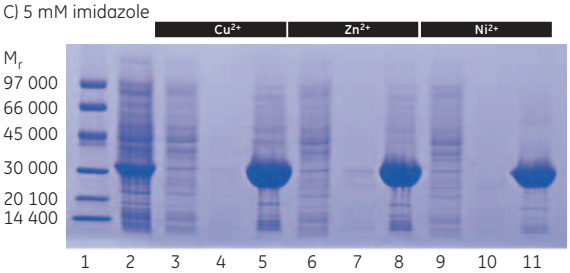
- At 20 mM imidazole, there was significant leakage of target protein in the wash with Cu^{2+} and Zn^{2+}, a sign that imidazole concentration was too high to allow maximal yield. Very little leakage was seen with Ni^{2+}. Purity was excellent in all three cases.
- At 10 mM imidazole, leakage of target protein in the wash was significantly reduced for Cu^{2+} and Zn^{2+}. The purity of the target protein in the eluted pool was similar with all three metal ions, but not as pure as with 20 mM imidazole.
- At 5 mM imidazole, no leakage occurred with any of the metal ions. Ni^{2+} provided the purest protein, though still not as pure as with 20 mM imidazole.



Lanes
1. LMW markers
2. Start material APB7, diluted 1:10
3. Flowthrough, diluted 1:10, Cu^{2+}
4. Wash, Cu^{2+}
5. Eluted pool, Cu^{2+}
6. Flowthrough, diluted 1:10, Zn^{2+}
7. Wash, Zn^{2+}
8. Eluted pool, Zn^{2+}
9. Flowthrough, diluted 1:10, Ni^{2+}
10. Wash, Ni^{2+}
11. Eluted pool, Ni^{2+}



Lanes
1. LMW markers
2. Start material APB7, diluted 1:10
3. Flowthrough, diluted 1:10, Cu^{2+}
4. Wash, Cu^{2+}
5. Eluted pool, Cu^{2+}
6. Flowthrough, diluted 1:10, Zn^{2+}
7. Wash, Zn^{2+}
8. Eluted pool, Zn^{2+}
9. Flowthrough, diluted 1:10, Ni^{2+}
10. Wash, Ni^{2+}
11. Eluted pool, Ni^{2+}



Lanes
1. LMW markers
2. Start material APB7, diluted 1:10
3. Flowthrough, diluted 1:10, Cu^{2+}
4. Wash, Cu^{2+}
5. Eluted pool, Cu^{2+}
6. Flowthrough, diluted 1:10, Zn^{2+}
7. Wash, Zn^{2+}
8. Eluted pool, Zn^{2+}
9. Flowthrough, diluted 1:10, Ni^{2+}
10. Wash, Ni^{2+}
11. Eluted pool, Ni^{2+}

Fig 47. SDS-PAGE analyses (reducing conditions) of fractions from the purification of APB7 using IMAC Sepharose 6 Fast Flow, prepacked in HiTrap IMAC FF 1-ml columns, charged with either Cu^{2+}, Zn^{2+}, or Ni^{2+}, and with (A) 20 mM imidazole in the sample, (B) 10 mM imidazole in the sample, or (C) 5 mM imidazole in the sample. The gels were stained with Coomassie.

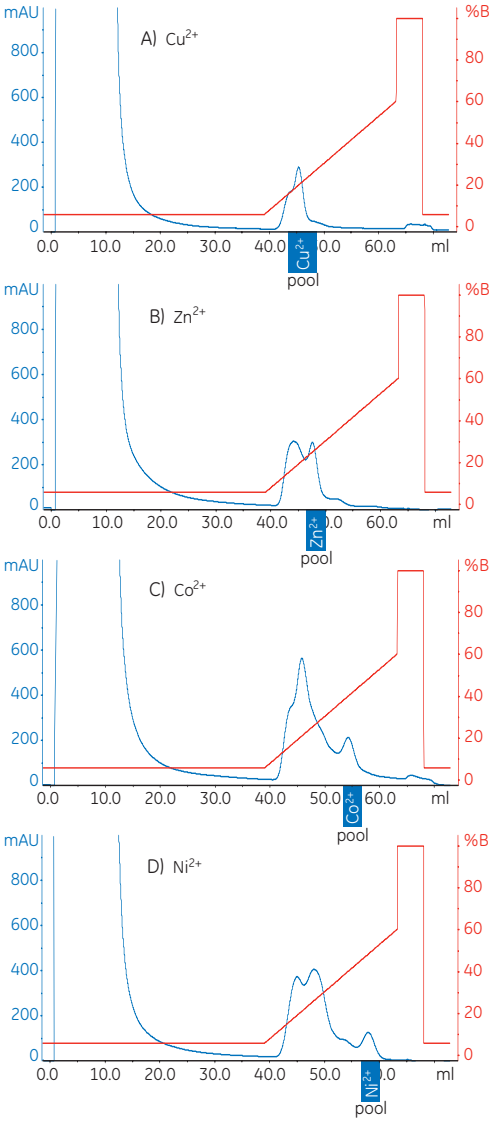
Note that a large amount of sample was applied in the SDS-polyacrylamide gel. Because of this, the gels show a number of contaminants in the eluted material.

The results illustrate that, for any given metal ion, imidazole concentration can be adjusted to achieve high yield, high purity, or a successful compromise. IMAC Sepharose media typically requires a slightly higher concentration of imidazole in the wash buffer than similar IMAC media on the market. A good starting point for most separations is to include 20 to 40 mM imidazole in the binding and wash buffers. Be sure to use highly pure imidazole, which gives essentially no absorbance at 280 nm. To remove imidazole from the protein, use HiTrap Desalting, PD-10 Desalting, or HiPrep 26/10 Desalting column, depending on the sample volume.

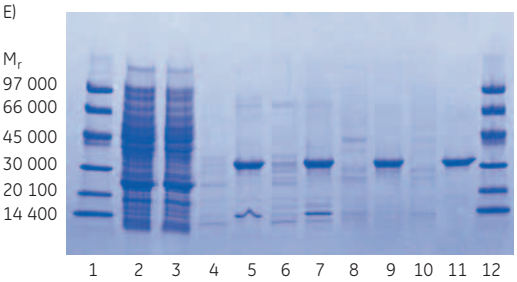
2. Screening for optimized purity using Cu^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} on HiTrap IMAC HP

Successful purifications require attention to the properties of the target protein and the affinity of the target protein toward the metal ion used. Four different HiTrap IMAC HP 1-ml columns prepacked with IMAC Sepharose High Performance were charged separately with four different metal ions: Cu^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}. Purification performance was assessed for the target protein: APB7, (M_r 28 000) expressed in *E. coli*.

The results demonstrate the importance of screening samples to determine the most suitable metal ion and purification conditions for specific target proteins. For APB7, the highest purity was achieved using Ni^{2+} or Co^{2+}, but the difference compared to the results for Zn^{2+} and Cu^{2+} was small (Fig 48). The gradient elutions with imidazole employed in these examples also offer a methodology for selecting the appropriate imidazole concentration for a given purification.



Columns:	HiTrap IMAC HP, 1 ml, charged with: A) Cu^{2+} B) Zn^{2+} C) Co^{2+} D) Ni^{2+} Conditions were otherwise the same for the four purifications
Sample:	*E. coli* extract with APB7, a $(histidine)_6$-tagged protein (M_r ~28 000), including 30 mM imidazole
Sample volume:	10 ml
Binding buffer:	20 mM sodium phosphate, 500 mM NaCl, 30 mM imidazole, pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4
Flow rate:	1 ml/min
Gradient:	6–60% elution buffer (30–300 mM imidazole) in 25 m 100% elution buffer (500 mM imidazole) in 5 ml
Detection:	Absorbance, 280 nm
System:	ÄKTAexplorer 10



Lanes

1. LMW markers
2. Start material, diluted 1:10
3. Flowthrough, diluted 1:10, Cu^{2+}
4. Wash, Cu^{2+}
5. Eluted pool, Cu^{2+}
6. Wash, Zn^{2+}
7. Eluted pool, Zn^{2+}
8. Wash, Co^{2+}
9. Eluted pool, Co^{2+}
10. Wash, Ni^{2+}
11. Eluted pool, Ni^{2+}
12. LMW markers

Fig 48. Purification of APB7, a $(histidine)_6$-tagged protein expressed in *E. coli* BL-21 on four different HiTrap IMAC HP 1-ml columns charged separately with metal ions. (A) Cu^{2+}, (B) Zn^{2+}, (C) Co^{2+}, or (D) Ni^{2+}. Pools selected after SDS-PAGE of individual 1-ml fractions (not shown) are indicated. (E) SDS-PAGE analysis: reducing conditions on ExcelGel SDS Gradient 8–18; Coomassie staining.

Optimizing using multistep purifications

Target protein can be further purified by adding one or more additional purification steps, as shown in the examples below. This topic is discussed in detail in Chapter 7.

Below we present two examples showing successful multistep purification of the target proteins.

1. Two-step purification of a high-molecular-weight (histidine)$_{10}$-tagged protein using affinity chromatography and gel filtration

In a two-step purification of (His)$_{10}$-trx-p450 (histidine-tagged at the N-terminus, 10 histidine residues) produced in *E. coli*, a HisTrap FF column was used in the first step. The eluted pool was then applied to a HiLoad 16/60 Superdex 200 pg column for further purification by gel filtration.

Three major bands were detected after the first purification step (Fig 49B, lane 5). Lane 6 (pool 2 from gel filtration) contains full-length target protein. Lanes 7 and 8 (pools 3 and 4, respectively) contain truncated forms of the target protein, as verified by N-terminal sequencing (data not shown). Lane 9 (pool 1) from gel filtration contains aggregated proteins. Thus subsequent gel filtration provided very good separation between the truncated forms and the full-length target protein, (His)$_{10}$-trx-p450.

Columns:	Affinity chromatography (AC): HisTrap FF 1-ml Gel filtration (GF): HiLoad 16/60 Superdex 200 pg
Sample:	*E. coli* extract with (His)$_{10}$-trx-p450, (M_r ~130 000)
Sample volume:	50 ml
Binding buffer:	AC: 20 mM sodium phosphate, 60 mM imidazole, 500 mM NaCl, pH 7.4
Elution buffer:	AC: 20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.4 GF: 20 mM phosphate, 0.28 M NaCl, 6 mM KCl, pH 7.4
Flow rate:	AC: 1 ml/min GF: 0.5 ml/min

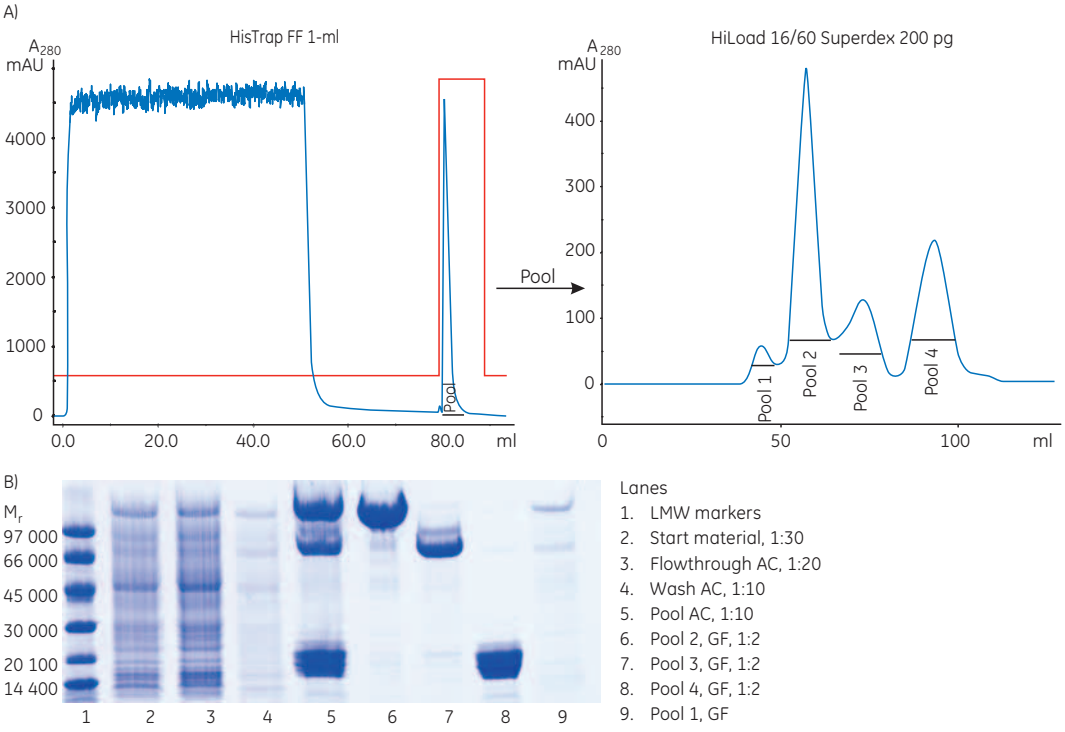


Fig 49. (A) Two-step purification of a high-molecular-weight (histidine)$_{10}$-tagged protein using affinity chromatography followed by gel filtration. (B) SDS-PAGE under reducing conditions and Coomassie staining.

2. Automatic three-step purification of unclarified cell lysate on ÄKTAxpress

An automated three-step protocol was used to purify histidine-tagged maltose binding protein from 100 ml of an unclarified *E. coli* cell lysate. The three steps were: Affinity chromatography (AC) using HisTrap FF crude (1-ml column), desalting (DS) using HiPrep 26/10 Desalting, and ion exchange chromatography (IEX) using Mono Q™ 5/50 GL. These are referred to as AC-DS-IEX in the image. As can be seen from the SDS-PAGE analysis, the target protein was obtained highly pure and in good yield.

Columns:	Affinity chromatography (AC):	HisTrap FF crude, 1 ml
	Desalting (DS):	HiPrep 26/10 Desalting
	Ion exchange (IEX):	Mono Q 5/50 GL
Sample:	Histidine-tagged Maltose Binding Protein, MBP-$(His)_6$, M_r 43 000, in *E. coli* DH5α extract	
Sample volume:	100 ml	
AC binding buffer:	50 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, pH 8.0	
AC elution buffer:	50 mM Tris-HCl, 0.5 M NaCl, 500 mM imidazole, pH 8.0	
DS/IEX binding buffer:	50 mM Tris-HCl, pH 8.0	
IEX elution buffer:	50 mM Tris-HCl, 1.0 M NaCl, pH 8.0	

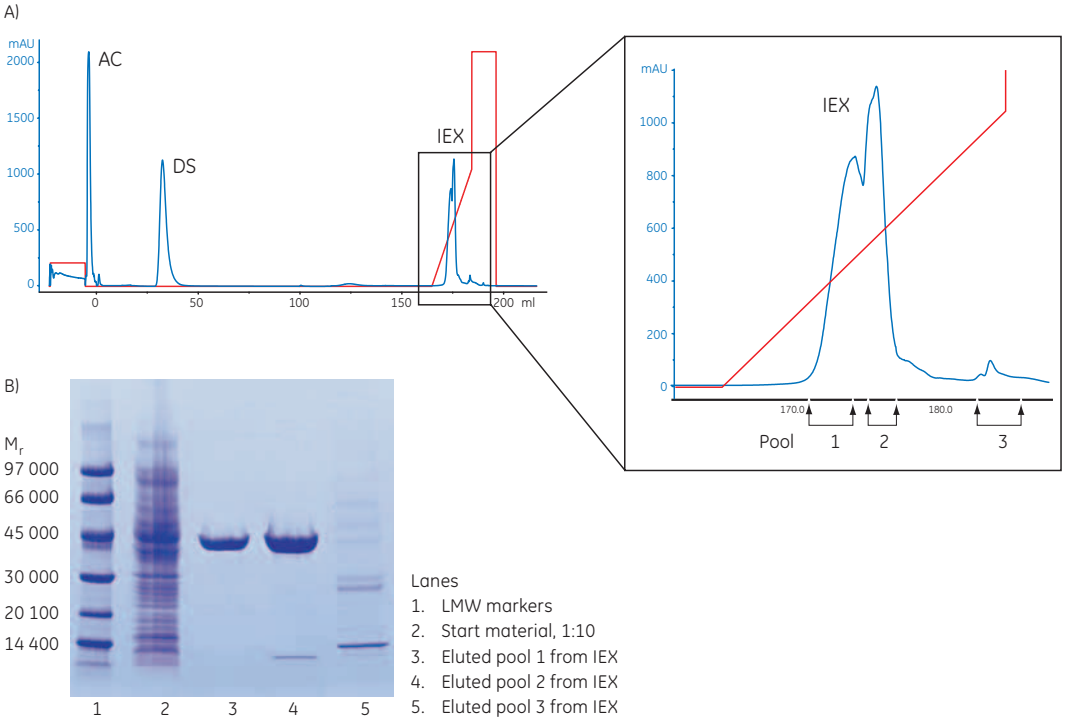


Fig 50. (A) AC-DS-IEX with an enlargement of the IEX peaks and the collected pools to the right. Yield: 9.4 mg in pools 1 + 2. (B) SDS-PAGE of eluted pools from IEX. The gel was stained with Coomassie.

Chapter 5
Purification of GST-tagged recombinant proteins

Introduction

Use of the Glutathione S-transferase (GST) affinity tag was first introduced in 1988 and has since become a popular choice when working with recombinant proteins. The method is based on the affinity of GST to the glutathione ligand coupled to a matrix. The binding of a GST-tagged protein to the ligand is reversible, and the protein can be eluted under mild, nondenaturing conditions by the addition of reduced glutathione to the elution buffer. The technique thus provides a purification process that preserves protein antigenicity and function.

The GST Gene Fusion System is a versatile system from GE Healthcare for the expression, purification, and detection of GST-tagged proteins produced in *E. coli*. The system consists of three major components: pGEX plasmid vectors, products for GST purification, and a GST detection kit. A series of site-specific proteases complements the system.

GST occurs naturally in most organisms. Tagged proteins that possess the complete amino acid sequence of GST also demonstrate GST enzymatic activity and can undergo dimerization similar to that observed in nature. The crystal structure of recombinant *Schistosoma japonicum* GST (M_r 26 000) from pGEX vectors has been determined.

The pGEX vectors are designed for inducible, high-level intracellular expression of genes or gene fragments as fusions with *S. japonicum* GST. Expression in *E. coli* yields tagged proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus.

A variety of affinity chromatography products are available from GE Healthcare that have glutathione immobilized to one of three Sepharose media: Sepharose High Performance (HP), Sepharose 6 Fast Flow (FF), or Sepharose 4B. The Glutathione Sepharose media are available in several formats, ranging from 96-well filter plates to prepacked HiTrap and HiPrep columns to lab packs (media packs in sizes from 25 ml to 500 ml). The media vary in their performance parameters, and the different formats provide options of scale and convenience.

If desired, cleavage of the protein from GST can be achieved using a site-specific protease whose recognition sequence is located immediately upstream from the multiple cloning site on the pGEX plasmids. Tagged proteins can be detected using colorimetric or immunological methods. Cleavage and detection options are discussed later in this chapter.

This chapter summarizes key aspects of working with GST-tagged proteins, with a focus on purification methodologies. For more detailed information on the GST system, refer to the *GST Gene Fusion System Handbook* (Code No. 18-1157-58); the handbook includes detailed information on expression, purification, detection, and removal of the GST tag and is an invaluable guide when working with the system.

Expression

Selecting an expression strategy begins with choosing the vector best-suited for your purpose, taking note of reading frame, cloning sites, and protease cleavage sites. Correct preparation of the insert is important and must take into account the reading frame and orientation, size, and compatibility of the fragment ends. Selection of host cells involves consideration of cloning and maintenance issues and anticipated expression levels. Finally, growth conditions must be evaluated in order to optimize expression. These topics are discussed below.

pGEX vectors

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

Because of the mild elution conditions for release of tagged proteins from the affinity medium, effects on antigenicity and functional activity of the protein are minimized. The vectors have a range of protease cleavage recognition sites as shown in Table 15.

Table 15. Protease cleavage sites of pGEX vectors.

Vector	Cleaved by
pGEX-6P-1, pGEX-6P-2, pGEX-6P-3	PreScission Protease
pGEX-4T-1, pGEX-4T-2, pGEX-4T-3	Thrombin
pGEX-5X-1, pGEX-5X-2, pGEX-5X-3	Factor Xa
pGEX-2TK Allows detection of expressed proteins by direct labeling *in vitro*	Thrombin

The vectors provide all three translational reading frames beginning with the *Eco*R I restriction site (see Appendix 7). The same multiple cloning sites in each vector ensure easy transfer of inserts. pGEX-6P-1, pGEX-4T-1, and pGEX-5X-1 can directly accept and express cDNA inserts isolated from λgt11 libraries.

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

Refer to Appendix 7 for a listing of the control regions of the pGEX vectors. Complete DNA sequences and restriction site data are available with each individual vector's product information, at the GE Healthcare Web site (***http://www.gehealthcare.com/lifesciences***) and also from GenBank™. GenBank accession numbers are listed in Appendix 7.

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

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

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

The host

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

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

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

E. coli BL21, a strain defective in *OmpT* and *Lon* protease production, gives high levels of expression of GST-tagged proteins. It is the host of choice for expression studies with GST-tagged proteins.

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

Insert DNA

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

Optimizing expression

Once it has been established that the insert is in the proper orientation and that the correct junctions are present, the next step is to optimize expression of tagged proteins. The capability to screen crude lysates from many clones is critical to this process, so that optimal expression levels and growth conditions can be readily determined. Once conditions are established, one is ready to prepare large-scale bacterial sonicates of the desired clones.

To screen many putative clones simultaneously, several purification methods are recommended. The first method uses GST MultiTrap FF or GST MultiTrap 4B 96-well plates, which are designed to allow parallel purification of GST-tagged proteins directly from unclarified cell lysates (maximum 600 µl per well). In the second method, a crude lysate suitable for screening from 2 to 3 ml of culture is prepared, using a batch purification method with one of the Glutathione Sepharose media. In the third method, the GST SpinTrap Purification Module is used. This module can isolate protein from up to 12 ml of culture using a standard microcentrifuge. All of these methods are presented later in this chapter.

In addition, several options are presented later in this chapter for determining expression levels.

Growth conditions should be evaluated for optimal expression: media, growth temperature, culture density, induction conditions, and other variables should be evaluated. It is important to assure sufficient aeration and to minimize the time spent in each stage of growth, as well as to use positive selection for the plasmid (antibiotic resistance). Formation of inclusion bodies should be monitored and possibly be avoided by optimizing expression. This topic is discussed in Chapter 8.

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

Purification

GST-tagged proteins are easily purified from bacterial lysates by affinity chromatography using glutathione immobilized to a matrix such as Sepharose (Fig 51). When applied to the affinity medium, tagged proteins bind to the ligand, and impurities are removed by washing with binding buffer. Tagged proteins are then eluted from the Glutathione Sepharose under mild, nondenaturing conditions that preserve both protein antigenicity and function.

If separation of the cloned protein from the GST affinity tag is desired, the tagged protein can be digested with an appropriate site-specific protease while the protein is bound to Glutathione Sepharose. Alternatively, the tagged protein can be digested following elution from the medium (see later in this chapter for both of these alternatives). Cleavage of the bound tagged protein eliminates the extra step of separating the released protein from GST because the GST moiety remains bound to the medium while the cloned protein is eluted using wash buffer.

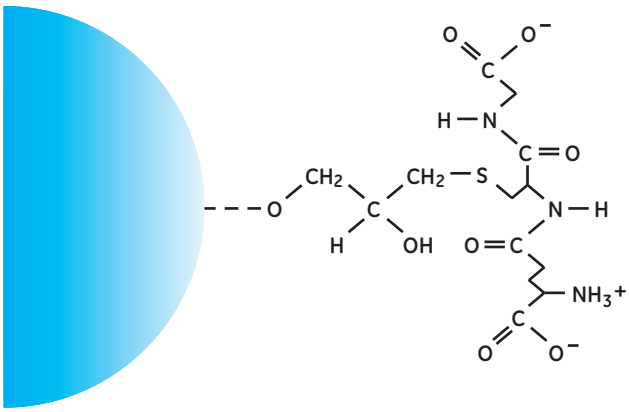


Fig 51. Terminal structure of Glutathione Sepharose. Glutathione is specifically and stably coupled to Sepharose by reaction of the SH-group with oxirane groups obtained by epoxy-activation of the Sepharose matrix. The structure of glutathione is complementary to the binding site of glutathione S-transferase.

Selecting a product for GST-tagged protein purification

Products designed to meet specific purification needs are available for purification of GST-tagged proteins, as shown in the selection guide in Table 16. These products rely on affinity chromatography using gravity flow, centrifugation, vacuum, syringe, or pump action to purify the protein. A comparison of the physical characteristics of the three Glutathione Sepharose media is given in Appendix 2.

Table 16. Selection guide summarizing purification options for GST-tagged proteins, including companion products.

Product	Plate/column size	Plate/column capacity	Description	Application
Glutathione Sepharose High Performance	25 ml 100 ml	Approx. 10 mg (rGST)	Lab pack	For high resolution and elution of a more concentrated sample (high-performance purification).
Glutathione Sepharose 4 Fast Flow	25 ml 100 ml 500 ml	Approx. 10 mg (rGST)	Lab pack	Excellent for scale-up due to good binding capacity and good flow properties.
Glutathione Sepharose 4B	10 ml 100 ml (function tested) 300 ml	> 5 mg (rGST)	Lab pack	Good binding capacity.
Glutathione Sepharose 4B	2 ml	Up to 10 mg (horse liver GST)	2 columns prepacked with Glutathione Sepharose 4B.	Gravity flow. No system needed.
GST MultiTrap FF	50 µl	Up to 0.5 mg (rGST)	Prepacked 96-well filter plates with Glutathione 4 Fast Flow.	For high-throughput expression screening. For use with robotics or manually by centrifugation or vacuum.
GST MultiTrap 4B	50 µl	Up to 0.5 mg (rGST)	Prepacked 96-well filter plates with Glutathione Sepharose 4B.	For high-throughput expression screening. For use with robotics or manually by centrifugation or vacuum.
GST SpinTrap Purification Module	50 µl	Up to 0.4 mg (rGST)	50 columns and reagents. Columns prepacked with Glutathione Sepharose 4B.	For use in a microcentrifuge. Medium throughput for expression screening and minipreps.
GSTrap HP	1 ml 5 ml	Approx. 10 mg Approx. 50 mg (rGST)	Columns prepacked with Glutathione Sepharose High Performance.	For use with a peristaltic pump or chromatography system in preference over syringe. For high resolution and elution of a more concentrated sample (high-performance purification).
GSTrap FF	1 ml 5 ml	Approx. 10 mg Approx. 50 mg (rGST)	Columns prepacked with Glutathione Sepharose 4 Fast Flow.	For use with syringe, peristaltic pump, or chromatography system. Provides good flow properties. Scale-up.
GSTrap 4B	1 ml 5 ml	> 5 mg > 25 mg (horse liver GST)	Columns prepacked with Glutathione Sepharose 4B.	For use with syringe, peristaltic pump or chromatography system.
GSTPrep™ FF 16/10	20 ml	Approx. 200 mg (rGST)	Columns prepacked with Glutathione Sepharose 4 Fast Flow.	For use with a chromatography system. Scale-up purification.

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Table 16. Selection guide summarizing purification options for GST-tagged proteins, including companion products (continued).

Product	Pack size	Capacity	Description	Application
pGEX Vectors (GST Gene Fusion System)	5 to 25 µg vector	N/A	Vector and *E. coli* BL21 cells.	A *tac* promoter for chemically inducible, high-level expression. PreScission, thrombin, or Factor Xa protease recognition sites.
Anti-GST Antibody	0.5 ml	50 detections	Anti-GST antibody.	Polyclonal. For sensitive and specific detection of GST-tagged proteins. For use with an enzyme-conjugated anti-goat antibody.
Anti-GST HRP Conjugate	75 µl	1:5000 dilution, typical concentration	Highly specific antibody to GST conjugated to HRP and optimized for use in Western blotting with ECL detection reagents.	Polyclonal. Offers speed, sensitivity, and safety for detection of GST-tagged proteins. Recognizes multiple epitopes of GST, thus not reliant on functional GST for detection.
GST 96-Well Detection Module	5 plates HRP conjugated Anti-GST Antibody and GST protein.	N/A	GST 96-Well Detection Module.	Plates precoated with Anti-GST antibody and blocked for the capture of GST-tagged proteins, which are then detected using HRP conjugated Anti-GST Antibody.
GST Detection Module	1-chloro-2-4-dinitro-benzene (CDNB), Anti-GST Antibody, and instructions.	50 detection reactions	GST Detection Module.	For the biochemical or immunological detection of GST-tagged proteins. Glutathione and CDNB serve as substrates to yield a yellow product detectable at 340 nm. The antibody is suitable for use in Western blots.
ECL GST Western Blotting Detection Kit	For 1000 or 3000 cm^2 membrane	ECL Plus Solution A and ECL Plus Solution B	Acridan-based substrate.	For chemiluminescent and chemifluorescent detection. Extended signal duration allows multiple exposures to be made.
PreScission Protease	500 units	One unit cleaves $\geq$ 90% of 100 µg of a test GST-tagged protein when incubated in 1 mM EDTA, 1 mM DTT, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.0) at 5°C for 16 h.	PreScission Protease.	For specific, low-temperature cleavage between Gln and Gly residues in the sequence Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro. A tagged protein consisting of human rhinovirus protease and GST. Can be used for tag cleavage when the PreScission protease recognition sequence occurs between the tag sequence and the protein of interest, e.g., the GST tag from proteins expressed using the pGEX-6P vector.

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Table 16. Selection guide summarizing purification options for GST-tagged proteins, including companion products (continued).

Product	Pack size	Capacity	Description	Application
Thrombin	500 units	One unit cleaves ≥ 90% of 100 µg of a test GST-tagged protein when incubated in 1x PBS at 22°C for 16 h.	Thrombin	For specific cleavage at the recognition sequence for thrombin. Can be used for tag cleavage when the thrombin recognition sequence occurs between the tag sequence and the protein of interest, e.g., the GST tag from proteins expressed using the pGEX-T vectors.
Factor Xa	400 units	One unit cleaves ≥ 90% of 100 µg of a test GST-tagged protein when incubated in 1 mM $CaCl_2$, 100 mM NaCl, and 50 mM Tris-HCl (pH 8.0) at 22°C for 16 h.	Factor Xa	For specific cleavage following the tetrapeptide Ile-Glu-Gly-Arg. Can be used for tag cleavage when the Factor Xa recognition sequence occurs between the tag sequence and the protein of interest, e.g., the GST tag from proteins expressed using pGEX-X vectors.
HiTrap Benzamidine FF (high sub)	1 ml 5 ml	≥ 35 mg trypsin ≥ 175 mg trypsin	Columns prepacked with Benzamidine Sepharose 4 Fast Flow (high sub).	Removal of serine proteases, e.g., thrombin and Factor Xa after tag cleavage.
Benzamidine Sepharose 4 Fast Flow (high sub)	25 ml	≥ 35 mg trypsin/ml medium	Lab pack	Removal of serine proteases, e.g., thrombin and Factor Xa after tag cleavage.
Collection Plate	96-well plates	500 µl	V-shaped bottom	For use with the GST MultiTrap products.

General considerations for purification of GST-tagged proteins

Yield of tagged protein is highly variable and is affected by the nature of the tagged protein, the host cell, and the expression and purification conditions used. Tagged protein yields can range from 1 mg/l up to 10 mg/l. Table 17 can be used to approximate culture volumes based on an average yield of 2.5 mg/l.

Table 17. Reagent volume requirements for different protein yields.

Tagged protein yield	50 mg	10 mg	1 mg	50 µg
Culture volume	20 l	4 l	400 ml	20 ml
Volume of extract	1 l	200 ml	20 ml	1 ml
Glutathione Sepharose bed volume	10 ml	2 ml	200 µl	10 µl
1× PBS[1]	100 ml	20 ml	2 ml	100 µl
Glutathione elution buffer	10 ml	2 ml	200 µl	10 µl

[1] This volume is per wash. Three washes are required per sample in the following procedures.

Use deionized (or double-distilled) water and chemicals for sample and buffer preparation. Samples should be centrifuged immediately before use and/or filtered through a 0.45 µm filter. If the sample is too viscous, to prevent it from clogging the column dilute it with binding buffer, increase lysis treatment (sonication, homogenization), or add DNase/RNase to reduce the size of nucleic acid fragments.

One of the most important parameters affecting the binding of GST-tagged proteins to Glutathione Sepharose is the flow rate. Because the binding kinetics between glutathione and GST are relatively slow, it is important to keep the flow rate low during sample application to achieve maximum binding capacity. Washing and elution can be performed at a slightly higher flow rate to save time. For batch purification, incubation time should be considered.

The binding properties of the target protein can be improved by adjusting the sample to the composition of the binding buffer. Dilute in binding buffer or perform a buffer exchange using a desalting column such as HiTrap Desalting 5 ml, PD-10 Desalting, or HiPrep 26/10 Desalting. Refer to Chapter 9 for use of desalting columns.

Volumes and times used for elution may vary among tagged proteins. Further elution with higher concentrations of glutathione (20 to 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 6.5 to 8. Flowthrough, wash, and eluted material from the column should be monitored for GST-tagged proteins using SDS-PAGE in combination with Western blot if necessary.

Following the elution steps, a significant amount of tagged protein may remain bound to the medium. Volumes and times used for elution may vary among tagged proteins. Additional elutions may be required. Eluates should be monitored for GST-tagged protein by SDS-PAGE or by 1-chloro-2,4 dinitrobenzene (CDNB) assay (see later in this chapter).

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

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

Purification steps should be monitored using one or more of the detection methods described later in this chapter. The GST Detection Module contains components that can be used for either enzymatic or immunochemical determination of concentrations of GST-tagged proteins in extracts as well as sample obtained during purification.

The yield of protein in purified samples can also be determined by standard chromogenic methods (e.g., Lowry, BCA, Bradford, etc.). If a Lowry or BCA type method is to be used, the glutathione in the purified material must be removed using, for example, HiTrap Desalting 5 ml or dialysis against 2000 volumes of PBS to reduce interference with the assay. The Bradford method can be performed in the presence of glutathione.

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

Selecting equipment for purification

The choice of equipment will depend on the specific purification. Many purification steps can be carried out using simple methods and equipment as, for example, step-gradient elution using a syringe in combination with prepacked HiTrap columns. Linear gradients may improve purity when GST-tagged proteins are purified from eukaryotic hosts because endogenous GST may be co-eluted in step-gradient elution. If the same column is to be used for many runs in series, it is wise to use a dedicated system. Table 8 in Chapter 2 provides a guide to aid in selecting the correct purification system.

For small-scale purifications or for high-throughput screening, we recommend GST MultiTrap FF or GST MultiTrap 4B 96-well filter plates, which can purify up to approximately 0.5 mg of GST-tagged protein per well. In addition, GST SpinTrap columns, each containing 50 µl of Glutathione Sepharose 4B, can purify up to 400 µg of recombinant GST.

For purification of larger quantities of GST-tagged proteins, prepacked columns such as GSTrap and GSTPrep FF 16/10 provide excellent formats. To increase capacity, use several GSTrap columns (1 ml or 5 ml) or two GSTPrep FF 16/10 columns (20 ml) in series or, for even larger capacity requirements, pack Glutathione Sepharose media into a suitable column.

For simple and rapid, one-step reproducible purification, use of a chromatography system such as ÄKTAprime plus is advantageous because it has preprogrammed methods for the most typical applications, including purification of histidine- and GST-tagged proteins. A UV and conductivity monitor and easy-to-use software enable automatic tracking of the protein. Monitoring is continuous in real time, thus eliminating manual errors. For laboratory environments in which all experimental data must be recorded and traceable, or where multistep purification schemes, method development, optimization, and/or scale-up are needed, ÄKTApurifier or ÄKTAexplorer chromatography system is recommended.

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

These three media are all used for the purification of GST-tagged recombinant proteins and other S-tranferases or glutathione-dependent proteins. They allow mild elution conditions that preserve protein antigenicity and function. All are supplied preswollen in 20% ethanol and are also available in various prepacked formats, such as GSTrap, as described later in this chapter. See Appendix 2 for the main characteristics of all Glutathione Sepharose media.

In Glutathione Sepharose High Performance, the glutathione ligand is coupled to highly cross-linked 6% agarose. The medium has an average bead size of 34 µm and is an excellent choice for high-resolution purification and elution of a more concentrated sample.

In Glutathione Sepharose 4 Fast Flow, the glutathione ligand is coupled to highly cross-linked 4% agarose. The medium has an average bead size of 90 µm. It is a very good choice for scale-up due to its good binding capacity and flow properties. This medium is also a good choice for batch and gravity-flow purifications.

In Glutathione Sepharose 4B, the glutathione ligand is coupled to 4% agarose. The medium has an average bead size of 90 µm. It provides good binding capacity, and is suitable for small-scale purification as well as batch and gravity-flow operations.

Glutathione Sepharose 4 Fast Flow and Glutathione Sepharose 4B are also available prepacked in 96-well filter plates (see page 120).

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

Fig 52. Glutathione Sepharose High Performance and Glutathione Sepharose 4 Fast Flow for purification of GST-tagged proteins.

Batch purification of GST-tagged proteins using Glutathione Sepharose HP, Glutathione Sepharose 4 FF, or Glutathione Sepharose 4B

Refer to page 112, General considerations, before beginning this procedure.

Sample preparation

1. Prepare the cell lysate.
2. Centrifuge the cell lysate at high speed for 10 min at 4°C and filter through a 0.45-µm filter before applying to the Glutathione Sepharose medium. If the sample is too viscous, dilute it with binding buffer, increase lysis treatment (sonication, homogenization), or add DNase/RNase to reduce the size of nucleic acid fragments.

Buffer preparation

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

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

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

1 to 20 mM DTT may be included in the binding and elution buffers to increase the purity. However, this may result in lower yield of GST-tagged protein.

Preparation of Glutathione Sepharose media for use in batch purification

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

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

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

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

For cleaning, storage, and handling information, refer to Appendix 2.

Batch purification

1. Add the cell lysate to the prepared Glutathione Sepharose medium and incubate for at least 30 min at room temperature, using gentle agitation such as end-over-end rotation.
2. Use a pipette or cylinder to transfer the mixture to an appropriate container/tube.
3. Sediment the medium by centrifugation at 500 × g for 5 min. Carefully decant the supernatant (= flowthrough) and save it for SDS-PAGE analysis to determine the binding efficiency of the GST-tagged protein to the medium.
4. Wash the Glutathione Sepharose medium by adding 5 ml of PBS per 1 ml of slurry (= 50% slurry). Invert to mix.
5. Sediment the medium by centrifugation at 500 × g for 5 min. Carefully decant the supernatant (= wash) and save it for SDS-PAGE analysis.

6. Repeat steps 4 and 5 twice for a total of three washes.
7. Elute the bound protein by adding 0.5 ml of elution buffer per 1 ml slurry of Glutathione Sepharose medium. Incubate at room temperature for 5 to 10 min, using gentle agitation such as end-over-end rotation.
8. Sediment the medium by centrifugation at 500 × g for 5 min. Carefully decant the supernatant (= eluted protein) and transfer to a clean tube.
9. Repeat steps 7 and 8 twice for a total of three elutions. Check the three eluates separately for purified protein and pool those eluates containing protein.

Column purification of GST-tagged proteins using Glutathione Sepharose HP, Glutathione Sepharose 4 FF, or Glutathione Sepharose 4B

Refer to page 112, General considerations, before beginning this procedure.

Sample preparation

1. Prepare the cell lysate.
2. Centrifuge the cell lysate at high speed for 10 min at 4°C and pass it through a 0.45 µm filter before applying to the Glutathione Sepharose column. If the sample is too viscous, to prevent it from clogging the column dilute it with binding buffer, increase lysis treatment (sonication, homogenization), or add DNase/RNase to reduce the size of nucleic acid fragments.

Buffer preparation

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

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

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

1 to 20 mM DTT may be included in the binding and elution buffers to increase the purity. However, this may result in lower yield of GST-tagged protein.

Column packing

Glutathione Sepharose media are supplied preswollen in 20% ethanol. Because of the nature of the media, the steps for packing columns with them vary and are presented separately below.

Packing a column containing Glutathione Sepharose High Performance

Refer to Appendix 4 for general guidelines for column packing. Recommended lab-scale columns and associated flow rates for Glutathione Sepharose High Performance are listed in Table 18.

Table 18. Recommended lab-scale columns for Glutathione Sepharose High Performance.

Empty column[1]	Packing flow rate (ml/min)		Max. recommended flow rate for purification (ml/min)
	first step	second step	
Tricorn 5/20	0.5	1	0.5
Tricorn 5/50	0.5	1	0.5
Tricorn 10/20	2	4	2
Tricorn 10/50	2	4	2
Tricorn 10/100	2	4	2
XK 16/20	5	9	5
XK 26/20	13	27	13

[1] *For inner diameter and maximum bed volumes and bed heights, refer to the catalog, BioDirectory, or Web site.*

1. Equilibrate all materials to the temperature at which the purification will be performed.
2. Prepare a slurry by decanting an appropriate amount of the 20% ethanol solution and replacing it with distilled water in a ratio of 75% settled medium to 25% distilled water.
3. Assemble the column (and packing reservoir if necessary).
4. Remove air from the end-piece and adapter by flushing with distilled water. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with water.
5. Resuspend the medium and pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.
6. If using a packing reservoir, immediately fill the remainder of the column and reservoir with distilled water. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.
7. Open the bottom outlet of the column and set the pump to run at the desired flow rate.

Ideally, Sepharose High Performance media are packed in XK or Tricorn columns in a two-step procedure: Do not exceed 1.0 bar (0.1 MPa) in the first step and 3.5 bar (0.35 MPa) in the second step. If the packing equipment does not include a pressure gauge, use a packing flow rate of 5 ml/min (XK 16/20 column) or 2 ml/min (Tricorn 10/100 column) in the first step, and 9 ml/min (XK 16/20 column) or 3.6 ml/min (Tricorn 10/100 column) in the second step. If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate your pump can deliver. This should also give a well-packed bed.

For subsequent chromatography procedures, do not exceed 75% of the packing flow rate.

8. Maintain packing flow rate for at least 3 bed volumes after a constant bed height is reached. Mark the bed height on the column.
9. Stop the pump and close the column outlet.
10. If using a packing reservoir, disconnect the reservoir and fit the adapter to the column.
11. With the adapter inlet disconnected, push the adapter down into the column until it reaches the mark. Allow the packing solution to flush the adapter inlet. Lock the adapter in position.
12. Connect the column to a pump or a chromatography system and start equilibration. Readjust the adapter if necessary.

Packing a column containing Glutathione Sepharose 4 Fast Flow

Refer to Appendix 4 for general guidelines for column packing.

Recommended columns:

- Tricorn 10/100 (10 mm i.d.) for bed volumes up to 8.5 ml at bed heights up to 10.8 cm.
- XK 16/20 (16 mm i.d.) for bed volumes up to 30 ml at bed heights up to 15 cm.
- XK 26/20 (26 mm i.d.) for bed volumes up to 80 ml at bed heights up to 15 cm.

1. Equilibrate all materials to the temperature at which the purification will be performed.
2. Prepare a slurry by decanting an appropriate amount of the 20% ethanol solution and replacing it with distilled water in a ratio of 75% settled medium to 25% distilled water.
3. Assemble the column (and packing reservoir if necessary).
4. Remove air from the end-piece and adapter by flushing with distilled water. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with distilled water.
5. Resuspend the medium and pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.

6. If using a packing reservoir, immediately fill the remainder of the column and reservoir with water. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.
7. Open the bottom outlet of the column and set the pump to run at the desired flow rate.
8. Maintain the packing flow for at least 3 bed volumes after a constant bed height is obtained. Mark the bed height on the column.

Ideally, Fast Flow media are packed at constant pressure not exceeding 1 bar (0.1 MPa) in XK columns. If the packing equipment does not include a pressure gauge, use a packing flow rate of maximum 15 ml/min, 450 cm/h (XK 16/20 column) or 6 ml/min, 450 cm/h (Tricorn 10/100 column). If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate the pump can deliver. This should also give a reasonably well-packed bed.

For subsequent chromatography procedures, do not exceed 75% of the packing flow rate.

9. Stop the pump and close the column outlet.
10. If using a packing reservoir, disconnect the reservoir and fit the adapter to the column.
11. With the adapter inlet disconnected, push the adapter down into the column until it reaches the mark. Allow the packing solution to flush the adapter inlet. Lock the adapter in position.
12. Connect the column to a pump or a chromatography system and start equilibration. Readjust the adapter if necessary.

Packing a column containing Glutathione Sepharose 4B

Refer to Appendix 4 for general guidelines for column packing.

Recommended columns:

- Tricorn 10/100 (10 mm i.d.) for bed volumes up to 8.5 ml at bed heights up to 10.8 cm.
- XK 16/20 (16 mm i.d.) for bed volumes up to 30 ml at bed heights up to 15 cm.
- XK 26/20 (26 mm i.d.) for bed volumes up to 80 ml at bed heights up to 15 cm.

1. Equilibrate all materials to the temperature at which the purification will be performed.
2. Prepare a slurry by decanting an appropriate amount of the 20% ethanol solution and replacing it with distilled water in a ratio of 75% settled medium to 25% distilled water.
3. Assemble the column (and packing reservoir if necessary).
4. Remove air from the end-piece and adapter by flushing with distilled water. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with distilled water.
5. Resuspend the medium and pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.
6. If using a packing reservoir, immediately fill the remainder of the column and reservoir with distilled water. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.
7. Open the bottom outlet of the column and set the pump to run at the desired flow rate.
8. Maintain the packing flow for at least 3 bed volumes after a constant bed height is obtained. Mark the bed height on the column.

Ideally, 4B media are packed at constant pressure not exceeding 1 bar (0.1 MPa) in XK columns. If the packing equipment does not include a pressure gauge, use a packing flow rate of maximum 2.5 ml/min, 75 cm/h (XK 16/20 column) or 1 ml/min, 75 cm/h (Tricorn 10/100 column). If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate the pump can deliver. This should also give a reasonably well-packed bed.

For subsequent chromatography procedures, do not exceed 75% of the packing flow rate.

9. Stop the pump and close the column outlet.
10. If using a packing reservoir, disconnect the reservoir and fit the adapter to the column.
11. With the adapter inlet disconnected, push the adapter down into the column until it reaches the mark. Allow the packing solution to flush the adapter inlet. Lock the adapter in position.
12. Connect the column to a pump or a chromatography system and start equilibration. Readjust the adapter if necessary.

Column purification

1. Equilibrate the column with approximately 5 column volumes of binding buffer.
2. Apply the pretreated sample.
3. Wash the column with 5 to 10 column volumes of binding buffer or until no material appears in the flowthrough. Save the flowthrough for SDS-PAGE analysis to measure the binding efficiency to the medium.
4. Elute the bound protein with 5 to 10 column volumes of elution buffer. Collect the fractions and check separately for purified protein. Pool those fractions containing the GST-tagged target protein.

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

GST MultiTrap FF and GST MultiTrap 4B (Fig 53) are prepacked, disposable 96-well filter plates for reproducible, high-throughput screening of GST-tagged proteins. Typical applications include expression screening of different constructs, screening for solubility of proteins, and optimization of the conditions for small-scale parallel purification. These filter plates simplify the purification screening and enrichment of up to 0.5 mg of GST-tagged proteins/well. After thorough cell disruption, it is possible to apply up to 600 µl of unclarified lysate directly to the wells in the 96-well filter plate without precentrifugation and/or filtration of the sample. It is recommended to extend the duration of mechanical/chemical lysis if the sample is too viscous after lysis; alternatively, include nucleases to disrupt nucleic acids. The GST-tagged proteins are eluted under mild, nondenaturing conditions that preserve protein function and antigenicity.

The plates are packed with the affinity media Glutathione Sepharose 4 Fast Flow (4% highly cross-linked agarose beads) and Glutathione Sepharose 4B (4% agarose beads), respectively. Each well contains 500 µl of a 10% slurry of Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B in storage solution (50 µl of medium in 20% ethanol). Note that binding depends on flow and may vary between proteins.

The 96-well filter plates with 800 µl wells are made of polypropylene and polyethylene. Characteristics of GST MultiTrap FF and GST MultiTrap 4B are listed in Appendix 2.

Prepacked GST MultiTrap FF and GST MultiTrap 4B plates give high consistency in reproducibility well-to-well and plate-to-plate. The repeatability of yield and purity of eluted protein is high. Automated robotic systems as well as manual handling using centrifugation or vacuum pressure can be used. The purification protocol can easily be scaled up because Glutathione Sepharose is available in larger prepacked formats: GSTrap FF, and GSTrap 4B (1-ml and 5-ml columns) and GSTPrep FF 16/10 (20-ml column). See later in this chapter for a discussion of these products.

Fig 53. GST MultiTrap FF and GST MultiTrap 4B 96-well filter plates.

Sample preparation

Refer to page 112, General considerations, before beginning this procedure.

Lysis with commercial kits could give large cell debris particles that may interfere with drainage of the wells during purification. This problem can be solved by centrifugation or filtration of the sample before adding it to the wells.

After thorough cell disruption, it is possible to apply unclarified lysate directly to the wells without pre-centrifugation and/or filtration of the sample. Apply the unclarified lysate to the wells directly after preparation, as the lysate may precipitate unless used immediately or frozen before use. New lysing of the sample can then prevent clogging of the wells when loading the plate.

If the sample is too viscous, an extension of the duration of mechanical treatment of the sample to ensure a more complete lysis is recommended (keep the sample on ice to prevent overheating).

Buffer preparation

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

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

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

1 to 20 mM DTT can be included in the binding and elution buffers. However, this may result in lower yield of GST-tagged protein.

Centrifugation procedure for high-throughput screening

Preparing the filter plate

1. Peel off the bottom seal from the 96-well filter plate. Be sure to hold the filter plate over a sink to accommodate any leakage of storage solution when removing the bottom seal.
2. Hold the filter plate upside down and gently shake it to dislodge any medium adhering to the top seal. Return the filter plate to an upright position.
3. Place the filter plate against the bench surface and peel off the top seal.

   Note: If the medium has dried out in one or several wells, add buffer to rehydrate it. The performance of the medium is not affected.
4. Position the filter plate on top of a collection plate.

   Note: Remember to change or empty the collection plate as necessary during the following steps.
5. Centrifuge the filter plate for 2 min at 500 × g to remove the ethanol storage solution from the medium.
6. Add 500 µl of deionized water to each well. Centrifuge the plate for 2 min at 500 × g.
7. Add 500 µl of binding buffer to each well to equilibrate the medium. Centrifuge for 2 min at 500 × g. Repeat this step once. The filter plate is now ready for use.

Centrifugation procedure

Do not apply more than 700 × g during centrifugation.

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

   Note: If the yield of protein is too low, increase the incubation time and/or gently agitate the filter plate to effect mixing.

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

   Note: An A_{280} reading of < 0.1 of the collected liquid is required to obtain high purity. If necessary, change the collection plate between each elution to prevent unnecessary dilution of the target protein.

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

   Note: The volume of elution buffer can be varied (50 µl to 100 µl per well), depending on the concentration of target protein required.

5. Change the collection plate and centrifuge at 500 × g for 2 min to collect the eluted protein. Repeat twice or until all of the target protein has been eluted.

If the yield of eluted target protein is low, the incubation time can be increased.

Vacuum procedure for high-throughput screening

Refer to page 112, General considerations, before beginning this procedure.

If problems with foaming, reproducibility, or bubbles in the collection plate occur using vacuum, the centrifugation procedure should be considered. The distance between the filter plate and the collection plate is critical; adjust the distance if necessary.

Preparing the filter plate

1. Peel off the bottom seal from the 96-well filter plate. Be sure to hold the filter plate over a sink to accommodate any leakage of storage solution when removing the bottom seal.
2. Hold the filter plate upside down and gently shake it to dislodge any medium adhering to the top seal. Return the filter plate to an upright position.
3. Place the filter plate against the bench surface and peel off the top seal.

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

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

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

5. Set the vacuum to -0.15 bar. Place the filter plate and collection plate on the vacuum manifold to remove the ethanol storage solution from the medium.
6. Add 500 µl of deionized water to each well. Apply a vacuum to remove the water from the wells.
7. Add 500 µl of binding buffer to each well to equilibrate the medium. Apply a vacuum as in step 5. Repeat this step once. The filter plate is now ready for use.

Vacuum procedure

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

Do not apply a pressure in excess of -0.5 bar during vacuum operation.

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

   Note: If the yield of protein is too low, increase the incubation time and/or gently agitate the filter plate.

2. Apply a vacuum of -0.15 bar until all the wells are empty. Slowly increase the vacuum to -0.30 bar and turn off the vacuum after approximately 5 sec. Discard the flowthrough.

Increasing the vacuum too quickly can result in foaming under the filter plate and subsequent cross-contamination of samples.

3. Add 500 µl of binding buffer per well to wash out any unbound sample. Apply a vacuum of -0.15 bar as in step 2. Repeat once or until all unbound sample is removed.

   Note: An A_{280} reading of < 0.1 of the collected liquid is required to obtain high purity. If necessary, change the collection plate between each elution to prevent unnecessary dilution of the target protein.

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

   Note: The volume of elution buffer can be varied (50 µl to 100 µl per well), depending on the concentration of target protein required.

5. Change the collection plate and apply a vacuum of -0.15 bar to collect the eluted protein. Repeat twice or until all of the target protein has been eluted.

If the yield of eluted target protein is low, the incubation time can be increased.

Application example

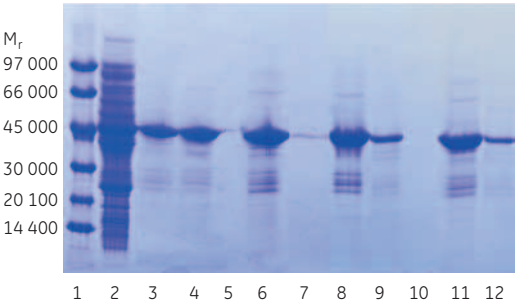
High-throughput screening and purification of GST-hippocalcin using GST MultiTrap FF

GST MultiTrap FF and GST MultiTrap 4B allow reproducible, high-throughput screening and rapid parallel purification of GST-tagged proteins, using robotic systems, centrifugation, or manual vacuum manifolds. In this example, the conditions for binding buffer were optimized for purification of GST-hippocalcin using GST MultiTrap FF. A buffer-screening study to determine optimal buffer conditions for purification was designed based on the parameters of buffer, pH, sodium chloride, glycerol, DTT, and glutathione. A comparison between sonication and use of a commercial cell lysis kit was also performed. Factorial design (design-of-experiments) and statistical analysis were performed using MODDE™ software (Umetrics). The different buffer conditions and sample preparation methods were applied randomly on the filter plate.

The presence of glutathione in sample and binding buffer (also used as wash buffer) decreased yield of purified GST-hippocalcin significantly, while the type of buffer used had no effect on yield. Low pH improved yield whereas high pH (8.0) affected yield negatively. No significant effect on purity (Fig 54) was seen with changing pH. Additives such as DTT, glycerol, and NaCl did not significantly affect yield or purity of this particular protein.

The screening results showed that the optimal buffer conditions for purifying GST-hippocalcin with highest yield and purity were: 10 to 20 mM sodium phosphate, 140 to 400 mM NaCl, pH 6.2 to 7.0 (data not shown). Results reflecting sample preparation showed in this case that both the commercial cell lysis kit and sonication can be used to lyse *E. coli* without significantly affecting the purification result.

96-well filter plate:	GST MultiTrap FF
Sample:	Unclarified *E. coli* BL21 lysate containing GST-tagged hippocalcin, M_r 43 000
Sample preparation:	Lysis using a commercial cell lysis kit and sonication were compared. Both methods were performed according to standard protocols.
Sample volume:	500 µl
Elution volume:	3 × 200 µl
Binding buffer:	Parameters varied and randomly tested: 10 to 20 mM PBS; 50 to100 mM Tris-HCl; pH 6.2 to 8.0; 140 to 400 mM NaCl; 0 to 5 mM DTT; 0% to 5% glycerol and 0 to 2 mM glutathione
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Elution method:	Centrifugation
Purification protocol:	According to GST MultiTrap instructions, 28-4070-75
Data evaluation:	MODDE software, UV-spectrometry (A_{280}), SDS-PAGE



Lanes

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

Fig 54. SDS-PAGE (reducing conditions, ExcelGel SDS Gradient 8–18; Coomassie staining) of collected fractions of eluted GST-hippocalcin from some of the GST MultiTrap FF filter plate wells.

Minipreps using the GST SpinTrap Purification Module

The GST SpinTrap Purification Module is useful for screening small or large numbers of bacterial lysates and for checking samples during the optimization of expression or purification conditions. Each module contains reagents sufficient for 50 purifications using SpinTrap columns prepacked with Glutathione Sepharose 4B. Sample application, washing, and elution can be performed using a standard microcentrifuge.

Purification of multiple samples using GST SpinTrap columns with a microcentrifuge

Refer to page 112, General considerations, before beginning this procedure.

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

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

Components in GST SpinTrap Purification Module

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

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

Dilution buffer: 50 mM Tris-HCl, pH 8.0

SpinTrap columns: 50 units

Procedure

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

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

Purification using GSTrap HP, GSTrap FF, and GSTrap 4B columns

GSTrap affinity columns are specially designed 1-ml and 5-ml HiTrap columns packed with Glutathione Sepharose HP, FF, or 4B media. Refer to the selection guide in Table 16 for a summary of their differences and to Appendix 2 for a list of key characteristics of each.

Sample application, washing, and elution can be performed using a syringe with a supplied connector, a peristaltic pump, or a liquid chromatography system such as ÄKTAdesign (see Table 8 for equipment choices). For easy scale-up, two to three columns can be connected together in series simply by screwing the end of one column into the top of the next.

Figure 55 shows a schematic representation of the simple steps needed for successful purification using GSTrap columns.

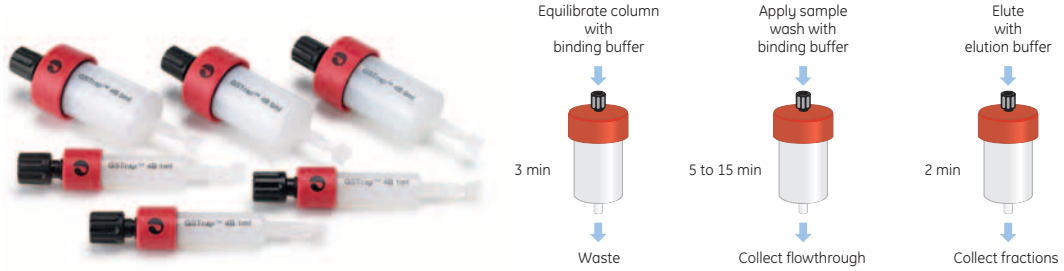


Fig 55. GSTrap HP, GSTrap 4B, and GSTrap FF 1-ml and 5-ml columns allow convenient and simple one-step purification of GST-tagged proteins. Simple purification of GST-tagged proteins is shown at right.

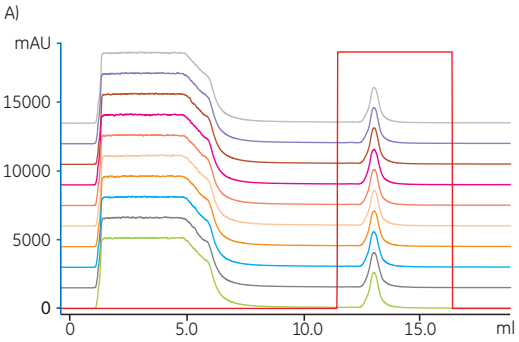
The GSTrap HP, FF, and 4B columns are made of polypropylene, which is biocompatible and non-interactive with biomolecules. The top and bottom frits are manufactured from porous polyethylene. Columns are delivered with a stopper on the inlet and a snap-off end on the outlet. Every package includes all necessary components for connection of the columns to different types of equipment. Note that GSTrap columns cannot be opened or refilled.

GSTrap columns are directly compatible with existing purification protocols for GST-tagged proteins, including on-column proteolytic cleavage methods. If removal of the GST moiety is required, the tagged protein can be digested with an appropriate site-specific protease while bound to the medium or, alternatively, after elution (see later in this chapter). On-column cleavage eliminates the extra step of separating the released protein from GST because the GST moiety remains bound. For quick scale-up of purifications, two or three GSTrap columns (1 ml or 5 ml) can be connected in series (back pressure will increase).

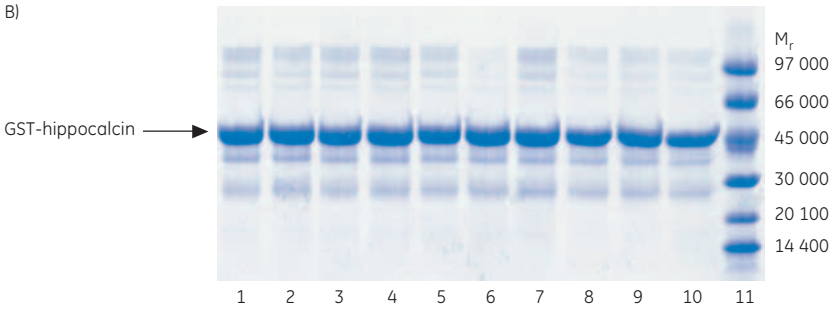
One of the three media, Glutathione Sepharose 4 Fast Flow, is also available in prepacked 20-ml GSTPrep FF 16/10 columns (see page 134). All three are available in lab packs (varying from 25 to 500 ml) for packing in a column of the user's choice.

For cleaning, storage, and handling information, refer to Appendix 2.

The media are very stable and the purification process very reproducible. This can be seen from the results of an experiment in which *E. coli* homogenates containing GST-hippocalcin (M_r 43 000) were repeatedly purified 10 times on the same column without cleaning between runs. The 10 overlaid chromatograms (Fig 56A) show a near perfect match, indicating little or no variation in binding capacity and stability of the medium. SDS-PAGE analysis (Fig 56B) also indicates no changes in purity or binding capacity after 10 runs.



Column: GSTrap HP 1 ml
Sample: Clarified *E. coli* homogenate containing expressed GST-hippocalcin, M_r 43 000
Binding buffer: 10 mM sodium phosphate, 0.14 M NaCl, 1 mM DTT, pH 7.5
Elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, 1 mM DTT, pH 8.0
Flow rate
sample loading: 0.3 ml/min
wash and elution: 1 ml/min
Running temperature: Room temperature



Lanes 1 to 10. Eluted pooled fractions from runs 1 to 10, diluted 1:8.
Lane 11. LMW marker.

Fig 56. (A) Confirmation of the stability of Glutathione Sepharose High Performance prepacked in 1-ml GSTrap HP columns. Chromatographic overlay of 10 repetitive purifications. (B) Coomassie-stained nonreduced SDS-PAGE (ExcelGel SDS Gradient 8–18) of pooled fractions from repetitive purification runs shown in (A).

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

The sample should be centrifuged and/or filtered through a 0.22-µm or a 0.45-µm filter immediately before it is applied to the column. If the sample is too viscous, dilute it with binding buffer or buffer exchange using HiTrap Desalting, PD-10 Desalting, or HiPrep 26/10 Desalting to prevent clogging the column.

Buffer preparation

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

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

Elution buffer: 50 mM Tris-HCl, 10 to 20 mM reduced glutathione, pH 8.0

1 to 20 mM DTT may be included in the binding and elution buffers to increase the purity. However, this may result in lower yield of GST-tagged protein.

Purification

1. Fill the syringe or pump tubing with binding buffer. Remove the stopper and connect the column to the syringe (use the connector supplied), laboratory pump, or chromatographic system "drop to drop" to avoid introducing air into the column.
2. Remove the snap-off end at the column outlet.
3. Equilibrate the column with 5 column volumes of binding buffer.
4. Apply the pretreated sample using a syringe fitted to the Luer connector or by pumping it onto the column. For best results, use a flow rate of 0.2 to 1 ml/min (1-ml column) and 0.5 to 5 ml/min (5-ml column) during sample application*.
5. Wash with 5 to 10 column volumes of binding buffer or until no material appears in the effluent. Maintain a flow rate of 1 to 2 ml/min (1-ml column) and 5 to 10 ml/min (5-ml column) for washing.
   Optional: Collect the flowthrough (in 1-ml fractions for the 1-ml column and 2-ml fractions for the 5-ml column) and reserve until the procedure has been successfully completed. Retain a sample for analysis by SDS-PAGE or by CDNB assay to measure the efficiency of protein binding to the medium.
6. Elute with 5 to 10 column volumes of elution buffer. Maintain a flow rate of 0.2 to 1 ml/min (1-ml column) and 0.5 to 5 ml/min (5-ml column) for elution.
7. After elution, regenerate the column by washing it with 3 to 5 column volumes of binding buffer. The column is now ready for a new purification.

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

Fig 57. Using a GSTrap column with a syringe. A) Prepare buffers and sample. Remove the column's top cap and snap off the end. B) Load the sample and begin collecting fractions. C) Wash and elute and continue collecting fractions.

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

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

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

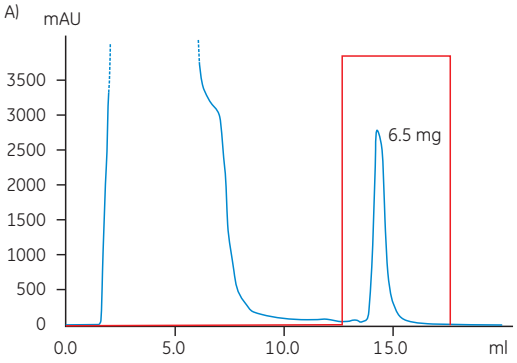
Application examples

1. High-performance purification of GST-hippocalcin using 1-ml and 5-ml GSTrap HP columns

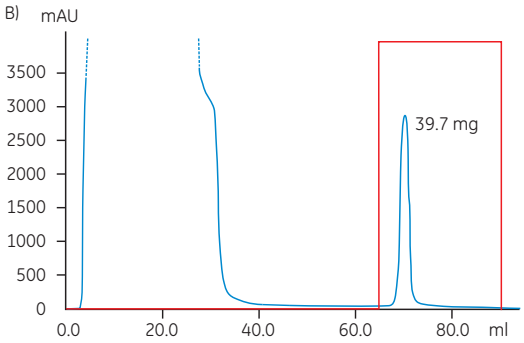
Glutathione Sepharose High Performance is easy to use for one-step purification of GST-tagged proteins. The following data shows the results from experiments using both GSTrap HP 1 ml and 5 ml.

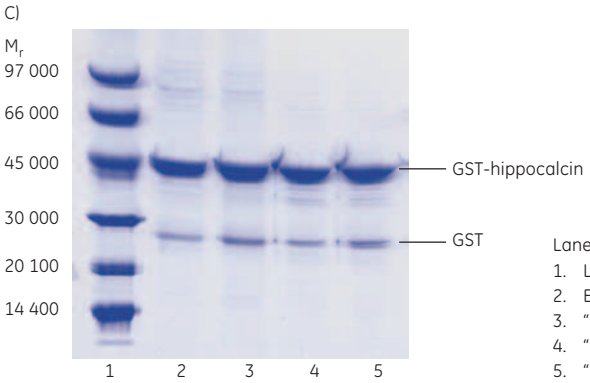
In this study, 5 ml and 25 ml of *E. coli* homogenate containing GST-hippocalcin was loaded on GSTrap HP 1-ml and 5-ml columns, respectively. Figure 58A–B shows the chromatograms from the two runs. The amount of protein in the eluted peaks was calculated as 6.5 mg and 39.7 mg, respectively.

The SDS-polyacrylamide gel shows GST-hippocalcin analyzed under nonreducing and reducing buffer conditions (Fig 58C). Each well was loaded with 10 µg of protein. The SDS-polyacrylamide gel also shows that free GST is expressed. The presence of reducing agent led to the removal of high-molecular-weight bands, which may correspond to GST-tagged protein that associated by oxidation of the free sulfhydryl groups.



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



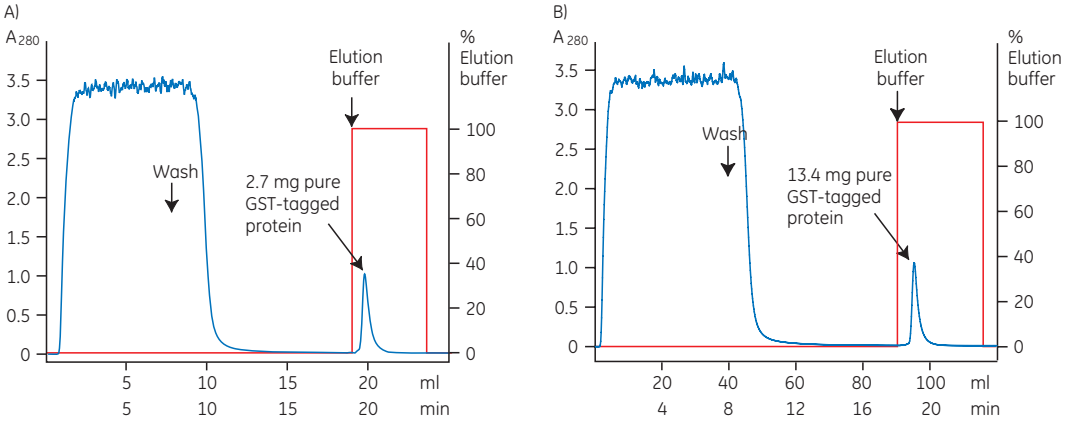


Lanes
1. LMW markers
2. Eluted pool from GSTrap HP 1 ml, nonreduced
3. " 5 ml, nonreduced
4. " 1 ml, reduced
5. " 5 ml, reduced

Fig 58. Scale-up from (A) GSTrap HP 1 ml to (B) GSTrap HP 5 ml. (C) Coomassie-stained reduced and nonreduced SDS-PAGE (ExcelGel SDS Gradient 8–18) of fractions from purification shown in Fig 58A–B.

2. Fast purification of a GST-tagged protein using GSTrap FF 1-ml and 5-ml columns

A GST-tagged protein was purified from 8 ml and 40 ml of a clarified cell lysate using GSTrap FF 1-ml and 5-ml columns, respectively. Samples were applied to columns preequilibrated with PBS (pH 7.3). After washing the columns with 10 column volumes of PBS, GST-tagged protein was eluted using reduced glutathione (Fig 59). Each run was completed in 25 min using ÄKTAexplorer 10. Analysis by SDS-PAGE indicated the isolation of highly pure GST-tagged protein (not shown). Yields of tagged proteins were 2.7 mg from GSTrap FF 1-ml and 13.4 mg from GSTrap FF 5-ml.



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

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

Fig 59. Purification of a GST-tagged protein on GSTrap FF 1-ml and 5-ml columns in combination with ÄKTAexplorer 10. Cytoplasmic extract (8 and 40 ml) from *E. coli* expressing a GST-tagged protein were applied to GSTrap FF 1-ml (A) and GSTrap FF 5-ml (B), respectively.

3. Two-step, automated purification using ÄKTAxpress

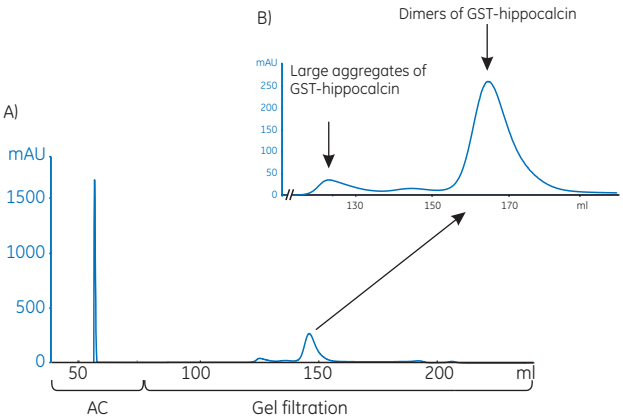
A two-step, automated purification of GST-hippocalcin from clarified *E. coli* lysate was performed on ÄKTAxpress. A GSTrap 4B 1-ml column was used in the first affinity chromatography capture step and a HiLoad 16/60 Superdex 200 pg column for the polishing step using gel filtration.

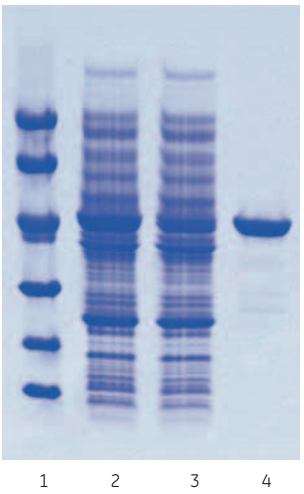
Reducing agent (DTT) was included in both sample and buffers. ÄKTAxpress enabled automated loading of eluted fractions of the target protein from the capture step (GSTrap 4B) onto the gel filtration column. Lysis of *E. coli* containing GST-hippocalcin was performed enzymatically followed by sonication. The lysate was clarified by centrifugation and filtration, and 5 ml of the clarified lysate was loaded on the 1-ml GSTrap 4B column. Chromatograms from the automated two-step purification, as well as SDS-PAGE of the eluted pool of target protein, are shown in Figure 60. Two peaks were obtained after gel filtration: one small and one large. According to SDS-PAGE (only the pool of the large peak is shown, Fig 60B), both peaks contained GST-hippocalcin. From evaluation of the gel filtration step, the large peak seemed to be the dimer of GST-hippocalcin. The small peak is possibly a larger aggregate of GST-hippocalcin. The purity of the GST-hippocalcin was good (Fig 60C).

Yield of eluted GST-hippocalcin, determined by absorbance at 280 nm using UNICORN software, was 6.4 mg.

This application shows the benefit of using a two-step purification for increasing the purity of GST-hippocalcin.

Columns:	GSTrap 4B, 1 ml HiLoad 16/60 Superdex 200 pg, 120 ml
Sample:	Clarified *E. coli* lysate containing expressed GST-hippocalcin, M_r 43 000
Sample volume:	5 ml (GSTrap 4B)
Binding buffer:	10 mM sodium phosphate, 140 mM NaCl, 20 mM DTT, pH 7.4
Elution buffer:	50 mM Tris-HCl, 20 mM glutathione, 20 mM DTT, pH 8.0
Buffer gel filtration:	10 mM sodium phosphate, 140 mM NaCl, 20 mM DTT, pH 7.4
Flow rate:	Sample loading, 0.3 ml/min (GSTrap 4B) Wash and elution, 1 ml/min (GSTrap 4B) 1.5 ml/min (HiLoad 16/60 Superdex 200 pg)
Running temperature:	22°C
System:	ÄKTAxpress





Lanes
1. LMW markers
2. Start material diluted 1:10
3. Flowthrough from affinity chromatography using GSTrap 4B, diluted 1:3
4. GST-hippocalcin pool from gel filtration, undiluted.

Fig 60. (A) Purification of GST-hippocalcin from *E. coli* lysate using an automated two-step purification on ÄKTAxpress. (B) Enlargement of the peak from the gel filtration step revealed large aggregates and dimers of purified GST-hippocalcin. (C) SDS-PAGE (ExcelGel SDS Gradient 8–18%) showing final purity of GST-hippocalcin (lane 4).

Purification of a GST-tagged protein using GSTrap FF 1 ml with ÄKTAprime plus

Refer to page 112, General considerations, before beginning this procedure.

This procedure uses a GSTrap FF 1-ml column but also can be used with GSTrap HP or GSTrap 4B 1-ml columns.

Sample preparation

The sample should be centrifuged and/or filtered through a 0.45-µm filter immediately before it is applied to the column. If the sample is too viscous, dilute it with binding buffer or buffer exchange using HiTrap Desalting, PD-10 Desalting, or HiPrep 26/10 Desalting to prevent clogging the column.

Buffer preparation

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

Binding buffer (port A1): 20 mM sodium phosphate, 0.15 M NaCl, pH 7.3

Elution buffer (port B): 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

Prepare at least 500 ml of eluent.

1 to 20 mM DTT may be included in the binding and elution buffers to increase the purity. However, this may result in lower yield of GST-tagged protein.

System preparation

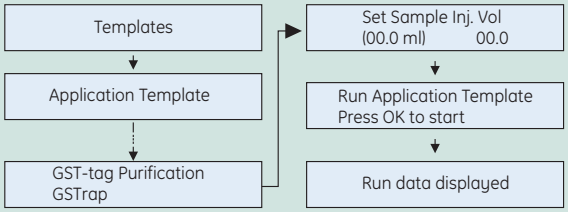
Once the system is prepared, the remaining steps (under Selecting Application Template and starting the method) will be performed automatically.

1. Place each inlet tubing from port A (8-port valve) in the binding buffer and the tubing from port B (2-port valve) in the elution buffer.
2. Place the three brown waste tubings in waste.
3. Connect the column between port 1 on the injection valve (7-port valve) and the UV flow cell.
4. Fill the fraction collector rack with 18-mm tubes (minimum 10) and position the white plate on the fractionation arm against the first tube.
5. Connect a sample loop large enough for your sample between port 2 and 6 on the injection valve. Use a syringe to manually fill the loop.

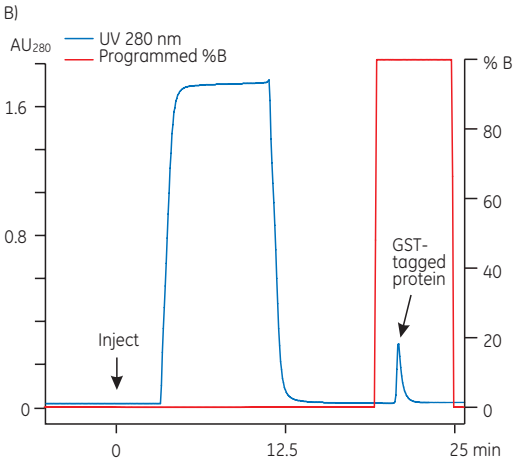
   Note: If a Superloop is needed, additional information is supplied in the instructions for Superloop.

Selecting Application Template and starting the method

1. Check the communication to PrimeView. At the lower right corner of the screen the text **Controlled By:** prime should be displayed.
2. Use the arrow and OK buttons to move in the menu tree until you find **GST-tag Purification GSTrap.**



3. Enter the sample volume and press **OK** to start the template.



Sample:	Clarified homogenate of *E. coli* expressing GST-tagged protein
Column:	GSTrap FF 1 ml
Binding buffer (port A1):	20 mM sodium phosphate, 0.15 M NaCl, pH 7.3
Elution buffer (port B):	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0

Fig 61. (A) Theoretical gradient in **GST-tag Purification GSTrap template**. Total separation time = 37 min + sample application time. (B) Typical results for purification of a GST-tagged protein.

Preparative purification using GSTPrep FF 16/10 column

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

Fig 62. GSTPrep FF 16/10 column.

The column is made of polypropylene, which is biocompatible and noninteractive with biomolecules. Separations can be easily achieved using a chromatography system such as ÄKTAdesign. Refer to Table 8 for a selection guide to purification equipment and to Appendix 2 for a list of GSTPrep FF 16/10 column parameters.

Glutathione Sepharose 4 Fast Flow is also available as prepacked 1-ml and 5-ml GSTrap FF columns, as prepacked 96-well filter plates, GST MultiTrap FF, and as a bulk medium in lab packs (25, 100, and 500 ml) for packing columns or batch purifications. Note that GSTPrep FF 16/10 columns cannot be opened or refilled.

Sample preparation

Refer to page 26 for a general procedure for sample preparation.

The sample should be centrifuged and/or filtered through a 0.22-µm or a 0.45-µm filter immediately before it is applied to the column. If the sample is too viscous, dilute it with binding buffer or buffer exchange using HiTrap Desalting, PD-10 Desalting, or HiPrep 26/10 Desalting to prevent clogging the column.

Buffer preparation

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

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

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

1 to 20 mM DTT may be included in the binding and elution buffers to increase the purity. However, this may result in lower yield of GST-tagged protein.

Purification

1. Apply the centrifuged and/or filtered sample (in binding buffer) to the column at a flow rate of 1 to 5 ml/min (30 to 150 cm/h).
2. Wash the column with 100 to 200 ml of binding buffer at 2 to 10 ml/min (60 to 300 cm/h).
3. Elute the bound protein with 100 to 200 ml of elution buffer at a flow rate of 2 to 10 ml/min (60 to 300 cm/h).
4. Equilibrate the column with 60 to 100 ml of binding buffer at a flow rate of 2 to 10 ml/min (60 to 300 cm/h). The column is now ready for a new purification.

Due to the relatively slow binding kinetics between GST and glutathione, it is important to keep the flow rate low during sample loading/elution. The binding capacity may be different for different proteins. The yield may therefore vary between proteins if sample load is close to the capacity of the column.

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

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

For cleaning, storage, and handling information, refer to Appendix 2.

Application example

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

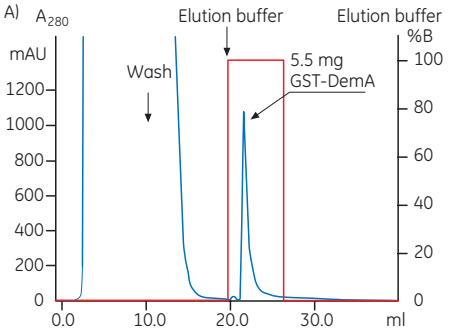
Glutathione Sepharose 4 Fast Flow is easy to use for one-step purification of GST-tagged proteins. Figures 63A-C and 64A-C show scale-up studies on GSTrap FF 1 ml, GSTrap FF 5 ml, and GSTPrep FF 16/10. Two different GST tagged proteins were purified: GST-DemA and GST-Purα. The gene encoding for DemA was isolated from *Streptococcus dysgalactiae*. DemA is a fibrinogen-binding protein that shows both plasma protein binding properties and sequence similarities with the M and M-like proteins of other streptococcal species. Purα has been shown to be involved in transcriptional regulation.

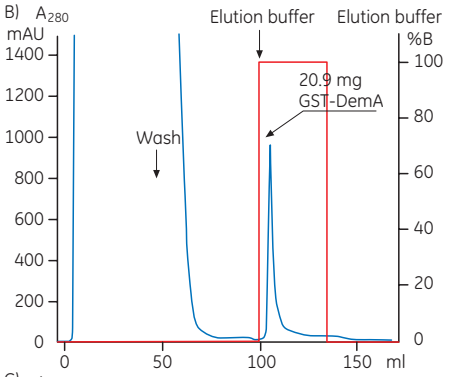
E. coli expressing the GST-tagged proteins was resuspended (1 g/5 ml) in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) supplemented with 1 mM PMSF, 1 mM DTT, 100 mM $MgCl_2$, 1 U/ml RNase A and 13 U/ml DNase I. The cells were lysed by sonication with a Vibracell™ ultrasonic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

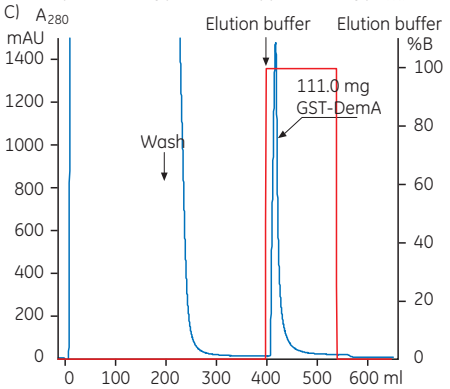
The following purification procedures were performed using ÄKTAexplorer 100 chromatography system. The columns, GSTrap FF 1 ml, GSTrap FF 5 ml, and GSTPrep FF 16/10 were equilibrated with 5 column volumes (CV) of PBS, pH 7.4 and the prepared sample was applied to the columns. The columns were washed with 10 CV of PBS (GST-DemA) and 20 CV of (GST-Purα) and eluted using 7 CV of Tris-HCl, pH 8.0 including 10 mM reduced glutathione. The purity of eluted proteins was analyzed by SDS-PAGE (see Figs 63D and 64D).

The main parameter in this scale-up study was the residence time (i.e., the period of time the sample is in contact with the medium). The residence time was the same for the GSTrap FF 1-ml and 5-ml columns whereas it was twice as long for the GSTPrep FF 16/10 column (20-ml column volume) compared with GSTrap FF 5-ml columns due to the difference in column length vs. column diameter. The amount of

protein bound differed between GST-DemA and GST-Purα, due to protein dependent binding characteristics. Some of the applied protein was found in the flowthrough as an effect of the low turnover rate number of GST. The amount of eluted GST-tagged proteins increased proportionally with increased column volume and sample load.







A) GSTrap FF 1 ml

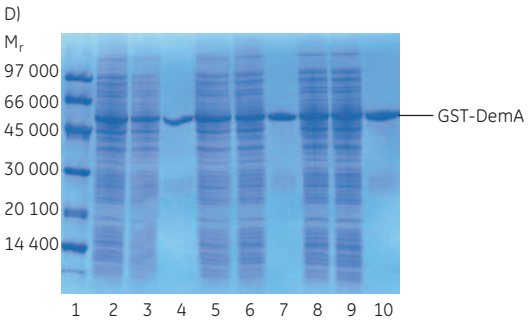
Column:	GSTrap FF 1 ml
Sample:	10 ml extract from *E. coli* expressing GST-DemA
Binding buffer:	PBS (pH 7.4)
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 0.5 ml/min Washing and elution: 1 ml/min
Chromatographic procedure:	5 CV (CV= Column Volume) binding buffer, 10 ml sample, 10 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTAexplorer 100

B) GSTrap FF 5 ml

Column:	GSTrap FF 5 ml
Sample:	50 ml extract from *E. coli* expressing GST-DemA
Binding buffer:	PBS (pH 7.4)
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 2.5 ml/min Washing and elution: 5 ml/min
Chromatographic procedure:	5 CV binding buffer, 50 ml sample, 10 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTAexplorer 100

C) GSTPrep FF 16/10

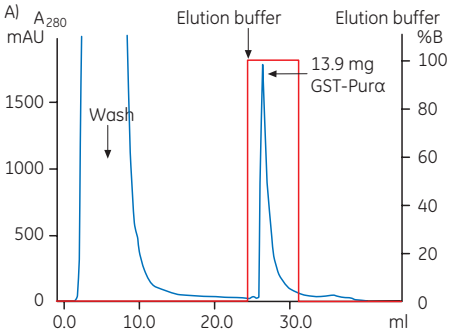
Column:	GSTPrep FF 16/10
Sample:	200 ml extract from *E. coli* expressing GST-DemA
Binding buffer:	PBS (pH 7.4)
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 5 ml/min Washing and elution: 10 ml/min
Chromatographic procedure:	5 CV binding buffer, 200 ml sample, 10 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTAexplorer 100



Lanes

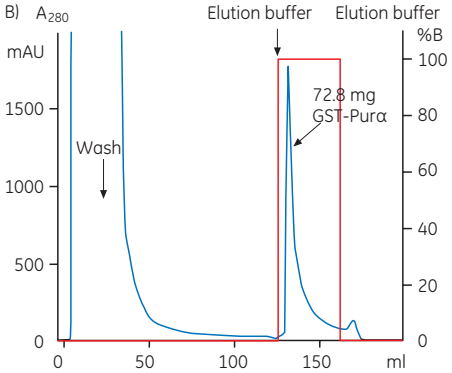
1. LMW markers, reduced
2. Extract of *E. coli* expressing GST-DemA, 1 g cell paste/5 ml
3. Flowthrough from GSTrap FF 1 ml
4. GST-DemA eluted from GSTrap FF 1 ml
5. Extract of *E. coli* expressing GST-DemA, 1 g cell paste/5 ml
6. Flowthrough from GSTrap FF 5 ml
7. GST-DemA eluted from GSTrap FF 5 ml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

Fig 63. Purification and scale-up of GST-DemA on (A) GSTrap FF 1 ml, (B) GSTrap FF 5 ml, and (C) GSTPrep FF 16/10. (D) SDS-PAGE analysis of GST-DemA on ExcelGel Homogeneous 12.5% using Multiphor™ II followed by Coomassie staining. Due to the relatively slow kinetics of low turnover rate number of GST and rather high load, some of the applied protein was found in the flowthrough.



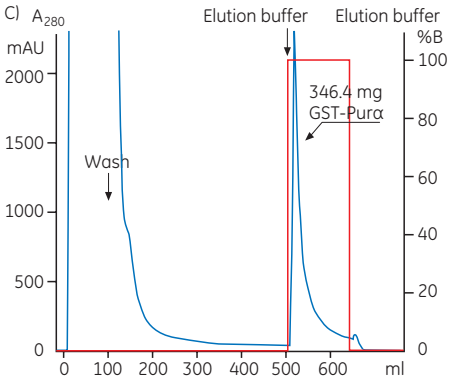
A) GSTrap FF 1 ml

Column:	GSTrap FF 1 ml
Sample:	5 ml extract from *E. coli* expressing GST-Purα
Binding buffer:	PBS (pH 7.4)
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 0.5 ml/min Washing and elution: 1 ml/min
Chromatographic procedure:	5 CV binding buffer, 5 ml sample, 20 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTAexplorer 100



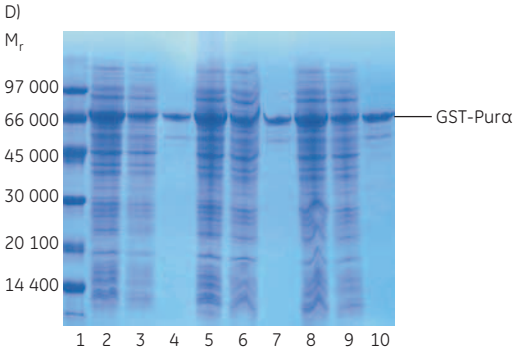
B) GSTrap FF 5 ml

Column:	GSTrap FF 5 ml
Sample:	25 ml extract from *E. coli* expressing GST-Purα
Binding buffer:	PBS (pH 7.4)
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 2.5 ml/min Washing and elution: 5 ml/min
Chromatographic procedure:	5 CV binding buffer, 25 ml sample, 20 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTAexplorer 100



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

Column:	GSTPrep FF 16/10
Sample:	100 ml extract from *E. coli* expressing GST-Purα
Binding buffer:	PBS (pH 7.4)
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow rate:	Sample loading: 5 ml/min Washing and elution: 10 ml/min
Chromatographic procedure:	5 CV binding buffer, 100 ml sample, 20 CV binding buffer, 7 CV elution buffer, 5 CV binding buffer
System:	ÄKTAexplorer 100



Lanes

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

Fig 64. Purification and scale-up of GST-Purα on (A) GSTrap FF 1 ml, (B) GSTrap FF 5 ml, and (C) GSTPrep FF 16/10. (D) SDS-PAGE analysis of GST-Purα on ExcelGel Homogeneous 12.5% using Multiphor II followed by Coomassie staining. Due to the low turnover rate number of GST, some of the applied protein was found in the flowthrough.

Troubleshooting of purification methods

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

Problem	Possible cause	Solution
GST-tagged protein does not bind or binds very poorly.	GST-tagged protein denatured by mechanical lysis (e.g., sonication). Too extensive lysis can denature the tagged protein and prevent it from binding.	Use mild mechanical/chemical lysis conditions during cell lysis. Conditions for lysis must be empirically determined.
	GST-tagged proteins have aggregated in the sample, causing precipitation.	Add DTT to the sample prior to cell lysis and also add DTT to the buffers. Adding DTT to a final concentration of 1 to 20 mM may significantly increase binding of some GST-tagged proteins.
	Concentration of tagged protein is too low.	Concentrate the sample. The binding capacity is concentration dependent. Low expressed proteins may not bind as efficiently as highly expressed proteins, therefore concentrating the sample may improve binding.
	The tagged protein may have altered the conformation of GST, thereby reducing the affinity for the GST-tagged protein.	Test the binding of GST from parental pGEX: Prepare a sonicate of cells harboring the parental pGEX plasmid and check binding to the medium. If GST produced from the parental plasmid binds with high affinity, the tagged protein may have altered the conformation of GST, thereby reducing the affinity for the GST-tagged protein. Adequate results may be obtained by reducing the temperature used for binding to 4°C, and by limiting washing.
	Equilibration time is too short.	Ensure that the column has been equilibrated with at least 5 column volumes of a buffer pH 6.5 to 8.0 (e.g., PBS).
	Binding of GST-tagged proteins is not efficient at pH less than 6.5 or greater than 8.	Equilibrate with a buffer pH 6.5 to 8.0 (e.g., PBS) before the clarified cell lysate is applied.
	GSTrap column: Column needs cleaning.	Clean the column according to the standard cleaning protocol (see Appendix 2). If the GSTrap column has already been used several times, it may be necessary to use a new one.
	Glutathione Sepharose medium has been used too many times.	Use fresh Glutathione Sepharose medium. See also cleaning procedures in Appendix 2.
	The flow rate used during sample loading is too high.	Decrease the flow rate during sample loading. One of the most important parameters affecting the binding of GST-tagged proteins to Glutathione Sepharose is the flow rate. Due to the relatively slow binding kinetics between GST and glutathione, it is important to keep the flow rate low during sample loading for maximum binding capacity.

continues on following page

Problem	Possible cause	Solution
	GSTrap columns on ÄKTAprime plus: The column or system is clogged, leading to high back pressure and no binding.	Clogged column: Clean the column according to instructions. Make sure the sample has been centrifuged and/or filtered through a 0.45 µm filter. Clogged system: Replace the column with a piece of tubing. Check pressure. If back pressure > 0.3 MPa, clean system according to manual.
	GSTrap columns on ÄKTAprime plus: The sample does not bind.	Check that the correct column is used. Check that the inlet tubing from each buffer is connected to the correct inlet port. Check that the composition and pH of the buffers are correct. Check that the sample has been adjusted to binding buffer conditions.
GST-tagged protein is not eluted efficiently.	The volume of elution buffer is insufficient.	Increase the volume of elution buffer used. In some cases, especially after on-column cleavage of a tagged protein, a larger volume of buffer may be necessary to elute the tagged protein.
	The time allowed for elution is insufficient.	Increase the time used for elution by decreasing the flow rate during elution. With GSTrap columns, for best results use a flow rate of 0.2 to 1 ml/min (1-ml HiTrap column) and 0.5 to 5 ml/min (5-ml HiTrap column) during sample application. For centrifugation methods, decrease the centrifugation speed during elution.
	The concentration of glutathione is insufficient.	Increase the concentration of glutathione in the elution buffer: The 10 mM recommended in this protocol should be sufficient for most applications, but exceptions exist. Try 50 mM Tris-HCl, 20 to 40 mM reduced glutathione, pH 8.0 as elution buffer.
	The pH of the elution buffer is too low.	Increase the pH of the elution buffer: Increasing the pH of the elution buffer to pH 8 to 9 may improve elution without requiring an increase in the concentration of glutathione used for elution.
	The ionic strength of the elution buffer is too low.	Increase the ionic strength of the elution buffer: Adding 0.1 to 0.2 M NaCl to the elution buffer may also improve results.
	The glutathione in the elution buffer is oxidized.	Use fresh elution buffer. Add DTT.
	Nonspecific hydrophobic interactions cause nonspecific interaction with the medium or aggregation of proteins, preventing solubilization and elution of tagged proteins.	Add a nonionic detergent to the elution buffer: Adding 0.1% Triton X-100 or 2% n-octylglucoside can significantly improve elution of some GST-tagged proteins.
	GSTrap columns on ÄKTAprime plus: Poor elution.	Check that the inlet tubing from each buffer is connected to the correct inlet port. Check that the composition and pH of the buffers are correct. Use alternative elution conditions according to the column instructions.

continues on following page

Problem	Possible cause	Solution
Multiple bands are observed after electrophoresis/Western blotting analysis of eluted target protein.	M_r 70 000 protein copurifies with the GST-tagged protein.	The M_r 70 000 protein is probably a protein product of the *E. coli* gene dnaK. This protein is involved in protein folding in *E. coli*. It has been reported that this association can be disrupted by incubating the tagged protein in 50 mM Tris-HCl, 2 mM ATP, 10 mM $MgSO_4$, pH 7.4 for 10 min. at 37°C prior to loading. Alternatively, remove the DnaK protein by passing the tagged protein solution through ATP-agarose or a similar purification medium, or perform ion exchange.
	Partial degradation of tagged proteins by proteases.	Add a protease inhibitor: Multiple bands may be a result of partial degradation of tagged proteins by proteases. Adding 1 mM PMSF to the lysis solution may improve results. A nontoxic, water-soluble alternative to PMSF is 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), commercially available as Pefabloc SC from Roche Biochemicals. *Note:* Serine protease inhibitors must be removed prior to cleavage by thrombin or Factor Xa. PreScission Protease is not a consensus serine protease and is insensitive to many of the protease inhibitors tested at GE Healthcare.  PMSF is toxic, with acute effects. Use Pefabloc whenever possible.
	Proteolysis in the host bacteria.	Use a protease-deficient host: Multiple bands may be the result of proteolysis in the host bacteria. If this is the case, the use of a host-deficient strain may be required (e.g., *lon*- or *ompT*). *E. coli* BL21 is provided with the pGEX vectors. This strain is defective in *OmpT* and *Lon* protease production.
	Cell disruption during mechanical lysis.	Decrease lysis time: Cell disruption is apparent by partial clearing of the suspension and can be checked by microscopic examination. Adding lysozyme (0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris-HCl, pH 8.0) prior to mechanical lysis may improve results. Avoid frothing as this may denature the tagged protein. Over-lysis can also lead to the copurification of host proteins with the GST-tagged protein.

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Problem	Possible cause	Solution
	Chaperonins may have copurified.	Include an additional purification step: Additional bands may be caused by the copurification of a variety of proteins known as chaperonins, which are involved in the correct folding of nascent proteins in *E. coli*. These include, but may not be limited to: DnaK (M_r ~ 70 000), DnaJ (M_r ~ 37 000), GrpE (M_r ~ 40 000), GroEL (M_r ~ 57 000), and GroES (M_r ~10 000). Several methods for purifying GST-tagged proteins from these copurifying proteins have been described.
	Antibodies that react with various *E. coli* proteins may be present in the tagged protein sample.	Cross-adsorb antibody with *E. coli* proteins: Depending on the source of the anti-GST antibody, it may contain antibodies that react with various *E. coli* proteins that may be present in the tagged protein sample. Cross-adsorb the antibody with an *E. coli* sonicate to remove anti-*E. coli* antibodies from the preparation. Anti-GST antibody from GE Healthcare has been cross-adsorbed against *E. coli* proteins and tested for its lack of nonspecific background binding in Western blots.
Multiple bands are observed after electrophoretic analysis of cleaved target protein.	Proteolysis has occurred in the host bacteria.	Determine when the bands appear: Test to be certain that additional bands are not present prior to PreScission Protease, thrombin, or Factor Xa cleavage. Such bands may be the result of proteolysis in the host bacteria. Tagged partner may contain recognition sequences for PreScission Protease, thrombin, or Factor Xa: Check the sequences. See the GST Gene Fusion System Handbook for details.

Detection of GST-tagged proteins

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

Much of the information presented below can also be applied to detection of histidine-tagged proteins, although the specific reagents will change.

GST 96-Well Detection Module for ELISA

The GST 96-Well Detection Module provides a highly sensitive enzyme-linked immunosorbent assay (ELISA) for testing clarified lysates and intermediate purification fractions for the presence of GST-tagged proteins (see Figs 65 and 66). Samples are applied directly into the wells of the plates, and GST-tagged proteins are captured by specific binding to anti-GST antibody that is immobilized on the walls of each well. After removal of unbound material by washing, the captured GST-tagged proteins are then detected with HRP/Anti-GST conjugate provided in the module. Standard curves for quantitation of tagged proteins can be constructed using purified recombinant GST, which is included as a control.

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

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

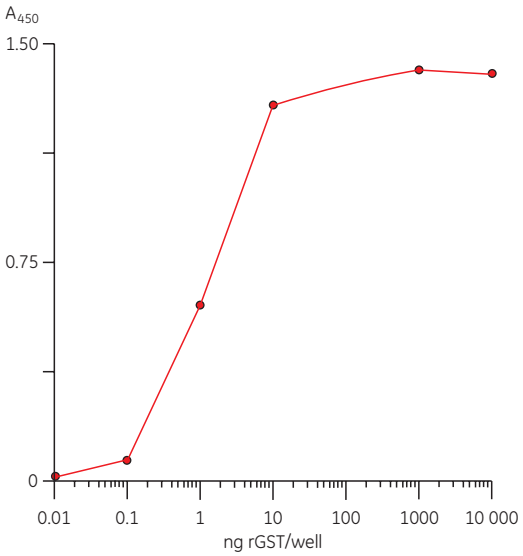


Fig 65. Sensitive detection of recombinant GST using the GST 96-Well Detection Module. Recombinant GST protein was prepared in 1× blocking buffer, and 100 µl volumes were applied directly to the wells of a GST 96-well capture plate. After 1 h binding at room temperature, the wells were washed in wash buffer and incubated with a 1:1000 dilution of HRP/Anti-GST conjugate for 1 h. Detection was performed using 3, 3',5,5'-tetramethyl benzidine (TMB) substrate, and the absorbance of each well was measured at 450 nm.

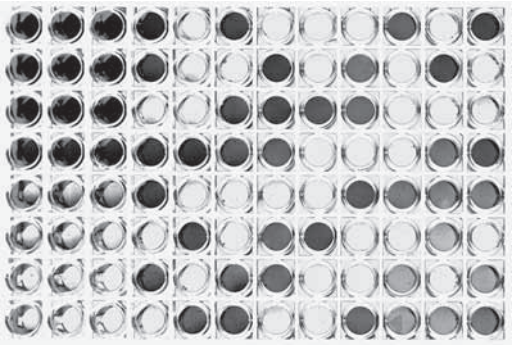


Fig 66. Screening of bacterial lysates for GST-tagged protein expression using the GST 96-Well Detection Module.

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

Prepare fresh buffers daily.

Components of GST 96-Well Detection Module

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

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

Purified recombinant GST standard protein

Additional reagents required for ELISA

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

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

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

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

Substrate

Procedure

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

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

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

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

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

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

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

    **3,3',5,5'-tetramethyl benzidine (A_{450}) and 2',2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS™) (A_{410}) have been used successfully.*
16. Read plate absorbance in a microplate reader or spectrophotometer.

GST Detection Module with CDNB enzymatic assay

GST-tagged proteins produced using pGEX vectors can be detected enzymatically using the GST substrate 1-chloro-2,4 dinitrobenzene (CDNB), included in the GST Detection Module. The GST-mediated reaction of CDNB with glutathione produces a conjugate that is measured by absorbance at 340 nm using either a plate reader or a UV/vis spectrophotometer. Assay results are available in less than 10 min for crude bacterial sonicates, column eluates, or purified GST-tagged protein. Figure 67 shows typical results from a CDNB assay. Each GST Detection Module contains reagents sufficient for 50 assays.

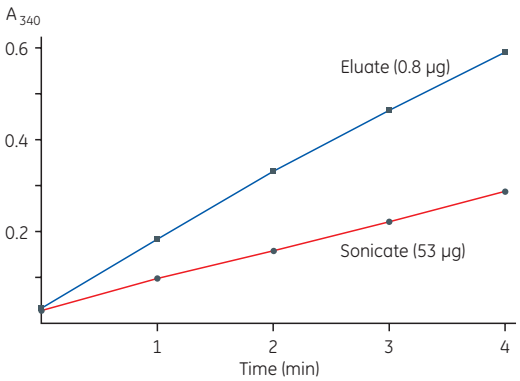


Fig 67. Typical results of a CDNB assay for GST-tagged proteins. 53 µg of total protein from an *E. coli* TG1/pGEX-4T-Luc sonicate and 0.8 µg of total protein eluted from Glutathione Sepharose were assayed according to instructions included with the GST Detection Module.

Components of GST Detection Module used with the CDNB enzymatic assay

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

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

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

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

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

Steps

1. In a microcentrifuge tube, combine the following:

Distilled water	880 µl
10× reaction buffer	100 µl
CDNB	10 µl
Glutathione solution	10 µl
Total volume	1000 µl

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

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

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

For analyses using 96-well plates, add samples to the cells first and add reagents second. Mix the content of the wells using the pipette. Start measuring the absorbance in the plate reader.

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

Calculations

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

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

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

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

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

The assay detects active GST. Additional GST-tagged protein may be present that is not active.

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

Western blot

Expression and purification of GST-tagged proteins can be monitored by Western blot analysis, using ECL, ECL Plus, or ECL Advance detection systems to enhance sensitivity. The combination of Western blot detection and total protein staining of the SDS-PAGE (Coomassie or silver staining) gives a powerful control of purification results.

Reagents required

Anti-GST antibody (goat polyclonal)

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

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

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

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

1. Separate the protein samples by SDS-PAGE.

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

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

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

Blocking of membrane

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

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

Incubation of membrane blot with primary antibody

1. Prepare an appropriate dilution of anti-GST antibody with blocking/incubation buffer (e.g., 5 to 10 µl of antibody in 50 ml of buffer).
2. Pour the antibody-buffer mixture into the container with the membrane.
3. Incubate for 1 h at ambient temperature with gentle shaking.
4. Decant and discard the antibody-buffer.
5. Rinse the membrane twice with 20 to 30 ml of wash buffer to remove most of the unbound antibody.
6. Decant and discard the rinse buffers.
7. Wash the membrane with 20 to 30 ml of blocking/incubation or wash buffer for 10 to 60 min at ambient temperature with gentle shaking.
8. Discard the buffer and repeat the wash from step 7.

Incubation of membrane blot with secondary antibody

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

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

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

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

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

GST Western Blotting Detection Kit

The GST Western Blotting Detection kit facilitates the detection of GST-tagged proteins on Western blots using chemiluminescence. This method may be used for crude bacterial sonicates, column eluates, or purified GST-tagged proteins. Unlike biochemical assays, this method is not dependent on the functional activity of GST, which can be affected by folding of the tagged protein.

Components of GST Western Blotting Detection Kit

Anti-GST-HRP conjugate: Horseradish peroxidase (HRP) conjugated to goat anti-GST polyclonal antibody. 75 µl supplied in PBS containing 50% glycerol.

rGST positive control: recombinant Glutathione S-transferase. 10 µl supplied at 5 mg/ml protein concentration in PBS.

ECL blocking agent: 20 g.

ECL detection reagents: detection reagent 1 (125 ml), detection reagent 2 (125 ml), sufficient for 2000 cm^2 membrane.

Additional reagents required

Phosphate-buffered saline (PBS), pH 7.5: For 1 l, dissolve 11.5 g sodium hydrogen orthophosphate anhydrous (80 mM), 2.96 g sodium dihydrogen orthophosphate (20 mM), and 5.84 g NaCl (100 mM) in 1 l of distilled water. Adjust pH.

Tris-buffered saline (TBS), pH 7.6: For 1 l add 20 ml 1 M Tris-HCl, pH 7.6 (20 mM) and 8 g NaCl to 1000 ml of distilled water. Adjust pH.

PBS-Tween (PBST) and TBS-Tween (TBST) Dilute the required amount of Tween 20 in the corresponding buffer. A 0.1% Tween 20 concentration is suitable for most blotting applications.

Do not use sodium azide as a bacteriocide as it inhibits horseradish peroxidase enzymes.

Gel electrophoresis

1. Perform SDS-PAGE according to standard techniques
2. Load 100 ng of the recombinant GST (included in the kit) as a positive control.

This conjugate has been used with both purified proteins and crude bacterial lysates. The anti-GST antibody has been cross-absorbed against *E. coli* proteins. However, this process may not remove all cross-reacting antibodies. We suggest that a sample of an *E. coli* lysate made from a culture that does not contain a pGEX plasmid be run as a control.

Western blotting

1. Transfer the proteins onto a membrane using standard protocols.

2a. Prewet Hybond ECL in distilled water and equilibrate in transfer buffer for 5 to 10 min before blotting.

2b. Briefly immerse Hybond PVDF in 100% methanol then rinse in distilled water before equilibrating in transfer buffer and blotting.

Nitrocellulose membrane is recommended with this protocol. However, PVDF membrane has been shown to give comparable results.

Immunodetection

1. Block nonspecific binding sites by immersing the membrane in 5% ECL blocking agent in PBST or TBS for 1 h on a platform shaker at room temperature.
2. Briefly rinse the membrane in two changes of PBST or TBST wash buffer.
3. Dilute the anti-GST-HRP conjugate in PBST or TBST. A 1:5000 dilution has been found to be satisfactory for many applications. Allow sufficient antibody solution to cover the membrane (at least 0.25 ml/cm^2 membrane). Incubate the membrane in diluted conjugate for 1 h at room temperature on a platform shaker.
4. Briefly rinse the membrane in two changes of PBST or TBST wash buffer. Wash the membrane in > 4 ml/cm^2 of wash buffer for 15 min at room temperature with gentle shaking.
5. Wash the membrane for 3 × 5 min with fresh changes of wash buffer at room temperature with gentle shaking.

This protocol has been optimized to provide good signal-to-noise ratios, resulting in intense signal and clean backgrounds. Users may find sensitivity is improved by increasing the concentration of conjugate used; however, this may result in increased background noise.

ECL detection reagents are supplied with this kit. However, ECL Plus detection reagents have also been used. When using ECL Plus reagents, increase conjugate dilution two-to-four-fold to reduce background noise to an acceptable level.

ECL detection

1. Prepare the ECL detection reagents by mixing an equal volume of solution 1 with solution 2.

Allow sufficient volume to cover the membrane (at least 0.125 ml/cm^2 is recommended). Although the mixed reagents are stable for 1 h at room temperature, it is advisable to mix the reagents immediately before use.

2. Drain the excess wash buffer from the washed membrane and place protein side up on a sheet of plastic wrap or other suitable clean surface. Pipette the mixed reagents onto the membrane and incubate for 1 min.
3. Work quickly once the membrane has been exposed to detection reagents. Drain off excess reagents by blotting the edge of the membrane on a tissue. Place the membrane on a fresh piece of plastic wrap, protein side down. Wrap the membrane, taking care to gently smooth out any air bubbles.
4. Place the wrapped membrane protein side up in an X-ray film cassette.
5. Complete further stages in a dark room using red safe lights. Place a sheet of autoradiography film on top of the membrane. Close the cassette and expose for 1 min.
6. Remove the film and replace with a second sheet of unexposed film. Develop the first piece of film immediately. Dependent on the appearance of the first film, estimate the exposure time for the second piece of film. This may vary from 5 min to 1 h.

SDS-PAGE with Coomassie blue or silver staining

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

Reagents required

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

Gel electrophoresis

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

The percentage of acrylamide in the SDS-polyacrylamide gel should be selected based on the expected molecular weight of the protein of interest (see Table 19).

Table 19. Selecting the appropriate gel composition for protein separation

Percent acrylamide in resolving gel		Separation size range (M_r ×10^3)
Single percentage	5%	36–200
	7.5%	24–200
	10%	14–200
	12.5%	14–100
	15%	14–60[1]
Gradient	5–15%	14–200
	5–20%	10–200
	10–20%	10–150

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

Troubleshooting of detection methods

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

Problem	Possible cause	Solution
Poor results with the GST Detection Module	The reaction rate is nonlinear.	The reaction rate of the CDNB assay is linear provided that an A_{340} of ~ 0.8 is not exceeded during the 5-min time course. Plot initial results to verify that the reaction rate is linear over the time course. Adjust the amount of sample containing the GST-tagged protein to maintain a linear reaction rate.
	The tagged protein is folded.	The tagged protein may have inhibited the correct folding of the GST moiety. The GST-tagged proteins will thus show very low activity with the CDNB assay. Whether for this or for any other reason, if a low absorbance is obtained using the CDNB assay, a Western blot using anti-GST antibody may reveal high levels of tagged protein expression.
	There is baseline drift.	Under standard assay conditions at 22°C and in the absence of GST, glutathione and CDNB react spontaneously to form a chemical moiety that produces a baseline drift at ΔA340 /min of ~ 0.003 (or 0.015 in 5 min). Correct for baseline drift by blanking the spectrophotometer with the blank cuvette before each reading of the sample cuvette. Alternatively, get slope directly from the spectrophotometer software. The slope will be the same as long as the spontaneous reaction is limited.
Poor results with the GST 96-Well Detection Module	Low absorbance is seen in the assay.	Check that host cells were sufficiently induced, that the samples were sufficiently lysed, and that inclusion bodies have not been formed. (See Troubleshooting purification methods).
	Concentration of blocking buffer is inadequate.	If clarified lysate is being tested, mix the initial GST sample with 2× blocking buffer to give a final concentration of 1× blocking buffer.
	There is poor day-to-day reproducibility.	Verify that all incubation times are consistent. GST capture incubation time can be decreased with slightly reduced signal, but do not incubate for less than 30 min. Every 15-min decrease in HRP/anti-GST conjugate incubation time can significantly reduce signal.

continues on following page

Problem	Possible cause	Solution
No signal in Western blotting	Proteins are not transferred during Western blotting.	Stain gel and membrane with total protein stain to check transfer efficiency. Optimize gel acrylamide concentration, time for transfer, and current.
		Ensure gel and membrane make proper contact during blotting and are orientated correctly with respect to the anode.
		Check that excess temperatures are not reached during electroblotting, producing bubbles or membrane distortion.
	Proteins are not retained on membrane.	Assess transfer of proteins (as above). Use a fresh supply of membrane.
	There are problems with detection reagents.	Ensure reagents are being used correctly. Prepare reagents freshly each time. Store reagents at correct temperature.
Weak signal in Western blotting	Protein transfer efficiency is poor.	Check transfer efficiency as above.
	Insufficient protein has been loaded.	Load more protein on gel.
	Exposure time is too short.	Increase film exposure time; up to 1 h may be required.
	Conjugate concentration is too low.	A 1:5000 dilution is recommended but a more concentrated solution may be required for some applications—try 1:1000.
Excessive diffuse signal in Western blotting	Too much protein has been loaded.	Reduce the amount of protein loaded.
	Conjugate concentration is too high.	A 1:5000 dilution is recommended, but a more dilute solution may be required for some applications—try 1:10 000.
High backgrounds in Western blotting	Washing is inadequate.	Ensure post conjugate washes are performed for a sufficient amount of time with an adequate volume of wash buffer (> 4 ml/cm^2 membrane).
	Blocking is inadequate.	Check the blocking buffer has been made correctly. Use freshly prepared blocking buffer each time.
		Increase the concentration of blocking reagent—try 10%.
		Use alternative blocking agent (e.g., 1% to 10% BSA, 0.5% to 3% gelatin.
		Increase incubation time with blocking buffer.
	Blotting equipment or buffers are contaminated.	Clean equipment. Prepare fresh buffers.
	Conjugate concentration is too high.	A 1:5000 dilution is recommended but further dilution may be required for some applications.
Multiple bands are seen in Western blotting	Conjugate is binding nonspecifically to other proteins.	Include a negative control of expression host not containing expression vector to determine nonspecific binding.

Removal of GST tag by enzymatic cleavage

Removal of the GST tag is often necessary to be able to perform functional or structural studies of the target protein. Tagged proteins containing a PreScission Protease, thrombin, or Factor Xa recognition site can be cleaved either while bound to Glutathione Sepharose or in solution after elution.

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

If thrombin or Factor Xa are used for cleavage of the tag, a convenient way to remove these enzymes is to connect in series one GSTrap FF column and one HiTrap Benzamidine FF (high sub) column. During the elution the cleaved product passes directly from the GSTrap into the HiTrap Benzamidine FF (high sub). The cleaved target protein passes through the HiTrap Benzamidine FF (high sub) column but the proteases bind. Thus in a single step the enzymes are removed and a pure cleaved target protein is achieved (see Fig 68 on following page). Note, however, that thrombin and Factor Xa may produce a less specific cleavage than PreScission Protease and that sometimes the target protein can be fragmented itself.

Table 20. Approximate molecular weights for SDS-PAGE analysis.

Protease	Molecular weight
PreScission Protease[1]	46 000
Bovine thrombin	37 000
Bovine Factor Xa	48 000

[1] PreScission Protease is a fusion protein of glutathione S-transferase and human rhinovirus type 14 3C protease.

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

If protease inhibitors (see Table 21) have been used in the lysis solution, they must be removed prior to cleavage with PreScission Protease, thrombin, or Factor Xa. (The inhibitors will usually be eluted in the flowthrough when sample is loaded onto a GSTrap column.)

Table 21. Inhibitors of the various proteases.

Enzyme	Inhibitor
PreScission Protease	100 mM $ZnCl_2$ (> 50% inhibition) 100 µM chymostatin 4 mM Pefabloc
Factor Xa and thrombin	AEBSF, APMSF, antithrombin III, Antipain, α_1-antitrypsin, aprotinin, chymostatin, hirudin, leupeptin, PMSF
Factor Xa only	Pefabloc FXa
Thrombin only	Pefabloc TH Benzamidine

Cleavage of GST tag using PreScission Protease

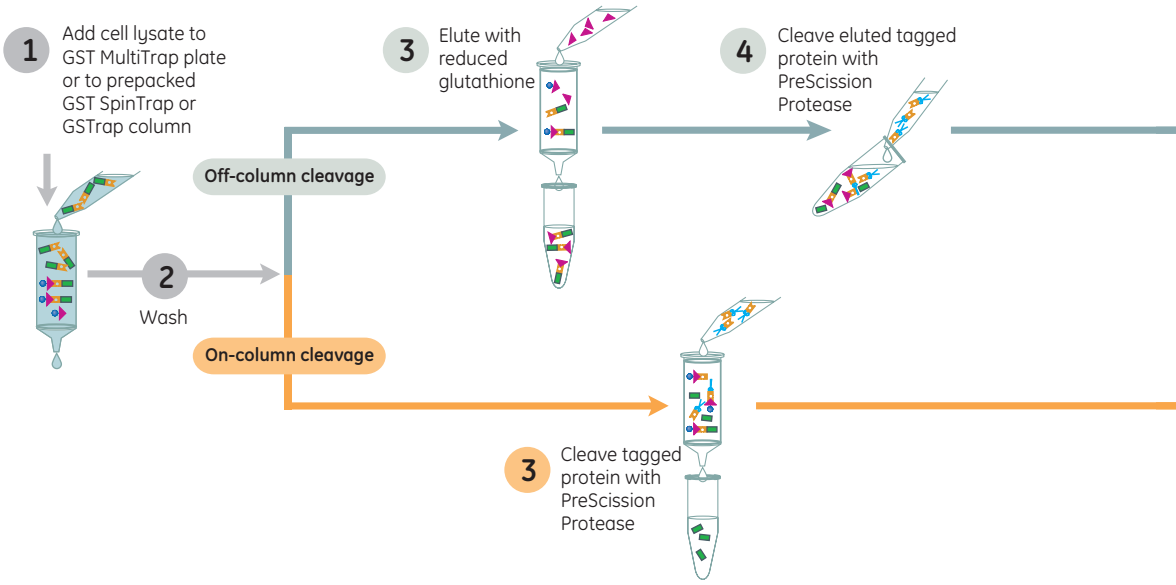


Fig 68A. Flow chart of the affinity purification procedure and PreScission Protease cleavage of GST-tagged proteins.

Cleavage of GST tag using thrombin or Factor Xa

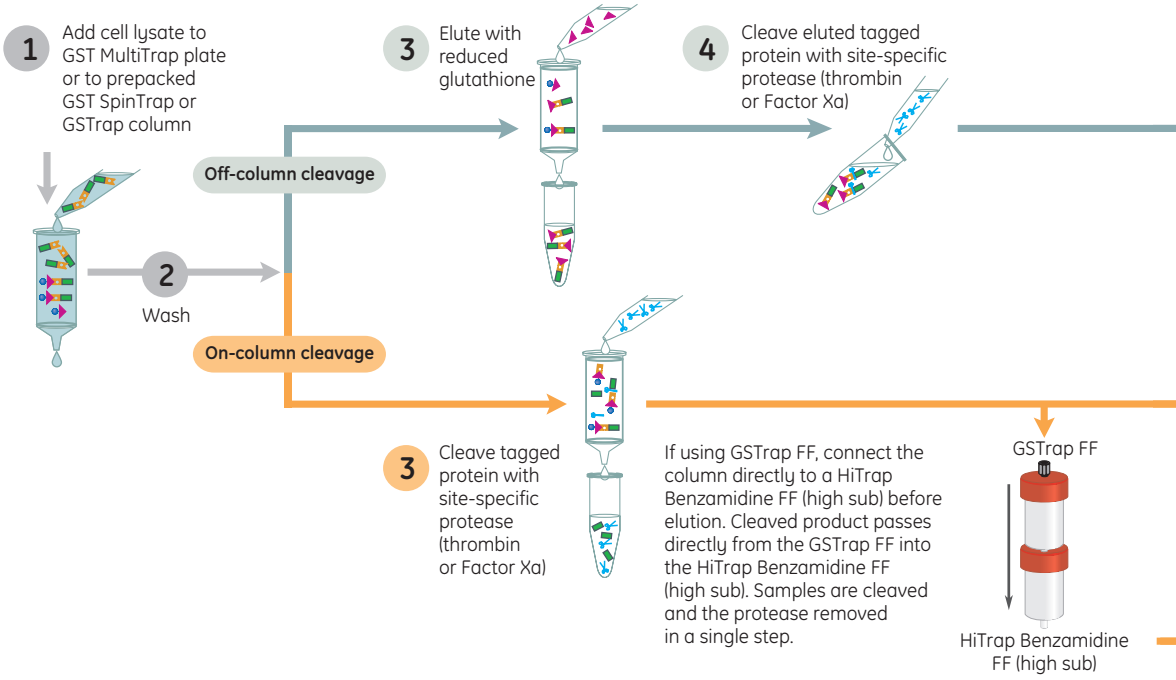
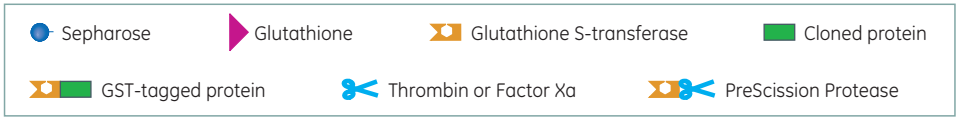
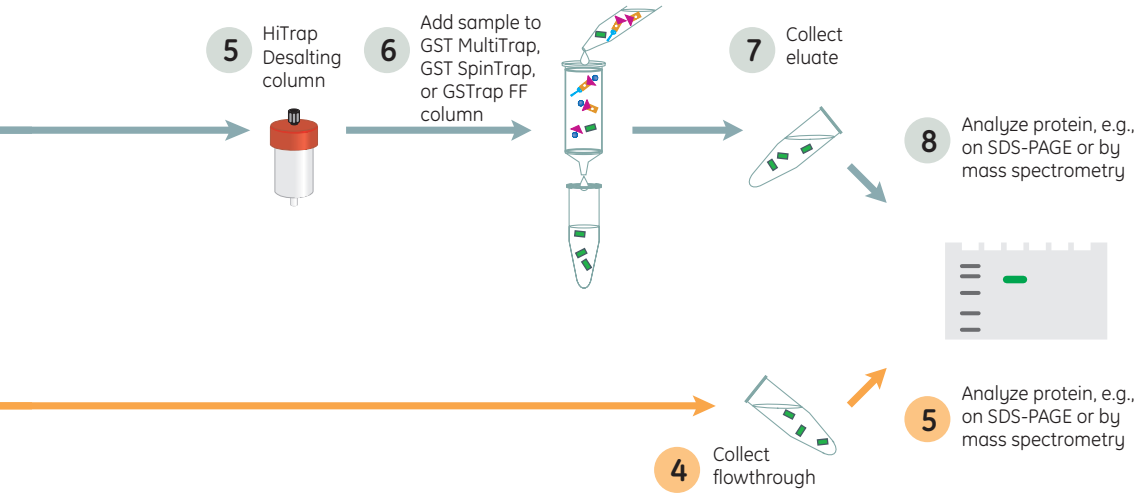
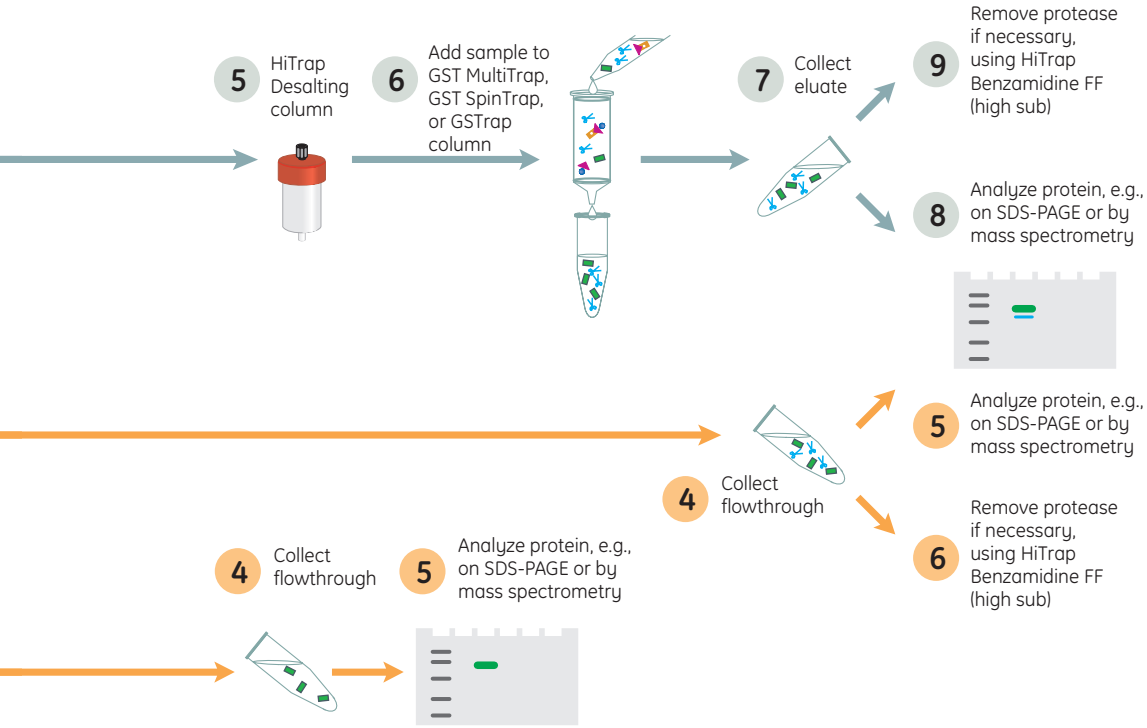


Fig 68B. Flow chart of the affinity purification procedure and thrombin or Factor Xa cleavage of GST-tagged proteins.




5 HiTrap Desalting column
6 Add sample to GST MultiTrap, GST SpinTrap, or GSTrap FF column
7 Collect eluate
8 Analyze protein, e.g., on SDS-PAGE or by mass spectrometry
4 Collect flowthrough
5 Analyze protein, e.g., on SDS-PAGE or by mass spectrometry


5 HiTrap Desalting column
6 Add sample to GST MultiTrap, GST SpinTrap, or GSTrap column
7 Collect eluate
9 Remove protease if necessary, using HiTrap Benzamidine FF (high sub)
8 Analyze protein, e.g., on SDS-PAGE or by mass spectrometry
4 Collect flowthrough
5 Analyze protein, e.g., on SDS-PAGE or by mass spectrometry
6 Remove protease if necessary, using HiTrap Benzamidine FF (high sub)
4 Collect flowthrough
5 Analyze protein, e.g., on SDS-PAGE or by mass spectrometry

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

For quick scale-up of purifications, two or three GSTrap columns can be connected in series (back pressure will be higher). Further scaling-up is possible using GSTPrep FF 16/10 columns or columns packed by the user. Protocols below are included for column or batch format using Glutathione Sepharose 4 Fast Flow, but this medium can easily be replaced with Glutathione Sepharose High Performance or Glutathione Sepharose 4B depending on what is the preferred media in the lab.

Cleavage and purification of GST-tagged protein bound to GSTrap FF

Recommended buffers

Binding buffer:	PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3
For PreScission Protease cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Cleavage buffer:	50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.0
PreScission Protease	
For thrombin cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Cleavage buffer:	PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3
Thrombin solution:	Dissolve 500 units in 0.5 ml of PBS prechilled to 4°C. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.
For Factor Xa cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Cleavage buffer:	50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5
Factor Xa solution:	Dissolve 400 units of Factor Xa in 4°C water to give a final solution of 1 unit/µl. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.

Purification and cleavage

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

1. Fill the syringe or pump tubing with distilled water. Remove the stopper and connect the column to the syringe (use the connector supplied), laboratory pump, or chromatography system "drop to drop" to avoid introducing air into the system.
2. Remove the snap-off end at the column outlet.
3. Wash out the ethanol with 3 to 5 column volumes of distilled water.
4. Equilibrate the column with at least 5 column volumes of binding buffer. Recommended flow rates are 1 ml/min (1-ml column) and 5 ml/min (5-ml column).
5. Apply the pretreated sample using a syringe fitted to the Luer connector or by pumping it onto the column. For best results, use a flow rate of 0.2 to 1 ml/min (1-ml column) and 0.5 to 5 ml/min (5-ml column) during sample application.

6. Wash with binding buffer (generally at least 5 to 10 column volumes) until the absorbance reaches a steady baseline or no material remains in the effluent. Maintain a flow rate of 1 to 2 ml/min (1-ml column) and 5 to 10 ml/min (5-ml column) for washing.

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

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

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

8a. Prepare the PreScission Protease mix:
–For GSTrap FF 1-ml columns, mix 80 µl (160 units) of PreScission Protease with 920 µl of PreScission cleavage buffer at 5°C.
–For GSTrap FF 5-ml columns, mix 400 µl (800 units) of PreScission Protease with 4.6 ml of PreScission cleavage buffer at 5°C.

8b. Prepare the thrombin mix:
–For GSTrap FF 1-ml columns, mix 80 µl (80 units) of thrombin solution with 920 µl of PBS.
–For GSTrap FF 5-ml columns, mix 400 µl (400 units) of thrombin solution with 4.6 ml of PBS.

8c. Prepare the Factor Xa mix:
–For GSTrap FF 1-ml columns, mix 80 µl (80 units) of Factor Xa solution with 920 µl of Factor Xa cleavage buffer.
–For GSTrap FF 5-ml columns, mix 400 µl (400 units) of Factor Xa solution with 4.6 ml of Factor Xa cleavage buffer.

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

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

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

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

11. Fill a syringe with 3 ml (1-ml column) or 15 ml (5-ml column) of cleavage buffer. Remove the top cap and stopper from the column and attach the syringe. Avoid introducing air into the column.

12. Begin elution of the cleaved target protein. Maintain flow rates of 1 to 2 ml/min (1-ml column) or (5-ml column), and collect the eluate (0.5 to 1 ml/tube for 1-ml column, 1 to 2 ml/tube for 5-ml column).

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

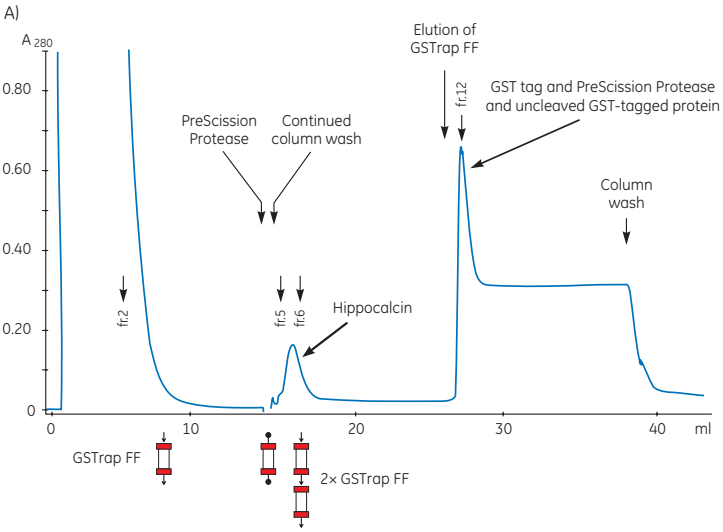
For thrombin and Factor Xa: The eluate will contain the protein of interest and thrombin or Factor Xa, respectively, while the GST moiety of the tagged protein will remain bound to the GSTrap column. The thrombin or Factor Xa can be removed from the protein of interest in one step using a HiTrap Benzamidine FF (high sub) column in series after the GSTrap column. In this process, the cleaved, tagged protein and thrombin or Factor Xa is washed from the GSTrap column onto the HiTrap Benzamidine FF (high sub) column. This second column captures the thrombin or Factor Xa, thus enabling the collection of pure protease-free protein in the eluent. Refer to the application on page 160 for an example of the purification and on-column cleavage of GST-tagged SH2 domain using thrombin and GSTrap FF, with sample clean-up accomplished using HiTrap Benzamidine FF (high sub) column in series with GSTrap FF.

See Appendix 2 for details on regenerating the GSTrap column for subsequent purifications.

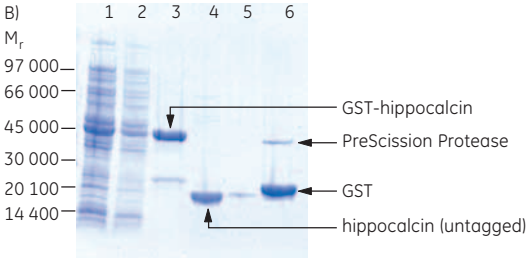
Application examples

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

The gene for human hippocalcin, a member of the neurone-specific calcium-binding protein family, was cloned into a pGEX vector containing a PreScission Protease site adjacent to the GST tag. The expressed tagged protein was captured on a GSTrap FF 1-ml column. The column was then incubated overnight at 4°C and for an additional 2 h at room temperature with PreScission Protease (which is GST-tagged itself). Following on-column cleavage, a second GSTrap FF 1-ml column was placed in series after the first to remove any PreScission Protease, uncleaved GST-tagged protein, or free GST tag that could co-elute with the sample during the additional wash with binding buffer (Fig 69). For every gram of wet *E. coli* cells, 10 mg of pure, untagged hippocalcin was obtained.



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



Lanes

1. Clarified *E. coli* homogenate containing expressed GST-hippocalcin
2. Flowthrough (fraction 2)
3. GST-hippocalcin
4. Pure hippocalcin after on-column cleavage (fraction 5)
5. Same as lane 4, but fraction 6
6. Eluted fraction from GSTrap FF containing PreScission Protease and GST-tag released by cleavage (fraction 12)

Fig 69. Purification of human hippocalcin-GST-tagged protein with on-column cleavage and post-cleavage removal of PreScission Protease using GSTrap FF columns. A) Chromatogram showing purification of hippocalcin. B) SDS-PAGE analysis of various sample processing steps. ExcelGel SDS Gradient, 8–18%, Coomassie blue staining.

2. Automatic removal of the GST tag with PreScission protease

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

The example in Figure 70 shows purification results for a GST-tagged protein, GST-purα (M_r 61 600), expressed in *E. coli*. The M_r of the cleaved product is 35 200. After harvest, cell lysis was performed by sonication. The samples were clarified by centrifugation prior to sample loading.

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

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

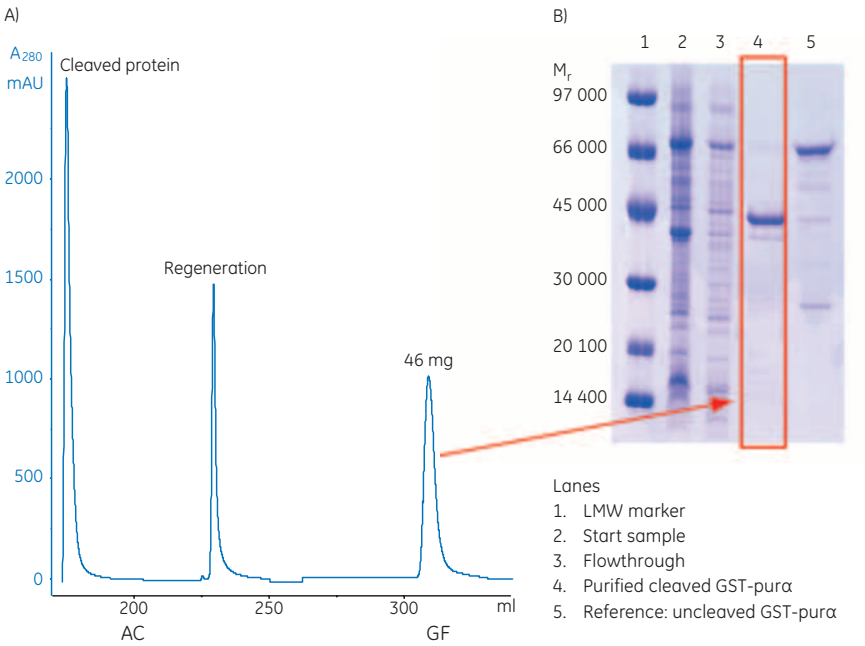
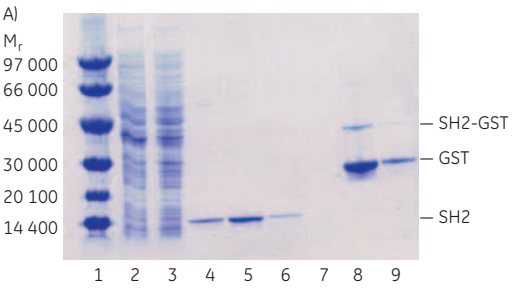


Fig 70. (A) Two-step protocol for automatic GST-tagged protein cleavage with PreScission Protease. (B) Analysis of the untagged target protein after purification and GST-tagged cleavage on SDS-PAGE and Coomassie staining.

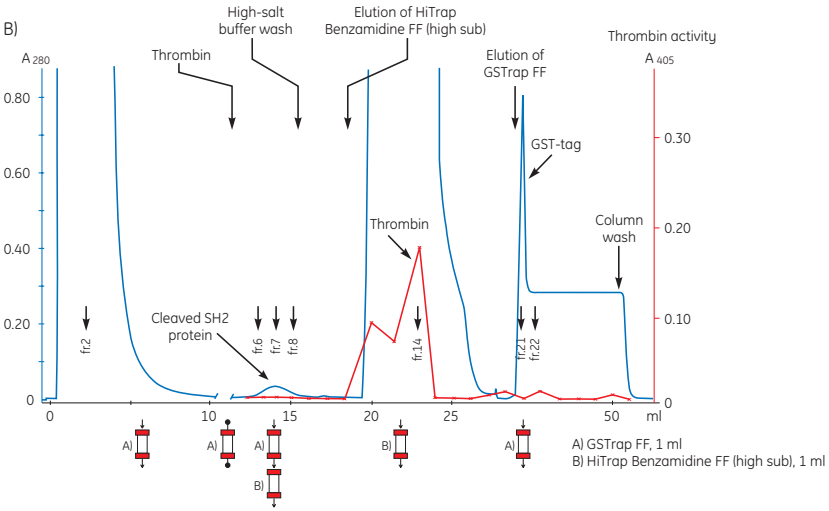
3. Purification and on-column cleavage of GST-tagged SH2 domain using thrombin and GSTrap FF. Direct removal of thrombin using HiTrap Benzamidine FF (high sub) column in series with GSTrap FF

The following application describes the purification of GST-SH2 (M_r 37 000) on a GSTrap FF-1 ml column, followed by on-column cleavage with thrombin (Fig 71). After the thrombin incubation step, a HiTrap Benzamidine FF (high sub) 1-ml column was placed in series below the GSTrap FF column. As the columns were washed with binding buffer and later with high salt buffer, the cleaved SH2 tagged protein and thrombin were washed from the GSTrap FF column onto the HiTrap Benzamidine FF (high sub) column. Thrombin was captured by this second column, thus enabling the collection of pure thrombin-free untagged target protein in the eluent (Fig 71A). Complete removal of thrombin was verified using the chromogenic substrate S-2238 (Chromogenix, Haemochrom Diagnostica AB; supplier in US is DiaPharma) for detection of thrombin activity (Fig 71B). This entire procedure could be completed in less than one day.



Lanes

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



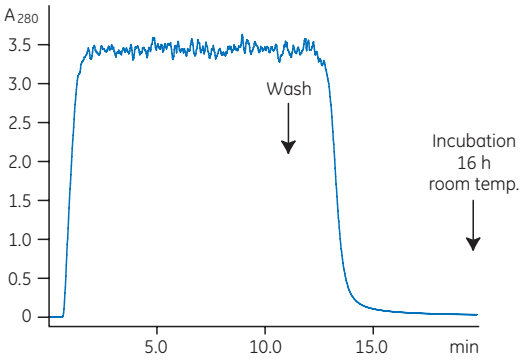
Sample:	2 ml clarified *E. coli* homogenate containing GST-SH2 (M_r 37 000) with a thrombin cleavage site
Columns:	GSTrap FF 1 ml and HiTrap Benzamidine FF (high sub) 1 ml
Binding buffer:	20 mM sodium phosphate, 0.15 M NaCl, pH 7.5
High salt wash buffer:	20 mM sodium phosphate, 1.0 M NaCl, pH 7.5
Benzamidine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

Fig 71. Purification of GST-SH2 GST-tagged protein with on-column cleavage and post-cleavage removal of thrombin using GSTrap FF and HiTrap Benzamidine FF (high sub) columns. (A) SDS-PAGE analysis of various sample processing steps. ExcelGel SDS Gradient 8–18%, Coomassie blue staining. (B) Chromatogram and thrombin activity curve demonstrating all steps in the purification of the SH2 domain.

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

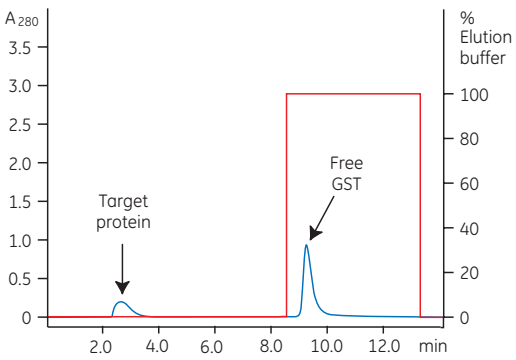
To demonstrate the efficiency of on-column cleavage in conjunction with purification, a GST-tagged protein containing the recognition sequence for thrombin was applied to GSTrap FF 1 ml. After washing, the column was filled by syringe with 1 ml of thrombin solution (20 U/ml in PBS, pH 7.3) and sealed using the supplied connectors. After incubation for 16 h at room temperature, the target protein minus the GST moiety was eluted using PBS, pH 7.3, and the bound GST was subsequently eluted using elution buffer (Fig 72). The cleavage reaction yield was 100%. Intact GST-tagged protein was not detected in the eluate by SDS-PAGE and silver staining (see Fig 72C, lane 5).

A)



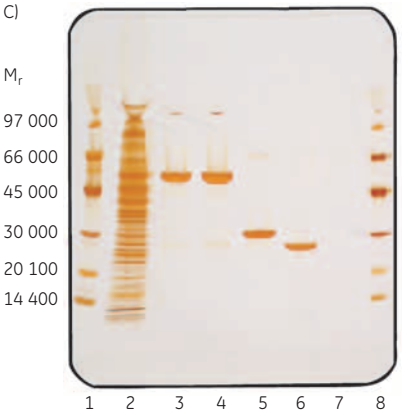
Sample: 10 ml clarified cytoplasmic extract from *E. coli* expressing a GST-tagged protein
Column: GSTrap FF 1 ml
Binding buffer: PBS, pH 7.3 (150 mM NaCl, 20 mM phosphate buffer)
Flow rate: 1 ml/min
Chromatographic procedure: 4 column volumes (CV) binding buffer, 10 ml sample, 10 CV binding buffer,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

B)



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

C)



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

Fig 72. On-column thrombin cleavage of a GST-tagged protein. (A) Equilibration, sample application, and washing of a GST-tagged protein on GSTrap FF 1-ml were performed using ÄKTAexplorer 10. After washing, the column was filled by syringe with 1 ml of thrombin (20 U/ml) and incubated for 16 h at room temperature. (B) GST-free target protein was eluted using PBS, pH 7.3. GST was eluted using 10 mM reduced glutathione. The GST-free target protein fraction also contained a small amount of thrombin not detectable by SDS-PAGE (lane 6). The thrombin can be removed using a HiTrap Benzamidine FF (high sub) column. (C) SDS-PAGE followed by silver staining.

Cleavage and purification of GST-tagged protein eluted from GSTrap FF

Recommended buffers

Binding buffer:	PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3
For PreScission Protease cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Cleavage buffer:	50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.0
PreScission Protease	
For thrombin cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Cleavage buffer:	PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3
Thrombin solution:	Dissolve 500 units in 0.5 ml of PBS prechilled to 4°C. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.
For Factor Xa cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Cleavage buffer:	50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5
Factor Xa solution:	Dissolve 400 units of Factor Xa in 4°C water to give a final solution of 1 unit/µl. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.

Purification and cleavage

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

1. Fill the syringe or pump tubing with distilled water. Remove the stopper and connect the column to the syringe (use the connector supplied), laboratory pump, or chromatography system "drop to drop" to avoid introducing air into the system.
2. Remove the snap-off end at the column outlet.
3. Wash out the ethanol with 3 to 5 column volumes of distilled water.
4. Equilibrate the column with at least 5 column volumes of binding buffer. Recommended flow rates are 1 ml/min (1-ml column) and 5 ml/min (5-ml column).
5. Apply the pretreated sample using a syringe fitted to the Luer connector or by pumping it onto the column. For best results, use a flow rate of 0.2 to 1 ml/min (1-ml column) and 0.5 to 5 ml/min (5-ml column) during sample application.
6. Wash with binding buffer (generally at least 5 to 10 column volumes) until the absorbance reaches a steady baseline or no material remains in the effluent. Maintain a flow rate of 1 to 2 ml/min (1-ml column) and 5 to 10 ml/min (5-ml column) for washing.
7. Elute the GST-tagged protein with 5 to 10 column volumes of elution buffer. Maintain flow rates of 1 to 2 ml/min (1-ml column) or 1 to 5 ml/min (5-ml column). Collect the eluate (0.5 to 1 ml/tube for 1-ml column, 1 to 2 ml/tube for 5-ml column). Pool fractions containing the GST-tagged protein (monitored by UV absorption at A_{280}).
8. Remove the free reduced glutathione from the eluate using a quick buffer exchange on HiTrap Desalting or HiPrep 26/10 Desalting column, depending on the sample volume.

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

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

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

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

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

11. Once digestion is complete, apply the sample to an equilibrated GSTrap FF column as described above (steps 1 to 7) to remove the GST moiety of the tagged protein.

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

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

See Appendix 2 for details on regenerating the GSTrap column for subsequent purifications.

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

Recommended buffers

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

Binding buffer:	PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3
For PreScission Protease cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Cleavage buffer:	50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.0
PreScission Protease	
For thrombin cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Cleavage buffer:	PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3
Thrombin solution:	Dissolve 500 units in 0.5 ml of PBS prechilled to 4°C. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.
For Factor Xa cleavage:	
Elution buffer:	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
Cleavage buffer:	50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5
Factor Xa solution:	Dissolve 400 units of Factor Xa in 4°C distilled water to give a final solution of 1 unit/µl. Swirl gently. Store solution in small aliquots at -80°C to preserve activity.

Preparation of Glutathione Sepharose media and binding of protein

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

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

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

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

Purification and cleavage

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

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

2a. Prepare the PreScission Protease mix:
For each ml of Glutathione Sepharose bed volume, prepare a mixture of 80 µl (160 units) of PreScission Protease and 920 µl of cleavage buffer at 5°C.

2b. Prepare the thrombin mix:
For each ml of Glutathione Sepharose bed volume, prepare a mixture of 80 µl (80 units) of thrombin and 920 µl of cleavage buffer.

2c. Prepare the Factor Xa mix:
For each ml of Glutathione Sepharose bed volume, prepare a mixture of 80 µl (80 units) of Factor Xa and 920 µl of cleavage buffer.

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

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

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

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

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

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

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

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

Reagents required

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

Elution buffer *alternatives* for eluting the protease:

0.05 M glycine-HCl, pH 3.0

10 mM HCl, 0.5 M NaCl, pH 2.0

20 mM p-Aminobenzamidine in binding buffer (competitive elution)

8 M urea or 6 M guanidine hydrochloride (denaturing solutions)

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

1. Fill the pump tubing or syringe with distilled water. Connect the column to the syringe, using the connector supplied, or to the pump tubing. Avoid introducing air into the column.
2. Remove the snap-off end.
3. Wash the column with five column volumes of distilled water to remove the storage buffer (0.05 M acetate buffer, pH 4, containing 20% ethanol).
4. Equilibrate the column with five column volumes of binding buffer.
5. Apply the sample using a syringe fitted to the Luer connector or by pumping it onto the column. Recommended flow rates for sample application are 1 ml/min for 1-ml column and 5 ml/min for 5-ml column. Collect the flowthrough and reserve. It contains the protease-depleted material to be saved. Apply a small volume of extra binding buffer to collect all desired material from the column.
6. Wash the column with 5 to 10 column volumes of binding buffer, collecting fractions (0.5 to 1 ml fractions for 1-ml column and 1 to 3 ml fractions for 5-ml column) until no material appears in the effluent (monitored by UV absorption at 280 nm).
7. Pool fractions from flowthrough and/or wash that contain the thrombin or Factor Xa free material (monitored by UV absorption 280 nm).
8. For reuse of column, elute the bound protease with 5 to 10 column volumes of the elution buffer of choice. If the eluted thrombin or Factor Xa is to be retained for reuse, buffer-exchange the fractions containing the protease using HiTrap Desalting or PD-10 Desalting column. If a low pH elution buffer has been used, collect fractions in neutralization buffer.
9. After all protease has been eluted, wash the column with binding buffer so it is ready for reuse.

If using low pH elution, collect protease fractions into neutralization buffer (60 to 200 µl of 1 M Tris-HCl, pH 9.0 per ml fraction collected), so that the final pH of the fractions will be approximately neutral.

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

Troubleshooting of cleavage methods

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

Situation	Possible cause	Remedy
GST-tagged proteins are not cleaved completely.	The ratios of PreScission Protease, thrombin, or Factor Xa to GST-tagged protein are not optimal.	Check the amount of tagged protein in the digest. Note that the capacity of the Glutathione Sepharose media for GST is ~ 10 mg/ml of medium for Glutathione Sepharose High Performance and Glutathione Sepharose 4 Fast Flow and ~ 5 mg/ml for Glutathione Sepharose 4B. In most purifications, however, the medium is not saturated with tagged protein. Verify that the correct ratios of enzyme to protein are used and adjust as necessary. For PreScission Protease and thrombin, use at least 10 units/mg of tagged protein. For Factor Xa, use an amount equivalent to at least 1% (w/w) of the weight of tagged protein. For some tagged proteins, up to 5% Factor Xa can be used. The optimal amount must be determined empirically. In some cases, optimal results have been achieved with a tagged protein concentration of 1 mg/ml. The addition of = 0.5% SDS (w/v) to the reaction buffer can significantly improve Factor Xa cleavage with some tagged proteins. Various concentrations of SDS should be tested to determine the optimal concentration.
	The incubation time and/or enzyme concentration is not sufficient for complete cleavage of the protein from the GST tag.	Increase the incubation time for the cleavage reaction. Increasing the reaction time to 20 h or more should improve cleavage as long as the tagged protein is not degraded by the extended incubation period. Alternatively, try increasing the amount of enzyme used for cleavage.
	Specific cleavage sites for the proteases have been altered during cloning of the tagged protein.	Verify the presence of specific enzyme cleavage sites. Check the DNA sequence of the construct and compare it with a known sequence to verify that the cleavage sites have not been altered.
	The presence of cleavage enzyme inhibitors is interfering with the cleavage reaction.	Remove any enzyme inhibitors that may interfere with the cleavage reaction. Prior to cleavage with PreScission Protease, buffer exchange or dialyze the tagged protein against 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5. Prior to cleavage with Factor Xa, buffer exchange the tagged protein on HiTrap Desalting, a PD-10 column, or HiPrep 26/10 Desalting, depending on the sample volume, or dialyze against 50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5.

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Problem	Possible cause	Solution
	Factor Xa is not properly activated.	Activate Factor Xa with Russell´s viper venom to generate functional enzyme. For activation of Factor Xa, incubate Russell´s viper venom with Factor Xa at a ratio of 1% in 8 mM Tris-HCl, 70 mM NaCl, 8 mM $CaCl_2$, pH 8.0. Incubate at 37°C for 5 min. Factor Xa from GE Healthcare has been preactivated by this procedure.
	The first amino acid after the Factor Xa recognition sequence is Arg or Pro.	Check the sequence of the tagged protein to verify that the first three nucleotides after the Factor Xa recognition sequence do not code for Arg or Pro.
Multiple bands are observed after electrophoresis/Western blotting analysis of the cleaved target protein	Proteolysis is occurring in the host bacteria prior to the cleavage reaction.	Determine when the extra bands appear. Verify that additional bands are not present prior to PreScission Protease, thrombin, or Factor Xa cleavage.
	The tagged protein itself contains recognition sequences for PreScission Protease, thrombin, or Factor Xa.	Check the sequence of the tagged protein to determine if it contains recognition sequences for the cleavage enzymes.
The tagged partner is contaminated with protease after purification	Glutathione Sepharose may have been saturated with GST-tagged protein during purification.	Pass the sample over a new GSTrap column or fresh Glutathione Sepharose to remove residual PreScission protease, or over a HiTrap Benzamidine (high sub) column in the case of thrombin or Factor Xa.

Chapter 6
Simple purification of other recombinant or native proteins

Numerous products are available, as discussed in previous chapters, that use affinity chromatography to isolate and purify a specific histidine- or GST-tagged protein. However, many other tagged and untagged proteins can also be isolated to a satisfactory degree of purity by a single-step purification using affinity chromatography. In fact, single-step purification saves time (personnel and equipment) and reduces both the risk of denaturation of the target protein and the loss of essential molecules that are weakly attached to the protein. For high-throughput purification platforms, the need for additional purification steps will increase the complexity of the task, and parallel formats become possible.

Fig 73. 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 22 shows the applications for which affinity purification with HiTrap and HiPrep columns are already available. All columns are supplied with a detailed protocol that outlines the buffers and steps required for optimal results. If higher binding capacity is needed, for larger-scale work, HiTrap columns can be linked together in series to increase the capacity of a single purification step. Media are also available for packing larger columns.

Table 22. Ready-to-use HiTrap and HiPrep columns for affinity purification.

Product	Application	Approx. binding capacity (mg/ml medium)	Average particle size	pH stability (long term)
MabSelect™	IgG, IgG subclasses, monoclonal	30 mg human IgG	85 µm	3–10
MabSelect SuRe™	IgG, IgG subclasses, monoclonal	30 mg human IgG	85 µm	3–13
MabSelect Xtra™	IgG, IgG subclasses, monoclonal	40 mg human IgG	75 µm	3–10
rProtein A FF	IgG, IgG subclasses, human IgG	50 mg human IgG	90 µm	3–10
Protein A HP	IgG, IgG subclasses, human IgG	20 mg human IgG	34 µm	3–9
Protein G HP	IgG, rat IgG, mouse IgG_1	25 mg human IgG	34 µm	3–9
IgM Purification HP	Monoclonal IgM from hybridoma supernatant	5 mg human IgM	34 µm	3–11
IgY Purification HP	IgY from egg yolk	20 mg pure IgY	34 µm	3–11

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Table 22. Ready-to-use HiTrap and HiPrep columns for affinity purification (continued).

Product	Application	Approx. binding capacity (mg/ml medium)	Average particle size	pH stability (long term)
Heparin HP	Antithrombin III and other coagulation factors, lipoprotein, lipases, DNA binding proteins, protein synthesis factors	3 mg AT III (bovine)	34 µm	5–10
Blue HP	Albumin, nucleotide-requiring enzymes, coagulation factors	20 mg human albumin	34 µm	4–12
HisTrap HP	Optimized high-performance purification of histidine-tagged proteins	At least 40 mg histidine-tagged protein	34 µm	3–12[1]
HisTrap FF	Optimized purification of histidine-tagged proteins	40 mg histidine-tagged protein	90 µm	3–12[1]
HisPrep FF 16/10	Larger-scale, optimized purification of histidine-tagged proteins	40 mg histidine-tagged protein	90 µm	3–12[1]
HisTrap FF crude	Optimized purification of histidine-tagged proteins. Optimized for direct purification from crude cell lysates.	40 mg histidine-tagged protein	90 µm	3–12[1]
IMAC HP	Purification of proteins and peptides with exposed histidine groups, histidine-tagged proteins. Uncharged medium.	40 mg histidine-tagged protein when charged with Ni^{2+}. Metal ion and protein dependent.	34 µm	3–12[2]
IMAC FF	Purification of proteins and peptides with exposed histidine groups, histidine-tagged proteins. Uncharged medium.	40 mg histidine-tagged protein when charged with Ni^{2+}. Metal ion and protein dependent.	90 µm	3–12[2]
HiPrep IMAC FF 16/10	Larger-scale, optimized purification of proteins and peptides with exposed histidine groups, histidine-tagged proteins. Uncharged medium.	40 mg histidine-tagged protein when charged with Ni^{2+}. Metal ion and protein dependent.	90 µm	3–12[2]
Chelating HP	Purification of proteins and peptides with exposed histidine groups, histidine-tagged proteins. Uncharged medium.	23 µmol Cu^{2+}	34 µm	3–13
NHS-activated HP	Coupling of own specific ligands via primary amino groups[3]		34 µm	3–12
Streptavidin HP	Biotinylated molecules, biotin-tagged proteins	> 300 nmol biotin	34 µm	4–9
GSTrap 4B	GST-tagged proteins, other glutathione S-transferases, or glutathione-dependent proteins	> 5 mg horse liver GST	90 µm	4–13
GSTrap HP	GST-tagged proteins, other glutathione S-transferases, or glutathione-dependent proteins. High-performance purifications.	10 mg recombinant GST	34 µm	3–12
GSTrap FF	GST-tagged proteins, other glutathione S-transferases, or glutathione-dependent proteins	10 mg GST, 11 mg GST-tagged protein, M_r 43 000	90 µm	3–12
GSTPrep FF 16/10	Larger-scale purification of GST-tagged proteins, other glutathione S-transferases, or glutathione-dependent proteins	10 mg GST, 11 mg GST-tagged protein, M_r 43 000	90 µm	3–12
Benzamidine FF (high sub)	Removal and/or purification of serine proteases	≥ 35 mg trypsin	90 µm	2–8

[1] Ni^{2+}- stripped medium.

[2] Uncharged medium.

[3] The medium is pre-activated and a suitable ligand must be coupled to obtain an affinity medium.

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, for example, by raising antibodies, tested for affinity to the target protein, and purified before immobilized to a chromatographic matrix. For further details on general purification strategies for proteins see Chapter 7 and the *Protein Purification Handbook* from GE Healthcare. A detailed account of the principles of affinity chromatography can be found in the *Affinity Chromatography, Principles and Methods Handbook* also available from GE Healthcare.

Use of HiTrap NHS-activated HP for simple preparation of an affinity purification column

NHS-activated Sepharose High Performance is a chromatographic matrix specifically designed for the covalent coupling of ligands containing primary amino groups. This is the most common method for coupling of proteins to chromatographic media. 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 µmol NHS-groups/ml medium. Nonspecific 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 an affinity medium using prepacked HiTrap NHS-activated HP column and is generally applicable to all NHS-activated Sepharose products.

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

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 $NaHCO_3$, 0.5 M NaCl, pH 8.3

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

Coupling within pH range 6.5 to 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 to 10 mg/ml (for protein ligands). If needed, perform a buffer exchange using a desalting column (see Chapter 9). The optimal concentration depends on the ligand. Optimal 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 snap-off end.

Ligand coupling

1. Wash out the isopropanol with 6 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 for 15 to 30 min at 25°C (or 4 h at 4°C).

If larger volumes of ligand solution are used, recirculate 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 to 30 min or, if using a peristaltic pump, simply recirculate the sample through the column.

If required, the coupling efficiency can be measured at this stage. These procedures are included in the instructions supplied with each HiTrap NHS-activated HP column package.

Washing and deactivation

This procedure deactivates any excess active groups that have not coupled to the ligand and washes out nonspecifically 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 × 2 column volumes of buffer A.
2. Inject 3 × 2 column volumes of buffer B.
3. Inject 3 × 2 column volumes of buffer A.
4. Seal and leave the column for 15 to 30 min.
5. Inject 3 × 2 column volumes of buffer B.
6. Inject 3 × 2 column volumes of buffer A.
7. Inject 3 × 2 column volumes of buffer B.
8. Inject 2 to 5 column volumes of a buffer with neutral pH.

The column is now ready for use.

Store the column in storage solution optimized for the specific column.

The presence of primary amines in the reaction mixture will inhibit the coupling reaction. Buffers (e.g., Tris) or additives must be avoided.

Purification

Optimal binding and elution conditions for purification of the target protein using a specific column must be determined separately for each ligand. Literature references and textbooks may offer good guidelines. Below is a general protocol that can be used initially.

Use double-distilled or deionized water and high-quality chemicals. We recommend passing the eluent through a 0.45 µm filter.

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, increase lysis treatment (sonication, homogenization), or add DNase/RNase to reduce the size of nucleic acid fragments. 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 9).

Prepare the column

Perform a blank run (use binding buffer instead of sample) to ensure that loosely bound ligand is removed (see below).

1. Wash with 3 column volumes of binding buffer.
2. Wash with 3 column volumes of elution buffer.
3. Equilibrate with 5 to 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 to 1 ml/min on a HiTrap 1-ml column.
2. Wash with 5 to 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, because this may decrease the yield.

3. Elute with 2 to 5 column volumes of elution buffer.
4. If required, purified fractions can be desalted and exchanged into the buffer of choice using prepacked desalting columns (see Chapter 9).

Reequilibrate the column

Reequilibrate the column by washing with 10 column volumes of binding buffer.

Chapter 7
Multistep purification of tagged and untagged recombinant proteins

Recombinant protein expression may allow production of large amounts of an affinity-tagged protein so that a single purification step using affinity chromatography is sufficient to achieve the desired level of purity. However, the purification obtained after a single step is frequently not sufficient, and affinity tags may sometimes interfere with the post-purification use of the protein. In these instances, multistep purification will be necessary.

A significant advantage when working with recombinant proteins is that there is often considerable information available about the product (amino acid sequence, M_r, pI, functional properties) and contaminants (the expression host may be well known). 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 74). 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 multistep protein purification. Appendix 9 provides useful background information describing the various techniques discussed herein. *The Protein Purification Handbook* (from GE Healthcare) is recommended as a guide to planning efficient and effective protein purification strategies.

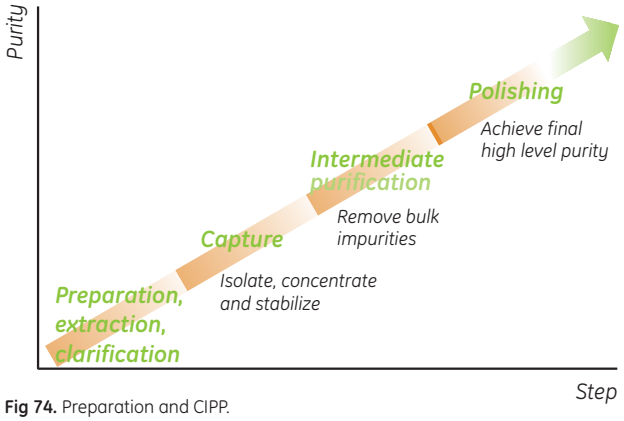


Fig 74. Preparation and CIPP.

CIPP is applied as follows:

- Imagine the purification has three phases—Capture, Intermediate Purification, and Polishing. Each phase may include one or more purification steps.
- Assign a specific objective to each step within the purification process.

The problem associated with a particular purification 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, that is, 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 optimal 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 23.

Table 23. Techniques for protein purification.

Protein property	Chromatographic technique
Charge	Ion exchange (IEX), Chromatofocusing
Size	Gel filtration (GF)
Hydrophobicity	Hydrophobic interaction (HIC), Reversed phase (RPC)
Biorecognition (ligand specificity)	Affinity (AC)

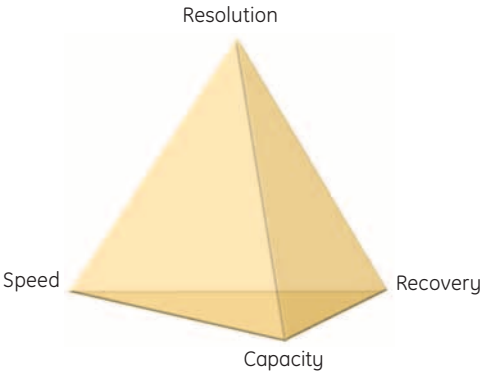


Fig 75. Every chromatographic technique offers a balance between resolution, capacity, speed, and recovery.

Resolution is achieved by the selectivity of the technique and the ability of the chromatographic medium 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 that can be loaded may be limited by volume (as in GF) or by large amounts of contaminants that also bind the column, rather than by the amount of the target protein.

Speed is of the highest importance at the beginning of purification, because the protein has not yet been stabilized.

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 unfavorable 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.

Combine techniques that are orthogonal to each other, that is, that apply very different separation mechanisms.

Keep in mind the interplay between "required purity" and "required yield." In general, every added purification step will increase purity and decrease yield (except for desalting).

A guide to the suitability of each purification technique for the stages in CIPP is shown in Table 24.

Table 24. Suitability of purification techniques for CIPP.

Technique	Main features	Capture	Inter-mediate	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, concentrated sample
HIC	good resolution good capacity high speed	++	+++	+	high ionic strength, sample volume not limiting, addition of salt needed	low ionic strength, concentrated sample
AC	high resolution high capacity high speed	+++	+++	++	specific binding conditions, sample volume not limiting	specific elution conditions, concentrated sample
GF	high resolution using Superdex		+	+++	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

Minimize sample handling between purification steps by combining techniques to avoid the need for sample conditioning before the next step. The product should be eluted from the first column in a buffer suitable for the start conditions required for the next technique (see Table 24).

HIC (which requires high salt to enhance binding to the media) is well-suited as the capture step after ammonium sulfate precipitation and clarification. 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 a nonbinding technique with limited volume capacity and is unaffected by buffer conditions. Because of its mechanism of acting, the sample zone in GF is broadened during passage through the column. Eluted material may sometimes thus need to be concentrated. GF is well suited for use after any of the concentrating techniques (IEX, HIC, AC).

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 76.

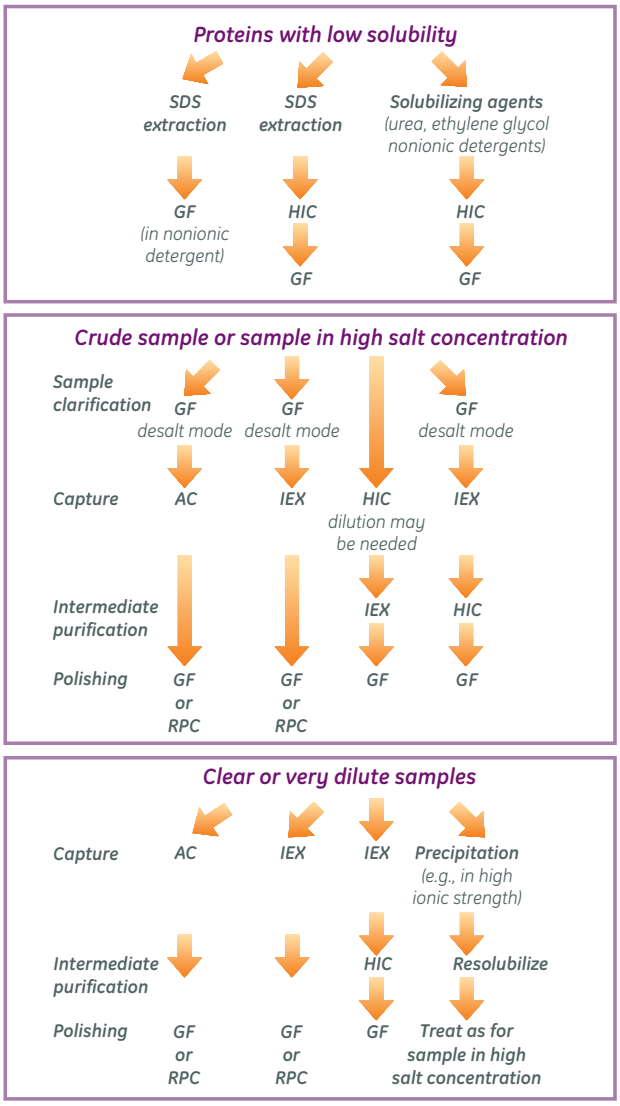


Fig 76. Example of logical combinations of chromatographic steps.

For the capture step, select a technique that binds the target protein and as few contaminants as possible. In some cases it may be advantageous to select a technique that does not bind the target protein but rather binds contaminants whose removal is critical, for example, proteases or major contaminants.

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). This orthogonality in separation mechanisms allows very powerful purification protocols for recombinant proteins without tags as well as for naturally abundant proteins.

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. Also consider the order of the techniques, as this will often make a great difference in purification.

IEX is a technique that offers different selectivities using either anion or cation exchangers. A target protein may very well bind to both exchangers at the same pH; alternatively, the pH can be changed. 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 and is not irreversibly bound or denatured by the matrix.

RPC separates proteins and peptides on the basis of hydrophobicity. RPC is a 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. Because 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 less commonly 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.

Application examples

1. Three-step purification of a recombinant enzyme using ÄKTAFPLC™ System

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 overexpression in soluble form in the cytoplasm of *E. coli* bacteria.

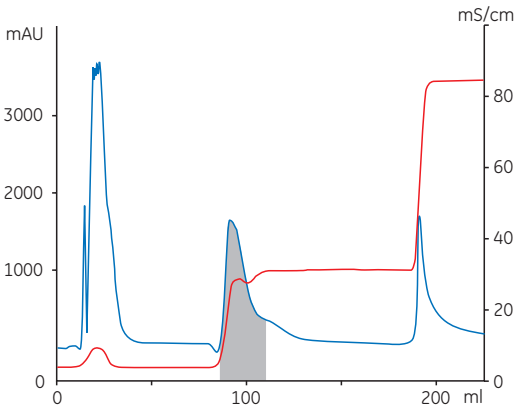
A more detailed description of this work can be found in Application Note 18-1128-91.

Sample extraction and clarification

Cells were suspended in Tris-based lysis buffer, pH 7.5 and lysed using ultrasonication. Streptomycin sulfate 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 optimal medium (results not shown). Optimization of the capture step (in Fig 77) allowed the use of a step elution at high flow rate to speed up the purification.

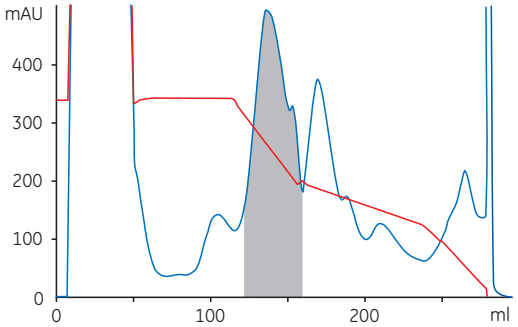


Column:	HiPrep 16/10 Q XL
Sample:	Clarified *E. coli* extract
Sample volume:	40 ml
Start buffer:	50 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, 0.2 M benzamidine-HCl, 0.2 mM PMSF, pH 7.5
Elution buffer:	Binding buffer + 1.0 M NaCl
Flow:	10 ml/min (300 cm/h)

Fig 77. Capture using IEX. The elution position of DAOCS is shaded.

Intermediate purification

HIC was selected because the separation principle is complementary to IEX and because a minimum amount of sample conditioning was required. Hydrophobic properties are difficult to predict, and it is always recommended to screen different media. After screening, RESOURCE ISO was selected on the basis of the resolution achieved. In this intermediate step, shown in Figure 78, the maximum possible speed for separation was sacrificed in order to achieve higher resolution and allow significant reduction of impurities.



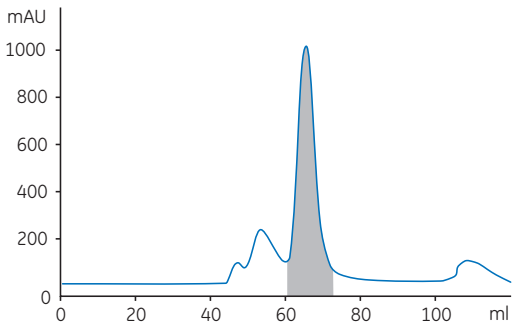
Column:	SOURCE™ 15ISO, packed in HR 16/10 column
Sample:	DAOCS pool from HiPrep 16/10 Q XL
Sample volume:	40 ml
Start buffer:	1.6 M ammonium sulfate, 10% glycerol, 50 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, 0.2 mM benzamidine-HCl, 0.2 mM PMSF, pH 7.5
Elution buffer (B):	50 mM Tris-HCl, 10% glycerol, 1 mM EDTA, 2 mM DTT, 0.2 mM benzamidine-HCl, 0.2 mM PMSF, pH 7.5
Gradient:	0–16%B in 4 CV, 16-24%B in 8 CV, 24–35%B in 4 CV, 100%B in 4 CV
Flow:	5 ml/min (150 cm/h)

CV = column volume

Fig 78. Intermediate purification using HIC. The elution position of DAOCS is shaded.

Polishing

The main goal of the polishing step, shown in Figure 79, 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. This data is presented in more detail in a *Nature* paper from 1998 [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)].



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

Fig 79. Polishing step using gel filtration. The elution position of DAOCS is shaded.

2. Three-step purification of a recombinant phosphatase using ÄKTAprime plus

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 *E. coli*. Using the preprogrammed method templates of ÄKTAprime plus with prepacked HiPrep and HiLoad columns ensured quick and easy method development. The purification strategy consisted of a capture step by IEX chromatography, intermediate purification by HIC, and polishing by GF. Active rPhosphatase (35 mg) was purified within 8 h.

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

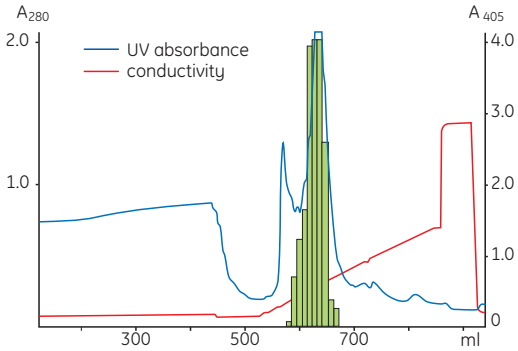
Sample preparation and extraction

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 ultrasonication, 6 × 20 bursts with 60 seconds cooling between each burst. DNA was removed by precipitation with 1% w/v streptomycin sulfate. The sample was clarified by centrifugation, 15 min 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 ÄKTAprime plus system pump was used to apply 200 ml of the clarified extract, diluted 1:2 with water, to a HiPrep 16/10 DEAE FF column. A preprogrammed method template for IEX chromatography was used for the separation.

Fractions of the eluate were collected and analyzed 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.

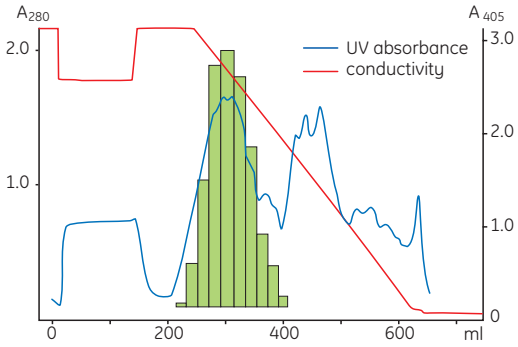


Sample:	200 ml clarified *E. coli* extract, diluted 1:2 with water, pH 6.6
Column:	HiPrep 16/10 DEAE FF, V_t = 20 ml
Start buffer:	25 mM Tris-HCl, pH 7.4, 10% glycerol, 1 mM EDTA, 2 mM DTT
Elution buffer (B):	1 M NaCl in start buffer
Flow:	5 ml/min (150 cm/h)
Run parameters:	Equilibration 0% B 2 CV Sample application Wash 1 0% B 4 CV Elution 0–50% B in 20 CV 50% B for 1 CV Wash 2 100% B 2 CV
	CV = column volume

Fig 80. Capture step using ion exchange. The phosphatase activity is represented by the green bars (absorbance at 405 nm).

Intermediate purification

HIC was used for intermediate purification because of its compatibility with samples containing a high salt concentration. The pooled fractions from the IEX column were purified on HiLoad 16/10 Phenyl Sepharose High Performance, using a preprogrammed method template in ÄKTAprime plus. The fractions containing rPhosphatase were pooled and concentrated to 10 ml on an Amicon™ 50 ml stirred-cell using a Diaflow™ PM10 filter. Reducing the sample volume enables a smaller GF column to be used for the final polishing step.



Sample: 170 ml rPhosphatase containing pool from HiPrep 16/10 DEAE FF in 1.6 M ammonium sulfate, pH 7.0
Column: HiLoad 16/10 Phenyl Sepharose HP, V_t = 20 ml
Start buffer: 25 mM Tris-HCl, pH 7.4 in1.4 M ammonium sulfate, 1 mM EDTA, 2 mM DTT
Elution buffer (B): 25 mM Tris-HCl in 10% glycerol, 1 mM EDTA, 2 mM DTT, pH 7.4
Flow: 5 ml/min (150 cm/h)
Run parameters:

Equilibration	0% B	2 CV
Sample application		
Wash 1	0% B	3 CV
Elution	0–100% B	in 20 CV
Wash 2	100% B	2 CV

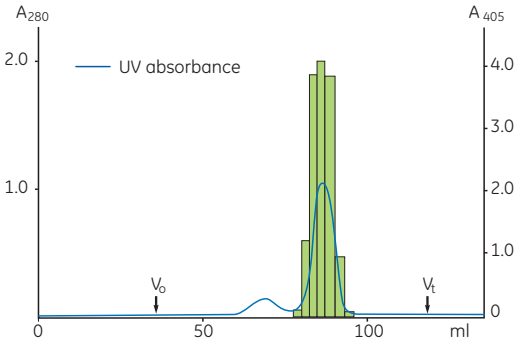
CV = column volume

Fig 81. 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 preprogrammed method template to run a GF separation on a HiLoad 16/60 Superdex 75 prep grade column. The purity of the fractions containing rPhosphatase was checked with SDS-PAGE (Fig 82) and by mass spectrometry (results not shown).

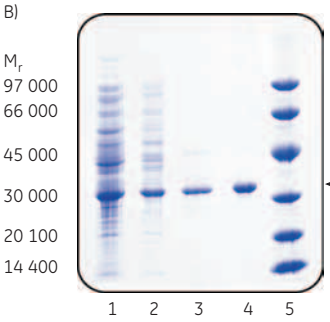
A)



Sample: 4 ml concentrated eluate, containing rPhosphatase from the HiLoad 16/10 Phenyl Sepharose HP
Column: HiLoad 16/60 Superdex 75 pg, V_t = 120 ml
Buffer: 25 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 2 mM DTT, pH 7.4
Flow: 0.5 ml/min (15 cm/h)

CV = column volume

B)



Lanes
1. *E. coli* extract
2. Eluate from the capture step (ion exchange chromatography)
3. Eluate from the intermediate step (hydrophobic interaction chromatography)
4. Eluate from the polishing step (gel filtration)
5. LMW markers

rPhosphatase

Fig 82. (A) Polishing step using gel filtration. The phosphatase activity is represented by the green bars (absorbance at 405 nm). (B) Purity check by SDS-PAGE. The proteins were stained with Coomassie Brilliant Blue.

Chapter 8
Handling inclusion bodies

Recombinant proteins are most often expressed in the intracellular space, but expression can also be controlled so that the protein is secreted into the periplasmic space or out into the culture medium. While secretion is advantageous in terms of protein folding, solubility, and disulfide bonding, the yield is generally much higher when using intracellular expression.

However, recombinant protein accumulated intracellularly is frequently deposited in the form of inclusion bodies, insoluble aggregates of misfolded protein lacking biological activity. The recombinant protein is often the major component of inclusion bodies. Therefore the presence of inclusion bodies can make preliminary isolation steps very simple, although the isolation of proteins from inclusion bodies often leads to difficulties with refolding and full recovery of biological activity. Table 25 summarizes the advantages and disadvantages of working with recombinant products expressed as inclusion bodies. Inclusion body formation frequently occurs when eukaryotic proteins are expressed in bacterial hosts.

Table 25. Advantages and disadvantages of inclusion bodies.

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

If the protein is expressed as inclusion bodies, there are several options to consider: optimize as much as possible for soluble expression, accept the formation of inclusion bodies but develop strategies to solubilize and refold the protein, try another expression host, or modify the plasmid construct.

Solubilization of inclusion bodies

The solubility of a recombinant protein can be made more favorable by modification of culture conditions.

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

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

In general, induction at lower cell densities (A_{600} = 0.5) usually results in greater yields of the tagged protein in a soluble form. However, in some cases it may be beneficial to grow the cells to a higher cell density (> 1 A_{600} unit) for a shorter period of time, or simply to induce for a shorter period of time.

Growing the cells to a higher cell density and either omitting induction (for example, by IPTG) or reducing the concentration of the inducing agent (for example, with IPTG, reducing the concentration to 0.1 mM) leads to lower yields, but more of the tagged protein is likely to be obtained in an intact form.

If culture modifications do not significantly improve the yield of soluble tagged proteins, then common denaturants such as 4 to 6 M guanidine hydrochloride, 4 to 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 the presence and concentration of reducing agent, time, temperature, ionic strength, and the ratio of denaturant to protein. Solubilized proteins can often be purified at this stage by using a separation technique that is compatible with the presence of the denaturant. Purification and refolding can often be combined in the same purification step, for example, by chromatographic on-column refolding.

Success of affinity purification in the presence of denaturing agents will depend on the nature of the tagged protein. Denaturants such as guanidine hydrochloride, urea, Tween 20, CTAB, or SDS have all been used, but it is important to test the chosen denaturant with the target protein before introducing it into the solubilization strategy.

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 26 compares conventional methods for refolding of insoluble recombinant proteins with on-column affinity purification and refolding.

Refolding usually requires extensive optimization. One should always consider other alternatives (as mentioned earlier), for example, optimizing expression parameters, making a new construct, or changing the expression host.

Table 26. Comparison of methods for protein refolding.

Refolding techniques	Advantages/Disadvantages
Step dialysis	Takes several days. Uses large volumes of buffer.
Dilution into near neutral pH	Simple technique. Gives extensive dilution, often several-hundred-fold.
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. Success varies and is dependent on the protein.

On-column refolding

Using a histidine-tagged 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 83 has been used successfully for several different histidine-tagged proteins.

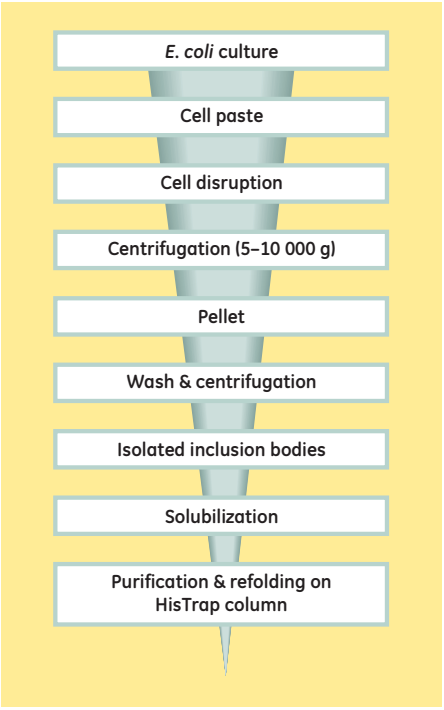


Fig 83. General scheme for the extraction, solubilization, and refolding of $(histidine)_6$-tagged proteins produced as inclusion bodies in *E. coli* cells.

High concentrations of chaotropic agents (such as urea or guanidine hydrochloride) enhance the binding of the histidine tag to immobilized divalent metal ions. Consequently, $(histidine)_6$-tagged proteins can be solubilized by chaotropic extraction and bound to Ni Sepharose. Removal of contaminating proteins and refolding by exchange to nondenaturing buffer conditions can be performed before elution of the protein from the column.

Once refolded, the protein may be purified further by other techniques (see Chapter 7) if a higher degree of purity is required.

Application

Purification and on-column refolding of an insoluble histidine-tagged protein from a 100 ml *E. coli* culture using HisTrap FF 1 ml with ÄKTAprime plus

This procedure uses a HisTrap FF 1-ml column but also can be used with a HisTrap HP 1-ml or a HisTrap FF crude 1-ml column.

Preparing the buffers

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

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 (port A1):	6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0
Solubilization buffer (port A2):	6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0
Elution buffer (port A3):	20 mM Tris-HCl, 0.5 M NaCl, 0.5 M imidazole, 1 mM 2-mercaptoethanol, pH 8.0
Refolding buffer (port B):	20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0
	Prepare at least 500 ml of each eluent.

Alternative binding buffers: 5 to 40 mM imidazole can be included in the binding buffer to reduce nonspecific binding of non-histidine-tagged proteins. The concentration of imidazole is protein dependent, and if the protein of interest elutes or does not bind at a certain imidazole concentration, reduce the concentration.

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. Remove supernatant and resuspend pellet in 3 ml of cold isolation buffer. 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 of binding buffer.
2. Stir for 30 to 60 min at room temperature.
3. Centrifuge for 15 min at high speed, 4°C.
4. Remove any remaining particles by passing sample through a 0.45 µm filter.

The optimal concentration of 2-mercaptoethanol (0 to 20 mM) must be determined experimentally for each individual protein.

If it has not been prepared as above, adjust the sample to the composition of binding buffer by diluting in binding buffer or by buffer exchange using HiTrap Desalting or HiPrep 26/10 Desalting, then pass the sample through a 0.45 µm filter.

Preparing the system

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

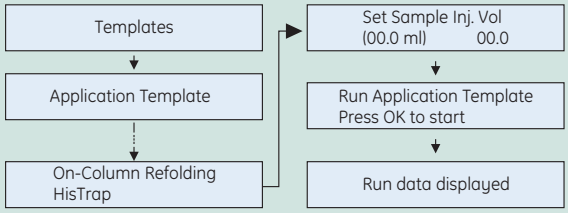
This example uses ÄKTAprime plus. Once the system is prepared, the remaining steps (under Selecting Application Template and starting the method) will be performed automatically.

1. Place each inlet tubing from port A (8-port valve) in eluents as given above and the tubing from port B (2-port valve) in the elution buffer.
2. Place the three brown waste tubings in waste.
3. Connect the column between port 1 on the injection valve (7-port valve) and the UV flow cell.
4. Fill the fraction collector rack with 18-mm tubes (minimum 40) and position the white plate on the fractionation arm against the first tube.
5. Connect a sample loop large enough for your sample between port 2 and 6 on the injection valve. Use a syringe to manually fill the loop.

   Note: If a Superloop is needed, additional information is supplied in the instructions for Superloop.

Selecting Application Template and starting the method

1. Check the communication to PrimeView. At the lower right corner of the screen the text **Controlled By:** prime should be displayed.
2. Use the arrow and OK buttons to move in the menu tree until you find **On-Column Refolding HisTrap**.



3. Enter the sample volume and press **OK** to start the template.

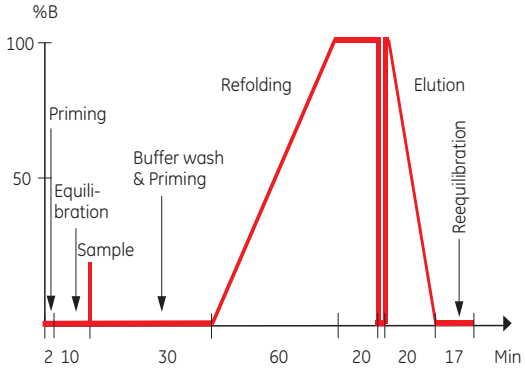
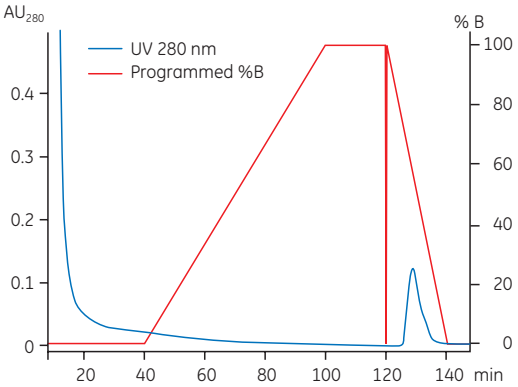


Fig 84. Theoretical gradient in **On-column Refolding HisTrap Application Template.**



Sample:	Clarified homogenate of *E. coli* expressing histidine-tagged protein
Column:	HisTrap FF 1 ml
Binding buffer (A1):	6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1mM 2-mercaptoethanol, pH 8.0
Solubilization buffer (port A2):	6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1mM 2-mercaptoethanol, pH 8.0
Elution buffer (port A3):	20 mM Tris-HCl, 0.5 M NaCl, 0.5 M imidazole, 1 mM 2-mercaptoethanol, pH 8.0
Refolding buffer (port B):	20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0

Fig 85. Typical results for on-column refolding of a histidine-tagged protein.

Troubleshooting

Situation	Possible cause	Remedy
High back pressure	Column clogged	Clean the column according to instructions or replace it with a fresh column. Make sure the sample has been centrifuged and/or filtered through a 0.45 µm filter.
	System clogged	Replace the column with a piece of tubing. Check pressure. If back pressure > 0.3 MPa, clean system according to manual.
No binding	–	Check that the correct column is used.
		Check that the inlet tubing from each buffer is connected to the correct inlet port.
		Check that correct "method program" was selected.
		Check that the composition and pH of the buffers are correct.
		Check that the sample has been adjusted to binding buffer conditions.
No elution	–	Check that the inlet tubing from each buffer is connected to the correct inlet port.
		Check that correct "method program" was selected.
		Check that the composition and pH of the buffers are correct.
		Use alternative elution conditions according to the column instructions.
		Check flow of buffer by looking for liquid coming from the outlet of the system.

Chapter 9
Desalting and buffer exchange

Desalting columns provide a simple and fast method to remove salt or other small molecules from a sample and exchange its buffer composition. The technique can also be used for adjusting to a higher salt concentration or for removal of additives that are not needed in subsequent use of the material. The columns are packed with Sephadex™ G-25, a gel filtration product that separates molecules on the basis of size. Desalting provides several advantages over dialysis, which is generally a slow technique that requires large volumes of buffer and carries a risk of losing material during handling or because of proteolytic breakdown, aggregation, or nonspecific binding of samples to the dialysis membranes.

With desalting columns, in a single step the sample is desalted, transferred into a new buffer, and low molecular weight materials are removed, all within minutes. The columns 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 approximately 70 mg/ml when using normal aqueous buffers, and provided that the protein is stable and soluble at the concentration used. Table 27 shows a selection guide for prepacked, ready-to-use desalting and buffer exchange columns.

Table 27. Selection guide for desalting/buffer exchange columns.

Column	Medium	Loaded volume (ml)	Eluted volume (ml)	Dilution factor	Operation
NAP™-5	Sephadex G-25 DNA Grade	0.1	0.5	5	gravity
		0.25	0.7	2.8	gravity
		0.5 (max.)	1.0	2	gravity
NAP-10	Sephadex G-25 DNA Grade	0.75	1.2	1.6	gravity
		1.0 (max.)	1.5	1.5	gravity
PD-10 Desalting	Sephadex G-25 Medium	1.5	3.5	1–1.5	gravity
		2.0	3.5	1–1.3	gravity
		2.5 (max.)	3.5	1–1.3	gravity
HiTrap Desalting	Sephadex G-25 Superfine	0.25	1.0	4	syringe/pump
		0.5	1.5	3	syringe/pump
		1.0	2.0	2	syringe/pump
		1.5 (max.)	2.0	1.3	syringe/pump
2x HiTrap Desalting	Sephadex G-25 Superfine	3.0 (max.)	4–5	1.3–1.7	syringe/pump
3x HiTrap Desalting	Sephadex G-25 Superfine	4.5 (max.)	6–7	1.3–1.7	syringe/pump

continues on following page

Table 27. Selection guide for desalting/buffer exchange columns (continued).

Column	Medium	Loaded volume (ml)	Eluted volume (ml)	Dilution factor	Operation
HiPrep 26/10 Desalting	Sephadex G-25 Fine	10	10–15	1–1.5	pump
		15 (max.)	15–20	1–1.3	pump
2x HiPrep 26/10 Desalting	Sephadex G-25 Fine	30 (max.)	30–40	1–1.3	pump
3x HiPrep 26/10 Desalting	Sephadex G-25 Fine	45 (max.)	45–55	1–1.2	pump
4x HiPrep 26/10 Desalting	Sephadex G-25 Fine	60 (max.)	60–70	1–1.2	pump

To desalt larger sample volumes:

Connect up to five HiTrap Desalting columns in series to increase the sample volume capacity, for example, two columns, sample volume 3 ml; five columns, sample volume 7.5 ml.

Connect up to four HiPrep 26/10 Desalting columns in series to increase the sample volume capacity, for example, two columns, sample volume 30 ml; four columns, sample volume 60 ml. Even with four columns in series the sample can be processed in 20 to 30 minutes.

Buffer preparation:

For substances carrying charged groups, an eluent containing a buffer salt is recommended. A salt concentration of at least 25 mM is recommended to prevent possible ionic interactions with the medium. Sodium chloride is often used for this purpose. Often a buffer with 25 to 50 mM concentration of the buffering substance is sufficient.

At salt concentrations above 1.0 M, hydrophobic substances may be retarded or bind to the medium. At even higher salt concentrations [> 1.5 M $(NH_4)_2SO_4$], the column packing shrinks.

Sample preparation:

The sample concentration does not influence the separation as long as the viscosity does not differ by more than a factor of 1.5 from that of the buffer used. This corresponds to a maximum concentration of 70 mg/ml for proteins or 5 mg/ml for high molecular weight polymers such as dextran, when normal aqueous buffers are used.

The sample should be fully solubilized. Centrifuge or filter (0.45 µm filter) immediately before loading to remove particulate material if necessary.

Protein solubility often depends on pH and/or ionic strength (salt concentration), and the exchange of buffer may therefore result in precipitation of the protein. Also, protein activity can be lost if the change of pH takes it outside of the range where the protein is active.

The protocols below describe desalting and buffer exchange using PD-10 Desalting, HiTrap Desalting, and HiPrep 26/10 Desalting columns.

PD-10 Desalting

PD-10 Desalting columns are packed with Sephadex G-25 medium for group separation of high (M_r > 5 000) from low molecular weight substances (M_r < 1 000) by desalting and buffer exchange. The medium is held within two sintered polyethylene frits. The frits protect the medium from running dry under gravitational buffer flow. The outlet to the column is sealed with a reusable cap.

Each column can process a sample volume up to 2.5 ml by gravity flow, and multiple samples can be processed in parallel. PD-10 columns are recommended for use with bulk media for affordable and versatile sample preparation.

The columns are packaged with a column stand—the PD-10 Desalting Workmate; LabMate buffer reservoir is available for easy equilibration.

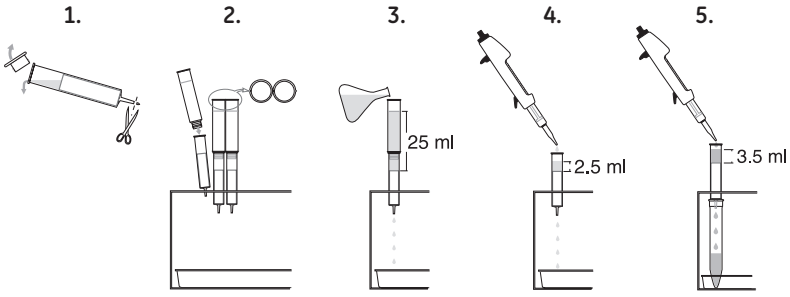
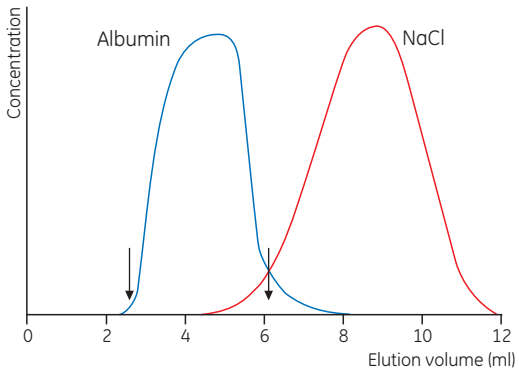


Fig 86. Schematic of the method used with PD-10 Desalting columns. (1) Preparation of the column; (2) attachment of the LabMate Buffer Reservoir, (3) column equilibration, (4) sample application, (5) elution and collection of sample.

1. Cut off bottom tip, remove top cap, and pour off excess liquid.
2. If available, mount the LabMate Buffer Reservoir on top of the PD-10 column and place the columns in the PD-10 Desalting Workmate.
3. Equilibrate the column with approximately 25 ml of buffer. Discard the flowthrough (you can use the plastic tray to collect the flowthrough).
4. Add sample of a total volume of 2.5 ml. If the sample is less than 2.5 ml, add buffer until the total volume of 2.5 ml is achieved. Discard the flowthrough.
5. Elute with 3.5 ml of buffer and collect the flowthrough. A typical separation is shown in Figure 87.



Column: PD-10
Sample: HSA, 25 mg in 2.5 ml 0.5 M NaCl
Equilibration: Distilled water

Fig 87. Removal of NaCl from albumin solution. A PD-10 Desalting column was equilibrated with distilled water. The sample contained human serum albumin (25 mg) dissolved in 2.5 ml 0.5 M NaCl solution. A total of 23.8 mg albumin was recovered in 3.5 ml eluent corresponding to a yield of 95.3% (between arrows). Initial total salt content of sample before desalting was 2.0%.

HiTrap Desalting

Fig 88. HiTrap Desalting allows efficient, easy-to-perform group separations with a syringe or pump.

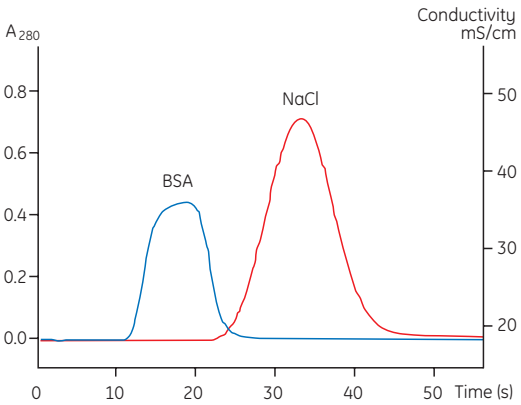
HiTrap Desalting is a 5-ml column packed with the well-known size-exclusion medium Sephadex G-25 Superfine. The medium is based on cross-linked dextran beads that allow excellent resolution and high flow rates. The fractionation range for globular proteins is between M_r 1 000 and 5 000, with an exclusion limit of approximately M_r 5 000. This ensures group separations of proteins/peptides larger than M_r 5 000 from molecules with a molecular weight less than M_r 1 000.

HiTrap Desalting can be used with aqueous solutions in the pH range 2 to 13. It is stable to all commonly used buffers, solutions of urea (8 M), guanidine hydrochloride (6 M), and all nonionic and ionic detergents. Lower alcohols (methanol, ethanol, propanol) may be used in the buffer or the sample, but we recommend that the concentration be kept below 25 v/v%. Prolonged exposure (hours) to pH values below 2 or above 13, or to oxidizing agents, should be avoided.

The recommended range of sample volumes is 0.1 to 1.5 ml when complete removal of low molecular weight components is desired. The separation is not affected by the flow rate, in the range 1 to 10 ml/min. The maximum recommended flow rate is 15 ml/min. The column cannot be opened or repacked.

Separations are easily performed with a syringe, a pump, or a chromatography system such as one of the ÄKTAdesign systems. Up to five columns can be connected in series, allowing larger sample volumes to be handled. Refer to Scaling up on page 197 for further discussion of these options.

Figure 89 shows a typical desalting and buffer exchange separation achieved using HiTrap Desalting and monitored by following changes in UV absorption and conductivity.



Column: HiTrap Desalting
Sample: 2 mg/ml BSA in 50 mM sodium phosphate, 0.5 M NaCl, pH 7.0
Sample volume: 1.4 ml
Buffer: 50 mM sodium phosphate, 0.15 M NaCl, pH 7.0
Flow rate: 10 ml/min
Detection: UV (280 nm, 5 mm cell) and conductivity

Fig 89. Highly efficient desalting in 30 seconds using HiTrap Desalting.

To avoid cross-contamination, use the column only with the same type of sample.

Column equilibration

1. Fill the syringe or pump tubing with buffer. Remove the stopper. To avoid introducing air into the column, connect the column "drop to drop" to either the syringe (via the connector) or to the pump tubing.
2. Remove the snap-off end at the column outlet.
3. Wash the column with 25 ml of 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 into 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.

Manual desalting using a syringe

1. To operate the column with a syringe, connect the syringe to the column using the supplied Luer connector.
2. Equilibrate the column; see above, Column equilibration.
3. Apply the sample using a 2- to 5-ml syringe at a flow rate between 1 and 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.
4. Elute the protein with the appropriate volume selected from Table 28. *Collect the desalted protein in the volume indicated.*

The maximum recommended sample volume is 1.5 ml (when using one HiTrap Desalting 5-ml column). See Table 28 for the effect of reducing the sample volume applied to the column.

Table 28. Recommended sample and elution volumes using a syringe, with examples of typical yields and remaining salt in the desalted sample.

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

The void volume of the column is 1.5 ml. High molecular weight components elute between 1.5 and 4.5 ml, depending on the sample volume. Low molecular weight components start to elute after 3.5 ml.

Note: Certain types of molecules, such as small heterocyclic or homocyclic aromatic compounds (purines, pyrimidines, dye stuffs) can interact with Sephadex and are therefore eluted later than expected. Larger sample volumes can be used in these cases, but the separation has to be optimized for each case.

Desalting using a pump

1. Equilibrate the column; see Column equilibration.
2. Apply up to 1.5 ml of sample. Monitor the effluent from the column with a UV monitor and/or a conductivity monitor. Keep the flow rate in the range 1 to 10 ml/min. Collect fractions.
3. Elute the column with approximately 10 ml of buffer before applying the next sample. Collect fractions.

Desalting with ÄKTAprime plus

ÄKTAprime plus contains preprogrammed templates for individual HiTrap Desalting and HiPrep Desalting 26/10 columns. The procedure below uses a HiTrap Desalting 5-ml column.

Buffer preparation

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

Buffer (port A1): 20 mM sodium phosphate, 0.15 M NaCl, pH 7.0
Prepare at least 500 ml of the required buffer.

Sample preparation

Pass the sample through a 0.45 µm filter.

The maximum recommended sample volume is 1.5 ml.

Preparing ÄKTAprime plus

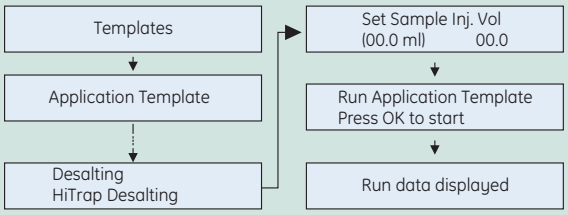
Once the system is prepared, the remaining steps (under Selecting Application Template and starting the method) will be performed automatically.

1. Place the inlet tubing from port A (8-port valve) and port B (2-port valve) in the buffer.
2. Place the three brown waste tubings in waste.
3. Connect the column between port 1 on the injection valve (7-port valve) and the UV flow cell.
4. Fill the fraction collector rack with 18-mm tubes (minimum 20) and position the white plate on the fractionation arm against the first tube.
5. Connect a sample loop large enough for your sample between ports 2 and 6 on the injection valve. Use a syringe to manually fill the loop.

   Note: If a Superloop is needed, additional information is supplied in the instructions for Superloop.

Selecting Application Template and starting the method

1. Check the communication to PrimeView. At the lower right corner of the screen the text **Controlled By:** prime should be displayed.
2. Use the arrow and OK buttons to move in the menu tree until you find **Desalting HiTrap Desalting.**



3. Enter the sample volume and press **OK** to start the template.

Figure 90 shows a theoretical desalting chromatogram, and Figure 91 shows typical results obtained for buffer exchange of a histidine-tagged protein. The UV and conductivity traces enable the appropriate desalted fractions to be pooled.

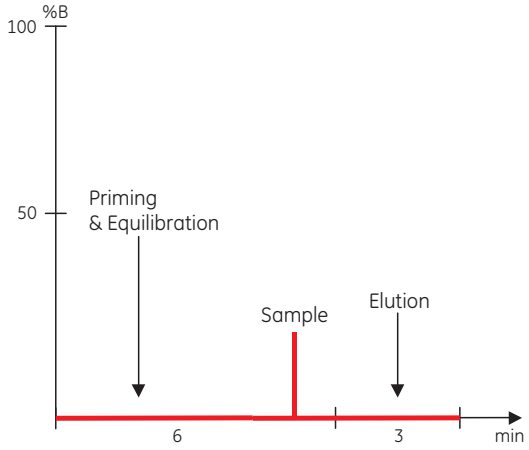
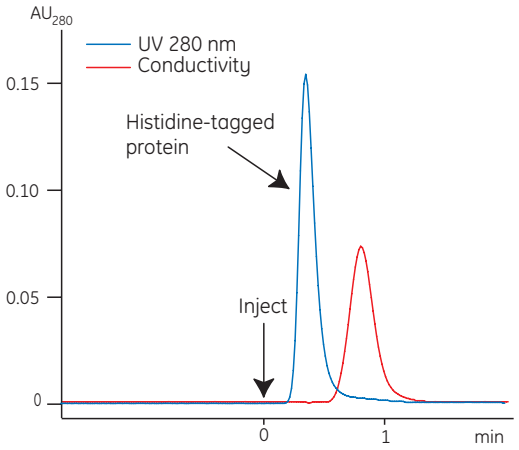


Fig 90. Theoretical gradient in **Desalting, HiTrap Desalting Application Template**. Total separation time = 9 min + sample application time.



Sample: Histidine-tagged protein in 20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4
Column: HiTrap Desalting 5 ml
Buffer (A1): 20 mM sodium phosphate, 0.15 M NaCl, pH 7.0

Fig 91. Typical results for buffer exchange of a histidine-tagged protein.

Scaling up

For separation of sample volumes larger than 1.5 ml, or to increase the resolution between high and low molecular weight components, up to five HiTrap Desalting columns can easily be connected in series (see Table 27). For syringe operations, the volumes suggested in Table 28 should be increased proportionally and the recommended flow rate maintained. The dilution of the sample is dependent on the sample volume and the number of columns used in series. Lower dilution factors than those proposed in Table 28 can be obtained, but the elution volumes have to be optimized for each combination of sample volume and number of columns in series. The back pressure for each column is approximately 0.25 bar at 10 ml/min. For sample volumes up to 15 ml, HiPrep 26/10 Desalting is available (see below). Up to four HiPrep 26/10 Desalting columns can be connected in series without passing the pressure limit (up to 60 ml sample volume), assuming that the sample has essentially the same viscosity as the eluent (see Table 27).

HiPrep 26/10 Desalting

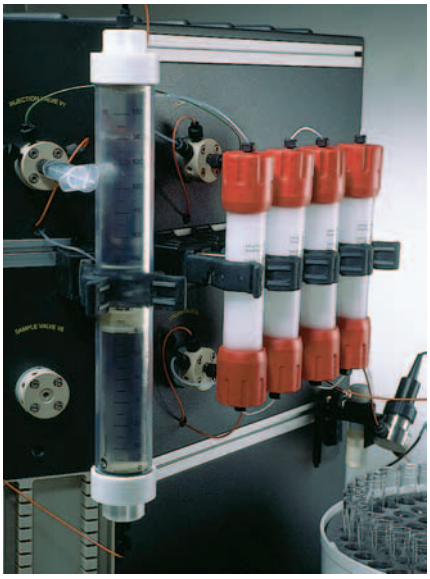


Fig 92. Sixty-ml sample sizes can be run on four HiPrep 26/10 Desalting columns coupled in series.

HiPrep 26/10 Desalting is packed with Sephadex G-25 Fine. It provides group separation of high (M_r > 5 000) from low molecular weight substances (M_r < 1 000), allowing reliable and reproducible desalting and buffer exchange with sample sizes of 15 ml per column. Two to four columns can be used in series for sample sizes of 30 to 60 ml. For more details, see Table 27.

Buffer exchange on HiPrep 26/10 Desalting *with* ÄKTAprime plus

Buffer preparation

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

Buffer (port A1): 20 mM sodium phosphate, 0.15 M NaCl, pH 7.0.
Prepare at least 500 ml of eluent.

Sample preparation

Pass the sample through a 0.45 µm filter.

The maximum recommended sample volume is 15 ml.

Preparing ÄKTAprime plus

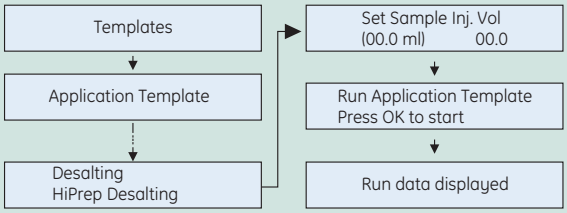
Once the system is prepared, the remaining steps (under Selecting Application Template and starting the method) will be performed automatically.

1. Place the inlet tubing from port A (8-port valve) and port B (2-port valve) in the buffer.
2. Place the three brown waste tubings in waste.
3. Connect the column between port 1 on the injection valve (7-port valve) and the UV flow cell.
4. Fill the fraction collector rack with 18-mm tubes (minimum 25) and position the white plate on the fractionation arm against the first tube.
5. Connect a sample loop large enough for your sample between port 2 and 6 on the injection valve. Use a syringe to manually fill the loop.

   Note: If a Superloop is needed, additional information is supplied in the instructions for Superloop.

Selecting Application Template and starting the method

1. Check the communication to PrimeView. At the lower right corner of the screen the text **Controlled By:** prime should be displayed.
2. Use the arrow and OK buttons to move in the menu tree until you find **Desalting HiPrep Desalting.**



3. Enter the sample volume and press **OK** to start the template.

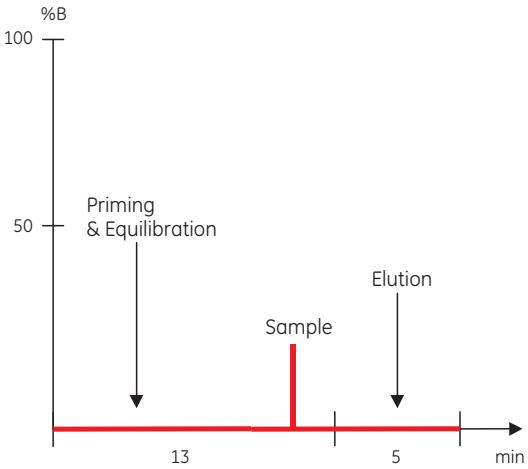
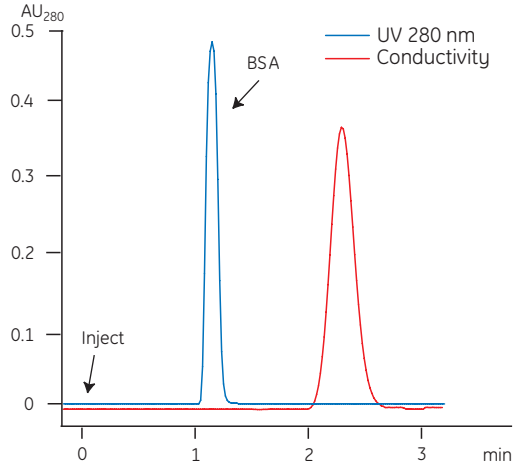


Fig 93. Theoretical gradient in **Desalting, HiPrep Desalting Application Template.** Total separation time = 18 min + sample application time.



Sample: BSA and sodium chloride
Column: HiPrep 26/10 Desalting
Buffer (port A1): 20 mM phosphate, 0.15 M NaCl, pH 7.0

Fig 94. Typical results for buffer exchange of BSA.

Appendix 1
Characteristics of Ni Sepharose and uncharged IMAC Sepharose products

Ni Sepharose products

Ni Sepharose High Performance is recommended for high-resolution purification of histidine-tagged proteins, providing sharp peaks and concentrated eluate. Ni Sepharose 6 Fast Flow is excellent for scaling up and batch purifications.

Table 29 summarizes key characteristics of bulk Ni Sepharose media, and Table 30 lists the stability of the media under various conditions. Tables 31 to 37 summarize the characteristics of these same media as prepacked columns and as prepacked 96-well plates. For more information, refer to Chapter 3.

Table 29. Characteristics of Ni Sepharose High Performance and Ni Sepharose 6 Fast Flow.

Characteristics	Ni Sepharose High Performance	Ni Sepharose 6 Fast Flow
Matrix	Highly cross-linked 6% agarose, precharged with Ni^{2+}	Highly cross-linked 6% agarose, precharged with Ni^{2+}
Metal ion capacity	Approx. 15 µmol Ni^{2+}/ml medium	Approx. 15 µmol Ni^{2+}/ml medium
Average particle size	34 µm	90 µm
Dynamic binding capacity[1]	At least 40 mg $(histidine)_6$-tagged protein/ml medium	Approx. 40 mg $(histidine)_6$-tagged protein/ml medium
Recommended flow rate[2]	< 150 cm/h	50–400 cm/h
Compatibility during use	Stable in all commonly used buffers, reducing agents, denaturing agents and detergents. See Table 30 for more information.	Stable in all commonly used buffers, reducing agents, denaturing agents and detergents. See Table 30 for more information.
Chemical stability[3]	For one week at 40°C: 0.01 M HCl, 0.1 M NaOH For 12 h: 1 M NaOH, 70% acetic acid 30 min tested: 30% 2-propanol 1 h tested: 2% SDS	For one week at 40°C: 0.01 M HCl, 0.1 M NaOH For 12 h: 1 M NaOH, 70% acetic acid 30 min tested: 30% 2-propanol 1 h tested: 2% SDS
pH stability[3]	Short term (≤ 2 hours) 2–14 Long term (≤ 1 week) 3–12	Short term (≤ 2 hours) 2–14 Long term (≤ 1 week) 3–12
Storage	20% ethanol	20% ethanol
Storage temperature	4–30°C	4–30°C

[1] Dynamic binding capacity conditions:

Sample: *1 mg/ml $(histidine)_6$-tagged pure protein (M_r 43 000) in binding buffer or $(histidine)_6$-tagged protein (M_r 28 000) bound from E. coli extract. Capacity at 10% breakthrough.*

Column volume: *0.25 ml or 1 ml*

Flow rate: *0.25 ml/min or 1 ml/min, respectively*

Binding buffer: *20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4*

Elution buffer: *20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4*

***Note**: Dynamic binding capacity is protein dependent.*

[2] H_2O at room temperature.

[3] Ni^{2+}-stripped medium.

Table 30. Compatibility guide: Ni Sepharose High Performance and Ni Sepharose 6 Fast Flow are stable toward these compounds at least at the concentrations given.

Compound	Concentration
Reducing agents[1]	5 mM DTE 5 mM DTT 20 mM β-mercaptoethanol 5 mM TCEP 10 mM reduced glutathione
Denaturing agents	8 M urea[2] 6 M guanidine-HCl[2]
Detergents	2% Triton X-100 (nonionic) 2% Tween 20 (nonionic) 2% NP-40 (nonionic) 2% cholate (anionic) 1% CHAPS (zwitterionic)
Other additives	20% ethanol 50% glycerol 500 mM imidazole 100 mM Na_2SO_4 1.5 M NaCl 1 mM EDTA[3] 60 mM citrate[2]
Buffers	50 mM sodium phosphate, pH 7.4 100 mM Tris-HCl, pH 7.4 100 mM Tris-acetate, pH 7.4 100 mM HEPES, pH 7.4 100 mM MOPS, pH 7.4 100 mM sodium acetate, pH 4[2]

[1] Before performing runs with sample/buffers containing reducing reagents, a blank run with binding and elution buffers excluding reducing agents is recommended, see page 33.

[2] Tested for one week at 40°C.

[3] The strong chelator EDTA has been used successfully in some cases, at 1 mM. Generally, chelating agents should be used with caution (and only in the sample, not in the buffers). Any metal-ion stripping may be counteracted by addition of a small excess of $MgCl_2$ before centrifugation/filtration of the sample. Note that stripping effects may vary with applied sample volume.

Table 31. Characteristics of His MultiTrap HP and His MultiTrap FF.

Media	His MultiTrap HP: Ni Sepharose High Performance His MultiTrap FF: Ni Sepharose 6 Fast Flow
Filter plate size[1]	127.8 × 85.5 × 30.6 mm
Filter plate material	Polypropylene and polyethylene
Binding capacity[2]	His MultiTrap HP: Up to 1 mg histidine-tagged protein/well His MultiTrap FF: Up to 0.8 mg histidine-tagged protein/well
Reproducibility between wells	+/- 10%
Volume packed medium/well	50 µl
Number of wells	96
Well volume	800 µl
Max. sample loading volume	600 µl
pH stability[3]	2–14 (short term), 3–12 (long term)
Storage	20% ethanol
Storage temperature	4–30°C

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

[2] Protein binding capacity is protein dependent.

[3] Ni^{2+}-stripped medium.

Table 32. Characteristics of His SpinTrap.

Medium	Ni Sepharose High Performance
Average particle size	34 µm
Bed volume	100 µl
Column material	Polypropylene barrel and polyethylene frits
Protein binding capacity[1]	Approx. 0.75 mg histidine-tagged protein/column
Compatibility during use	Stable in all commonly used buffers, reducing agents, denaturants and detergents. See Table 30 for more information.
Storage	0.15% Kathon CG
Storage temperature	4–30°C

[1] Protein binding capacity is protein dependent.

Table 33. Characteristics of HisTrap HP and HisTrap FF.

Media	HisTrap HP: Ni Sepharose High Performance HisTrap FF: Ni Sepharose 6 Fast Flow
Column volume	1 ml and 5 ml
Column dimensions	0.7 × 2.5 cm (1 ml); 1.6 × 2.5 cm (5 ml)
Dynamic binding capacity[1]	HisTrap HP: At least 40 mg histidine-tagged protein/ml medium HisTrap FF: Approx. 40 mg histidine-tagged protein/ml medium
Recommended flow rate	1 ml/min (1 ml); 5 ml/min (5 ml)
Max. flow rate[2]	4 ml/min (1 ml); 20 ml/min (5 ml)
Max. pressure[2]	0.3 MPa, 3 bar
pH stability[3]	2–14 (short term), 3–12 (long term)
Compatibility	Stable in all commonly used buffers, reducing agents, denaturants and detergents. See Table 30 for more information.
Chemical stability[3]	For one week at 40°C: 0.01 M HCl, 0.1 M NaOH For 12 h: 1 M NaOH, 70% acetic acid 30 min tested: 30% 2-propanol 1 h tested: 2% SDS
Storage	20% ethanol
Storage temperature	4–30°C

[1] Dynamic binding capacity conditions:

Sample: *1 mg/ml (histidine)$_6$-tagged pure protein (M_r 43 000) in binding buffer or (histidine)$_6$-tagged protein (M_r 28 000) bound from E. coli extract. Capacity at 10% breakthrough.*

Column volume: *0.25 ml or 1 ml*

Flow rate: *0.25 ml/min or 1 ml/min, respectively*

Binding buffer: *20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4*

Elution buffer: *20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4*

***Note**: Dynamic binding capacity is protein dependent.*

[2] H_2O at room temperature.

[3] Ni^{2+}-stripped medium.

Table 34. Characteristics of HisTrap FF crude.

Medium	Ni Sepharose 6 Fast Flow
Average particle size	90 µm
Column volume	1 ml and 5 ml
Column dimensions	0.7 × 2.5 cm (1 ml); 1.6 × 2.5 cm (5 ml)
Dynamic binding capacity[1]	Approx. 40 mg histidine-tagged protein/ml medium
Recommended flow rate[2]	1 ml/min (1 ml); 5 ml/min (5 ml)
Max. pressure[2]	3 bar (0.3 MPa, 42 psi)
Compatibility during use	Stable in all commonly used buffers, reducing agents, denaturing agents and detergents. See Table 30 for more information.
Chemical stability[3]	For one week at 40°C: 0.01 M HCl, 0.1 M NaOH For 12 h: 1 M NaOH, 70% acetic acid 30 min tested: 30% 2-propanol 1 h tested: 2% SDS
pH stability[3]	2–14 (short term), 3–12 (long term)
Storage	20% ethanol
Storage temperature	4–30°C

[1] Dynamic binding capacity conditions:

Sample: *1 mg/ml $(histidine)_6$-tagged pure protein (M_r 43 000) in binding buffer or $(histidine)_6$-tagged protein (M_r 28 000) bound from E. coli extract. Capacity at 10% breakthrough.*

Column volume: *0.25 ml or 1 ml*

Flow rate: *0.25 ml/min or 1 ml/min, respectively*

Binding buffer: *20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4*

Elution buffer: *20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4*

***Note**: Dynamic binding capacity is protein dependent.*

[2] H_2O at room temperature.

[3] Ni^{2+}-stripped medium.

Table 35. Characteristics and contents of HisTrap FF crude Kit.

Contents of kit	3 × 1 ml HisTrap FF crude columns[1] 2 × 50 ml phosphate buffer, 8× stock, pH 7.4 50 ml 2 M imidazole, pH 7.4 1 syringe, 5 ml Connectors Instructions

[1] See Table 34 for the characteristics of HisTrap FF crude columns.

Table 36. Characteristics of His GraviTrap.

Medium	Ni Sepharose 6 Fast Flow
Average particle size	90 µm
Bed volume	1 ml
Column material	Polypropylene barrel, polyethylene frits
Protein binding capacity[1]	Approx. 40 mg histidine-tagged protein/column
Compatibility during use	Stable in all commonly used buffers, reducing agents, denaturing agents and detergents. See Table 30 for more information.
Chemical stability[2]	For one week at 40°C: 0.01 M HCl, 0.1 M NaOH For 12 h: 1 M NaOH, 70% acetic acid 30 min tested: 30% 2-propanol 1 h tested: 2% SDS
Storage	20% ethanol
Storage temperature	4–30°C

[1] Protein binding capacity is protein dependent.

[2] Ni^{2+}-stripped medium.

Table 37. Characteristics of HisPrep FF 16/10.

Medium	Ni Sepharose 6 Fast Flow
Column volume	20 ml
Column dimensions	1.6 × 10 cm
Dynamic binding capacity[1]	Approx. 40 mg histidine-tagged protein/ml medium
Recommended flow rate[2]	2–10 ml/min (60–300 cm/h)
Max. flow rate[2]	10 ml/min (300 cm/h)
Max. pressure over the packed bed during operation[2]	1.5 bar (0.15 MPa, 22 psi)
Column hardware pressure limit	5 bar (0.5 MPa, 73 psi)
Compatibility during use	Stable in all commonly used buffers, reducing agents, denaturing agents and detergents. See Table 30 for more information.
Chemical stability[3]	For one week at 40°C: 0.01 M HCl, 0.1 M NaOH For 12 h: 1 M NaOH, 70% acetic acid 30 min tested: 30% 2-propanol 1 h tested: 2% SDS
Storage	20% ethanol
Storage temperature	4–30°C

[1] Dynamic binding capacity conditions:

Sample: *1 mg/ml (histidine)$_6$-tagged pure protein (M_r 43 000) in binding buffer or (histidine)$_6$-tagged protein (M_r 28 000) bound from E. coli extract. Capacity at 10% breakthrough.*

Column volume: *0.25 ml or 1 ml*

Flow rate: *0.25 ml/min or 1 ml/min, respectively*

Binding buffer: *20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4*

Elution buffer: *20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4*

***Note**: Dynamic binding capacity is protein dependent.*

[2] H_2O at room temperature.

[3] Ni^{2+}-stripped medium.

Stripping, recharging, and cleaning of Ni Sepharose products

Stripping and recharging

Ni Sepharose High Performance and Ni Sepharose 6 Fast Flow do not have to be stripped and recharged between each purification if the same protein is to be purified. It may be sufficient to strip and recharge it after approximately two to five purifications, depending on the specific sample, sample pretreatment, sample volume, etc.

Stripping buffer: 20 mM sodium phosphate, 500 mM NaCl, 50 mM EDTA, pH 7.4

1. Strip the media by washing with at least 5 to 10 column volumes of stripping buffer.
2. Wash with at least 5 to 10 column volumes of binding buffer.
3. Immediately wash with 5 to 10 column volumes of distilled water.
4. Recharge the water-washed column by loading 0.5 column volumes of 0.1 M $NiSO_4$ in distilled water onto the column.
5. Wash with 5 column volumes of distilled water, and 5 column volumes of binding buffer (to adjust pH) before storage in 20% ethanol. Salts of other metals, chlorides, or sulfates may also be used.

It is important to wash with binding buffer as the last step to obtain the correct pH before storage.

Washing with buffer before applying the metal ion solution may cause unwanted precipitation.

Cleaning-in-place

When an increase in back pressure is seen, the medium should be cleaned. Before cleaning, strip off metal ions using the recommended procedure described above. The **stripped** medium can be cleaned by the following methods:

To remove ionically bound protein:

1. Wash with several column volumes of 1.5 M NaCl.
2. Immediately wash with approximately 10 column volumes of distilled water.

To remove precipitated proteins, hydrophobically bound proteins, and lipoproteins:

1. Wash the column with 1 M NaOH, contact time usually 1 to 2 h (12 h or more for endotoxin removal).
2. Immediately wash with approximately 10 column volumes of binding buffer, followed by 5 to10 column volumes of distilled water.

To remove hydrophobically bound proteins, lipoproteins, and lipids:

1. Wash with 5 to 10 column volumes of 30% isopropanol for about 15 to 20 min.
2. Immediately wash with approximately 10 column volumes of distilled water.

2a. Alternatively, wash with 2 column volumes of detergent in a basic or acidic solution. Use, for example, 0.1 to 0.5% nonionic detergent in 0.1 M acetic acid, contact time 1 to 2 h. After treatment, always remove residual detergent by washing with at least 5 column volumes of 70% ethanol. Then wash with approximately 10 column volumes of distilled water.

Reversed flow may improve the efficiency of the cleaning-in-place procedure. After cleaning, store in 20% ethanol (wash with 5 column volumes) or recharge with Ni^{2+} prior to storage in ethanol.

Uncharged IMAC Sepharose products

IMAC Sepharose High Performance is recommended for high-resolution purifications, providing sharp peaks and concentrated eluate. IMAC Sepharose 6 Fast Flow is excellent for scaling up.

Table 38 summarizes key characteristics of IMAC Sepharose media, and Table 39 lists the stability of the media under various conditions. Tables 40 and 41 summarize the characteristics of the media as prepacked columns. For more information, refer to Chapter 3.

Table 38. Characteristics of IMAC Sepharose High Performance and IMAC Sepharose 6 Fast Flow.

Characteristics	IMAC Sepharose High Performance	IMAC Sepharose 6 Fast Flow
Matrix	Highly cross-linked 6% spherical agarose	Highly cross-linked 6% spherical agarose
Metal ion capacity	Approx. 15 µmol Ni^{2+}/ml medium	Approx. 15 µmol Ni^{2+}/ml medium
Average particle size	34 µm	90 µm
Dynamic binding capacity[1]	At least 40 mg $(histidine)_6$-tagged protein/ml medium (Ni^{2+}-charged)	*Histidine-tagged protein:* Approx. 40 mg $(histidine)_6$-tagged protein/ml medium (Ni^{2+}-charged) *Untagged protein:* Approx. 25 mg/ml medium (Cu^{2+}-charged); approx. 15 mg/ml medium (Zn^{2+}- or Ni^{2+}-charged).
Recommended flow rate[2]	< 150 cm/h	150 cm/h
Compatibility during use	Stable in all commonly used buffers, reducing agents, denaturing agents and detergents. See Table 39 for more information.	Stable in all commonly used buffers, reducing agents, denaturing agents and detergents. See Table 39 for more information.
Chemical stability[3]	For one week at 40°C: 0.01 M HCl, 0.1 M NaOH For 12 h: 1 M NaOH, 70% acetic acid 30 min tested: 30% 2-propanol 1 h tested: 2% SDS	For one week at 40°C: 0.01 M HCl, 0.1 M NaOH For 12 h: 1 M NaOH, 70% acetic acid 30 min tested: 30% 2-propanol 1 h tested: 2% SDS
pH stability[3]	Short term (≤ 2 hours): 2–14 Long term (≤ 1 week): 3–12	Short term (≤ 2 hours): 2–14 Long term (≤ 1 week): 3–12
Storage	20% ethanol	20% ethanol
Storage temperature	4–30°C	4–30°C

[1] Conditions for determining dynamic binding capacity:

Samples: *$(Histidine)_6$-tagged proteins: Capacity data were obtained for a protein (M_r 28 000) bound from an E. coli extract, and a pure protein (M_r 43 000) applied at 1 mg/ml in binding buffer; capacity at 10% breakthrough. Untagged protein (IMAC Sepharose 6 Fast Flow only): Capacities determined at 10% breakthrough for human apotransferrin applied at 1 mg/ml in binding buffer.*

Column volume: 0.25 ml or 1 ml

Flow rate: *0.25 ml/min or 1 ml/min, respectively*

Binding buffer: *20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole (1 mM for untagged protein, IMAC Sepharose 6 Fast Flow only), pH 7.4*

Elution buffer: *20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole (50 mM for untagged protein, IMAC Sepharose 6 Fast Flow only), pH 7.4*

Note: *Dynamic binding capacity is metal ion and protein dependent.*

[2] H_2O at room temperature.

[3] Uncharged medium only. See Table 39 for more information.

Table 39. Compatibility guide: IMAC Sepharose High Performance and IMAC Sepharose 6 Fast Flow are stable toward these compounds at least at the concentrations given.

Compound	Concentration
Reducing agents[1]	5 mM DTE 5 mM DTT 20 mM β-mercaptoethanol 5 mM TCEP 10 mM reduced glutathione
Denaturing agents	8 M urea[2] 6 M guanidine-HCl[2]
Detergents	2% Triton X-100 (nonionic) 2% Tween 20 (nonionic) 2% NP-40 (nonionic) 2% cholate (anionic) 1% CHAPS (zwitterionic)
Other additives	20% ethanol 50% glycerol 500 mM imidazole 100 mM Na_2SO_4 1.5 M NaCl 1 mM EDTA[3] 60 mM citrate[2]
Buffers	50 mM sodium phosphate, pH 7.4 100 mM Tris-HCl, pH 7.4 100 mM Tris-acetate, pH 7.4 100 mM HEPES, pH 7.4 100 mM MOPS, pH 7.4 100 mM sodium acetate, pH 4[2]

[1] Before performing runs with sample/buffers containing reducing reagents, a blank run with binding and elution buffers excluding reducing agents is recommended (see page 79).

[2] Tested for one week at 40°C.

[3] The strong chelator EDTA has been used successfully in some cases at 1 mM. Generally, chelating agents should be used with caution (and only in the sample, not in the buffers). Any metal-ion stripping may be counteracted by adding a small excess of $MgCl_2$ before centrifuging/filtrating the sample. Note that stripping effects may vary with applied sample volume.

Table 40. Characteristics of HiTrap IMAC HP and HiTrap IMAC FF.

Media	HiTrap IMAC HP: IMAC Sepharose High Performance HiTrap IMAC FF: IMAC Sepharose 6 Fast Flow
Column volume	1 ml or 5 ml
Dynamic binding capacity[1]	At least 40 mg histidine-tagged protein/ml medium when charged with Ni^{2+}. For untagged proteins, HiTrap FF can bind approx. 25 mg/ml medium charged with Cu^{2+} or approx. 15 mg/ml medium charged with Zn^{2+} or Ni^{2+}.
Column dimensions	0.7 × 2.5 cm (1 ml); 1.6 × 2.5 cm (5 ml)
Recommended flow rate	1 ml/min (1 ml); 5 ml/min (5 ml)
Max flow rate[2]	4 ml/min (1 ml); 20 ml/min (5 ml)
Max. back pressure[2]	0.3 MPa, 3 bar
pH stability[3]	2–14 (short term), 3–12 (long term)
Compatibility during use	Stable in all commonly used buffers, reducing agents, denaturants and detergents. See Table 39 for more information.

continues on following page

Table 40. Characteristics of HiTrap IMAC HP and HiTrap IMAC FF (continued).

Chemical stability[3]	For one week at 40°C: 0.01 M HCl, 0.1 M NaOH For 12 h: 1 M NaOH, 70% acetic acid 30 min tested: 30% 2-propanol 1 h tested: 2% SDS
Storage	20% ethanol
Storage temperature	4–30°C

[1] Conditions for determining dynamic binding capacity:

Samples: *(Histidine)$_6$-tagged proteins: Capacity data were obtained for a protein (M_r 28 000) bound from an E. coli extract, and a pure protein (M_r 43 000) applied at 1 mg/ml in binding buffer; capacity at 10% breakthrough.*
Untagged protein (IMAC Sepharose 6 Fast Flow only): Capacities determined at 10% breakthrough for human apotransferrin applied at 1 mg/ml in binding buffer.

Column volume: 0.25 ml or 1 ml

Flow rate: *0.25 ml/min or 1 ml/min, respectively*

Binding buffer: *20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole (1 mM for untagged protein, IMAC Sepharose 6 Fast Flow only), pH 7.4*

Elution buffer: *20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole (50 mM for untagged protein, IMAC Sepharose 6 Fast Flow only), pH 7.4.*

Note: *Dynamic binding capacity is metal ion and protein dependent.*

[2] H_2O at room temperature.

[3] Uncharged medium only. See Table 39 for more information.

Table 41. Characteristics of HiPrep IMAC FF 16/10.

Medium	IMAC Sepharose 6 Fast Flow
Column volume	20 ml
Column dimensions	1.6 × 10 cm
Dynamic binding capacity[1]	Approx. 40 mg histidine-tagged protein/ml medium when charged with Ni^{2+}. For untagged proteins, HiTrap FF binds approx. 25 mg/ml medium charged with Cu^{2+} or approx. 15 mg/ml medium charged with Zn^{2+} or Ni^{2+}.
Recommended flow rate[2]	2–10 ml/min (60–300 cm/h)
Max. flow rate[2]	10 ml/min (300 cm/h)
Max. pressure over the packed bed during operation[2]	0.15 MPa, 1.5 bar
Column hardware pressure limit	0.5 MPa, 5 bar
Compatibility during use	Stable in all commonly used buffers, reducing agents, denaturants and detergents. See Table 39 for more information.
Chemical stability[3]	For one week at 40°C: 0.01 M HCl, 0.1 M NaOH For 12 h: 1 M NaOH, 70% acetic acid 30 min tested: 30% 2-propanol 1 h tested: 2% SDS
Storage	20% ethanol
Storage temperature	4–30°C

[1] Conditions for determining dynamic binding capacity:

Samples: *(Histidine)$_6$-tagged proteins: Capacity data were obtained for a protein (M_r 28 000) bound from an E. coli extract, and a pure protein (M_r 43 000) applied at 1 mg/ml in binding buffer; capacity at 10 % breakthrough.*
Untagged protein: Capacities determined at 10 % breakthrough for human apo-transferrin applied at 1 mg/ml in binding buffer.

Column volume: 0.25 or 1 ml

Flow rate: *0.25 or 1 ml/min, respectively*

Binding buffer: *20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, (1 mM for untagged protein) pH 7.4*

Elution buffer: *20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, (50 mM for untagged protein) pH 7.4*

Note: *Dynamic binding capacity is metal ion and protein dependent.*

[2] H_2O at room temperature.

[3] Uncharged medium only. See Table 39 for more information.

Stripping, recharging, and cleaning of IMAC Sepharose products

IMAC Sepharose High Performance and IMAC Sepharose 6 Fast Flow do not have to be stripped and recharged between each purification if the same protein is to be purified. It may be sufficient to strip and recharge medium after approximately two to five purifications, depending on the specific sample, sample pretreatment, sample volume, etc.

Stripping and recharging

Stripping buffer: 20 mM sodium phosphate, 500 mM NaCl, 50 mM EDTA, pH 7.4

1. Strip the medium by washing with at least 5 to 10 column volumes of stripping buffer.
2. Wash with at least 5 to 10 column volumes of binding buffer.
3. Immediately wash with 5 to 10 column volumes of distilled water.
4. Prepare a 0.1 M solution of the chosen metal ion in distilled water. Salts of chlorides, sulfates, etc., can be used: e.g., 0.1 M $CuSO_4$ or 0.1 M $NiSO_4$.
5. Recharge the water-washed column by loading at least 0.5 column volume of 0.1 M metal ion/salt solution.
6. Wash with 5 column volumes of distilled water, and 5 column volumes of binding buffer (to adjust pH) before storing column in 20% ethanol.

It is important to wash with binding buffer as the last step to obtain the correct pH before storage.

Washing with buffer before applying the metal ion solution may cause unwanted precipitation.

Cleaning-in-place

When an increase in back pressure is seen, the medium should be cleaned. Before cleaning, strip off metal ions using the recommended procedure described above. The **stripped** medium can be cleaned by the following methods:

To remove ionically bound protein:

1. Wash with several column volumes of 1.5 to 2.0 M NaCl.
2. Immediately wash with approximately 3 to 10 column volumes of distilled water.

To remove precipitated proteins, hydrophobically bound proteins, and lipoproteins:

1. Wash the column with 1 M NaOH, contact time usually 1 to 2 h (12 h or more for endotoxin removal).
2. Immediately wash with approximately 10 column volumes binding buffer, followed by 5 to 10 column volumes distilled water.

To remove hydrophobically bound proteins, lipoproteins, and lipids:

1. Wash with 5 to 10 column volumes of 30% isopropanol for about 15 to 20 min.
2. Immediately wash with approximately 10 column volumes of distilled water.

2a. Alternatively, wash with 2 column volumes of detergent in a basic or acidic solution. Use, for example, 0.1 to 0.5% nonionic detergent in 0.1 M acetic acid, contact time 1 to 2 h. After treatment, always remove residual detergent by washing with at least 5 column volumes of 70% ethanol. Then wash with approximately 10 column volumes of distilled water.

Reversed flow may improve the efficiency of the cleaning-in-place procedure. After cleaning, store column in 20% ethanol (wash with 5 column volumes) or recharge with metal ions prior to storing in ethanol.

Appendix 2
Characteristics of Glutathione Sepharose products

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

Table 42 summarizes key characteristics of these three Glutathione Sepharose media, and Table 43 lists the stability of the media toward various compounds under various conditions. Tables 44 to 45 summarize the characteristics of the same media prepacked as GSTrap HP, GSTrap FF, and GSTrap 4B in columns and as 96-well plates. For more information, refer to Chapter 5.

Table 42. Characteristics of Glutathione Sepharose High Performance, Glutathione Sepharose 4 Fast Flow, and Glutathione Sepharose 4B.

Characteristics	Glutathione Sepharose High Performance	Glutathione Sepharose 4 Fast Flow	Glutathione Sepharose 4B
Matrix	Highly cross-linked 6% agarose	Highly cross-linked 4% agarose	4% agarose
Average particle size	34 µm	90 µm	90 µm
Ligand concentration	1.5–3.5 mg glutathione/ml medium (based on Gly)	120–320 µmol glutathione/ml medium	200–400 µmol glutathione/g washed and dried medium
Binding capacity[1]	> 10 mg recombinant glutathione S-transferase/ml medium	> 10 mg recombinant glutathione S-transferase/ml medium	> 5 mg recombinant glutathione S-transferase/ml medium
Recommended flow rate[2]	< 150 cm/h	50–300 cm/h	< 75 cm/h
Chemical stability	Stable to all commonly used aqueous buffers, e.g. 1 M acetate, pH 4.0 and 6 M guanidine hydrochloride for 1 h at room temperature	Stable to all commonly used aqueous buffers, e.g. 1 M acetate, pH 4.0, and 6 M guanidine hydrochloride for 1 h at room temperature	Stable to all commonly used aqueous buffers. Exposure to 0.1 M NaOH, 70% ethanol, or 6 M guanidine hydrochloride for 2 h at room temperature or to 1% (w/v) SDS for 14 d causes no significant loss of activity.
pH stability	3–12	3–12	4–13
Storage temperature	4–30°C	4–30°C	4–8°C
Storage buffer	20% ethanol	20% ethanol	20% ethanol

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

[2] H_2O at room temperature.

Table 43. Characteristics of GST MultiTrap FF and GST MultiTrap 4B.

Media	GST MultiTrap FF: Glutathione Sepharose 4 Fast Flow GST MultiTrap 4B: Glutathione Sepharose 4B
Filter plate size[1]	127.8 × 85.5 × 30.6 mm
Filter plate material	Polypropylene and polyethylene
Binding capacity	GST MultiTrap FF: Up to 0.5 mg GST-tagged protein/well GST MultiTrap 4B: Up to 0.5 mg GST-tagged protein/well
Reproducibility between wells[2]	+/- 10%
Volume packed medium/well	50 µl (500 µl of 10% slurry)
Number of wells	96
Centrifugation speed: recommended maximum	Depends on sample pretreatment and sample properties 100–500 × g 700 × g
Vacuum pressure: recommended maximum	Depends on sample pretreatment and sample properties -0.1 to -0.3 bar -0.5 bar
pH stability	Glutathione Sepharose 4 Fast Flow: 3–12 Glutathione Sepharose 4B: 4–13
Storage	20% ethanol
Storage temperature	4–8°C

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

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

Table 44. Characteristics of prepacked GSTrap HP, GSTrap HP, and GSTrap 4B columns.

Characteristics	GSTrap HP	GSTrap FF	GSTrap 4B
Media	Glutathione Sepharose High Performance	Glutathione Sepharose 4 Fast Flow	Glutathione Sepharose 4B
Average particle size	34 µm	90 µm	90 µm
Dynamic binding capacity[1,2]	Approx. 10 mg GST-tagged protein/ml medium, M_r 63 000	Approx. 11 mg GST-tagged protein/ml medium, M_r 43 000	Approx. 10 mg recombinant glutathione S-transferase (M_r 26 000)/ml medium
Max. back pressure[3]	0.3 MPa, 3 bar	0.3 MPa, 3 bar	0.3 MPa, 3 bar
Recommended flow rate[3]	Sample loading: 0.2–1 ml/min (1 ml) and 1–5 ml (5 ml) Washing and elution: 1–2 ml/min (1 ml) and 5–10 ml/min (5 ml)	Sample loading: 0.2–1 ml/min (1 ml) and 1–5 ml (5 ml) Washing and elution: 1–2 ml/min (1 ml) and 5–10 ml/min (5 ml)	Sample loading: 0.2–1 ml/min (1 ml) and 0.5–5 ml/min (5 ml) Washing and elution: 1 ml/min (1 ml) and 5 ml/min (5 ml)
Chemical stability	Stable to all commonly used aqueous buffers, e.g. 1 M acetate, pH 4.0 and 6 M guanidine hydrochloride for 1 h at room temperature	Stable to all commonly used aqueous buffers, e.g. 1 M acetate, pH 6.0, and 6 M guanidine hydrochloride for 1 h at room temperature	Stable to all commonly used aqueous buffers. Exposure to 0.1 M NaOH, 70% ethanol, or 6 M guanidine hydrochloride for 2 h at room temperature or to 1% (w/v) SDS for 14 d causes no significant loss of activity.

continues on following page

Table 44. Characteristics of prepacked GSTrap HP, GSTrap HP, and GSTrap 4B columns (continued).

Characteristics	GSTrap HP	GSTrap FF	GSTrap 4B
pH stability	3–12	3–12	4–13
Storage temperature	4–30°C	4–30°C	4–8°C
Storage buffer	20% ethanol	20% ethanol	20% ethanol

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

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

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

[3] H_2O at room temperature.

Table 45. Characteristics of GSTPrep FF 16/10.

Characteristics	GSTPrep FF 16/10
Medium	Glutathione Sepharose 4 Fast Flow
Column volume	20 ml
Column dimensions	1.6 × 10 cm
Dynamic binding capacity[1,2]	Approx. 11 mg GST-tagged protein/ml medium, M_r 43 000
Recommended flow rate[3]	1–10 ml/min (30–300 cm/h)
Max. flow rate[3]	10 ml/min (300 cm/h)
Max. pressure over the packed bed during operation[3]	1.5 bar (0.15 MPa, 22 psi)
Column hardware pressure limit	5 bar (0.5 MPa, 73 psi)
Storage	20% ethanol
Storage temperature	4–30°C

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

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

[3] H_2O at room temperature.

Cleaning of Glutathione Sepharose products

The procedure below is appropriate for use with both bulk media and prepacked columns.

Reuse 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 High Performance, Glutathione Sepharose 4 Fast Flow, and Glutathione Sepharose 4B media and prepacked columns can be regenerated for reuse as follows:

1. Wash with 2 to 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. Reequilibrate with 3 to 5 column volumes of PBS, pH 7.3.

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

To remove precipitated or denatured substances:

1. Wash with 2 column volumes of 6 M guanidine hydrochloride.
2. Immediately wash with 5 column volumes of PBS, pH 7.3.

To remove hydrophobically bound substances:

1. Wash with 3 to 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 7.3.

For long-term storage (> 1 month):

1. Wash the column twice with 5 to 10 column volumes of PBS, pH 7.3.
2. Repeat washes using 20% ethanol.
3. Store at 4 to 8°C.
4. Reequilibrate the column with 5 to 10 column volumes of PBS, pH 7.3 before reuse.

Appendix 3
Precipitation and resolubilization

Specific sample preparation steps may be required if the crude sample is known to contain contaminants such as lipids, lipoproteins, or phenol red that may build up on a column or if certain gross impurities, such as bulk protein, should be removed before any chromatographic step.

Fractional precipitation

Fractional precipitation is occasionally used at laboratory scale to remove gross impurities from small sample volumes and also in small-scale commercial production. When using a HiTrap affinity purification column, for example, a HisTrap or GSTrap column, at laboratory scale, it is unlikely that fractional precipitation will be required.

Precipitation techniques separate fractions by the principle of differential solubility. For example, 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 95.

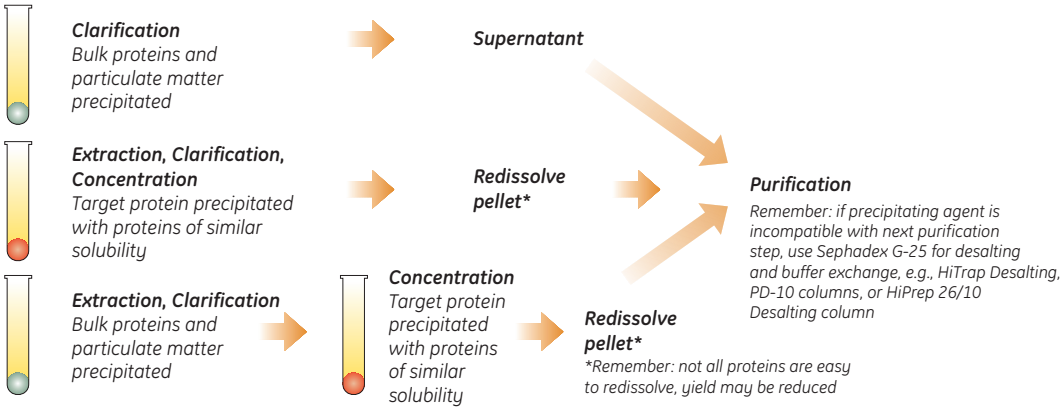


Fig 95. Three ways to use precipitation.

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

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

Examples of precipitation agents are reviewed in Table 46. The most common precipitation method using ammonium sulfate is described in more detail.

Table 46. Examples of precipitation techniques.

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulfate	As described below.	> 1 mg/ml proteins especially immuno-globulins.	Stabilizes proteins, no denaturation; supernatant can go directly to HIC. Helps to reduce lipid content.
Dextran sulfate	Add 0.04 ml of 10% dextran sulfate and 1 ml of 1 M $CaCl_2$ per ml of sample, mix 15 min, centrifuge at 10 000 × g, discard pellet.	Samples with high levels of lipoprotein, e.g., ascites.	Precipitates lipoprotein.
Polyvinylpyrrolidine	Add 3% (w/v), stir 4 h, centrifuge at 17 000 × g, discard pellet.	Samples with high levels of lipoprotein, e.g., ascites.	Alternative to dextran sulfate.
Polyethylene glycol (PEG, M_r > 4000)	Up to 20% (w/v)	Plasma proteins.	No denaturation, supernatant goes directly to IEX or AC, complete removal may be difficult. Stabilizes proteins.
Acetone (cold)	Up to 80% (v/v) at 0°C. Collect pellet after centrifugation at full speed in an Eppendorf centrifuge.		May denature protein irreversibly. Useful for peptide precipitation or concentration of sample for electrophoresis.
Polyethyleneimine	0.1% (w/v)		Precipitates aggregated nucleoproteins.
Protamine sulfate	1% (w/v)		Precipitates aggregated nucleoproteins.
Streptomycin sulfate	1% (w/v)		Precipitates nucleic acids.
Caprylic acid	(X/15) g where X = volume of sample.	Antibody concentration should be > 1 mg/ml.	Precipitates bulk of proteins from sera or ascites, leaving immunoglobulins in solution.

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

Ammonium sulfate precipitation

Ammonium sulfate precipitation is frequently used for initial sample concentration and clean-up. As the concentration of the salt is increased, proteins will begin to "salt out." Different proteins salt out at different concentrations, a process that can be taken advantage of to remove contaminating proteins from the crude extract. The salt concentration needs to be optimized to remove contaminants and not the desired protein. An additional step with increased salt concentration should then precipitate the target protein. If the target protein cannot be safely precipitated and redissolved, only the first step should be employed. HIC is often an excellent followup, as the sample already contains a high salt concentration and can be applied directly to the HIC column with little or no additional preparation. The elevated salt level enhances the interaction between the hydrophobic components of the sample and the chromatography medium.

Solutions needed for precipitation:

Saturated ammonium sulfate solution (add 100 g ammonium sulfate to 100 ml distilled water, stir to dissolve).

1 M Tris-HCl, pH 8.0.

Buffer for first purification step.

Some proteins may be damaged by ammonium sulfate. Take care when adding crystalline ammonium sulfate: high local concentrations may cause contamination of the precipitate with unwanted proteins.

It may be practical to use HIC as second step after an initial ammonium sulfate precipitation.

For routine, reproducible purification, precipitation with ammonium sulfate should be avoided in favor of chromatography.

In general, precipitation is rarely effective for protein concentrations below 1 mg/ml.

1. Filter (0.45 µm) or centrifuge the sample (10 000 × g at 4°C).
2. Add 1 part 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently. Add ammonium sulfate solution, drop by drop. Add up to 50% saturation[1]. Stir for 1 h.
4. Centrifuge 20 min at 10 000 × g.
5. Remove supernatant. Wash the pellet twice by resuspension in an equal volume of ammonium sulfate solution of the same concentration (i.e., a solution that will not redissolve the precipitated protein or cause further precipitation). Centrifuge again.
6. Dissolve pellet in a small volume of the buffer to be used for the next step.
7. Ammonium sulfate is removed during clarification/buffer exchange steps with Sephadex G-25, using desalting columns (see Chapter 9).

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

The quantity of ammonium sulfate required to reach a given degree of saturation varies according to temperature. Table 47 shows the quantities required at 20°C.

Table 47. Quantities of ammonium sulfate required to reach given degrees of saturation at 20°C.

	Final percent saturation to be obtained																
	20	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**	**65**	**70**	**75**	**80**	**85**	**90**	**95**	**100**
Starting percent saturation	**Amount of ammonium sulfate to add (grams) per liter 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

Appendix 4
Column packing and preparation

Prepacked columns from GE Healthcare will ensure reproducible results and the highest performance.

Use small prepacked columns or 96-well filter plates for media screening and method optimization to increase efficiency in method development.

Efficient column packing is essential for a good separation, especially when using gradient elution. A poorly packed column gives rise to poor and uneven flow, peak broadening, and loss of resolution. If column packing is required, the following guidelines will apply at all scales of operation:

- When using a binding technique, use short, wide columns (typically 5 to 15 cm bed height) for rapid purification, even with low linear flow.
- The amount of medium required will depend on the binding capacity of the medium and the amount of sample. The binding capacity of a medium is always significantly influenced by the hydrophobic nature of the sample as well as the medium itself and must be determined empirically. Estimate the amount of medium required to bind the sample of interest and use five times this amount to pack the column. The amount of medium required can be reduced if resolution is satisfactory.
- Once separation parameters have been determined, scale up a purification by increasing the diameter of the column to increase column volume. Avoid increasing the length of the column as this will alter separation conditions.

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

Fig 96. "Column Packing — The Movie" provides a step-by-step demonstration of column packing.

1. Equilibrate all materials to the temperature at which the separation will be performed.
2. Eliminate air by flushing column end pieces with the 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 medium.

Note that affinity media from GE Healthcare are supplied ready to use. Decanting of fines that could clog the column is unnecessary.

Avoid using magnetic stirrers because they may damage the matrix.

4. Estimate the amount of slurry (resuspended medium) required on the basis of the recommendations supplied.
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).

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

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

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

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

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

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

The medium must be thoroughly washed to remove the storage solution, usually 20% ethanol. Residual ethanol may interfere with subsequent procedures.

Many media equilibrated with sterile phosphate-buffered saline containing an antimicrobial 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

Tricorn and XK columns are fully compatible with the high flow rates achievable with modern media, and a broad range of column dimensions are available (see Table 48). In most cases the binding capacity of the medium and the amount of sample to be purified will determine the column size required. Also, Empty Disposable PD-10 Columns are available for single-use applications using gravity flow. For a complete listing of available columns, refer to the GE Healthcare catalog, BioDirectory, or ***www.gehealthcare.com/protein-purification***

Table 48. Column bed volumes and heights[1].

	Column Size		Bed Volume (ml)	Bed Height (cm)
	i.d. (mm)	Length		
Tricorn 5/20	5	20 mm	0.31–0.55	1.6–2.8
Tricorn 5/50	5	50 mm	0.90–1.14	4.6–5.8
Tricorn 10/20	10	20 mm	1.26–2.20	1.6–2.8
Tricorn 10/50	10	50 mm	3.61–4.56	4.6–5.8
Tricorn 10/100	10	100 mm	7.54–8.48	9.6–10.8
XK 16/20	16	20 cm	5–31	2.5–15
XK 16/40	16	40 cm	45–70	22.5–35
XK 26/20	26	18 cm	5.3–66	1–12.5
XK 26/40	26	40 cm	122–186	23–35
XK 50/20	50	18 cm	0–274	0–14
XK 50/30	50	30 cm	265–559	13.5–28.5
Empty Disposable PD-10[2]	15	7.4 cm	8.3	4.8–5

[1] All Tricorn and XK column specifications apply when one adapter is used.

[2] For gravity-flow applications. Together with LabMate Buffer Reservoir, up to 25 ml of buffer and/or sample can be applied, which reduces handling time considerably.

Appendix 5
Conversion data: proteins, column pressures

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

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

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

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

Column pressures

The maximum operating back pressure refers to the pressure above which the column contents may begin to compress.

Pressure units may be expressed in megaPascals, bar or pounds per square inch and can be converted as follows: 1 MPa = 10 bar = 145 psi

Appendix 6
Converting from linear flow (cm/h) to volumetric flow rates (ml/min) and vice versa

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

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

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

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

where

Y = linear flow in cm/h
d = column inner diameter in cm

Example:

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

Y = linear flow = 150 cm/h
d = inner diameter of the column = 1.6 cm

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

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

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

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

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

where

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

Example:

What is the linear flow in a Tricorn 5/50 column (i.d. 0.5 cm) when the volumetric flow rate is 1 ml/min?

Z = Volumetric flow rate = 1 ml/min
d = column inner diameter = 0.5 cm

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

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

From ml/min to using a syringe

1 ml/min = approximately 30 drops/min on a HiTrap 1 ml column
5 ml/min = approximately 120 drops/min on a HiTrap 5 ml column

Appendix 7
GST vectors

pGEX-2TK (27-4587-01)

Thrombin — Kinase

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

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

Thrombin

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

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

Thrombin

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

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

Thrombin

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

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

Factor Xa

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

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

Factor Xa

Ile Glu Gly Arg↓Gly Ile Pro Gly Ile Pro Gly Ser Thr Arg Ala Ala Ala Ser
ATC GAA GGT CGT GGG ATC CCC GGA ATT CCC GGG TCG ACT CGA GCG GCC GCA TCG TGA
BamH I · EcoR I · Sma I · Sal I · Xho I · Not I · Stop codon

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

Factor Xa

Ile Glu Gly Arg↓Gly Ile Pro Arg Asn Ser Arg Val Asp Ser Ser Gly Arg Ile Val Thr Asp
ATC GAA GGT CGT GGG ATC CCC AGG AAT TCC CGG GTC GAC TCG AGC GGC CGC ATC GTG ACT GAC TGA
BamH I · EcoR I · Sma I · Sal I · Xho I · Not I · Stop codons

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

PreScission Protease

Leu Glu Val Leu Phe Gln↓Gly Pro Leu Gly Ser Pro Glu Phe Pro Gly Arg Leu Glu Arg Pro His
CTG GAA GTT CTG TTC CAG GGG CCC CTG GGA TCC CCG GAA TTC CCG GGT CGA CTC GAG CGG CCG CAT
BamH I · EcoR I · Sma I · Sal I · Xho I · Not I

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

PreScission Protease

Leu Glu Val Leu Phe Gln↓Gly Pro Leu Gly Ser Pro Gly Ile Pro Gly Ser Thr Arg Ala Ala Ala Ser
CTG GAA GTT CTG TTC CAG GGG CCC CTG GGA TCC CCA GGA ATT CCC GGG TCG ACT CGA GCG GCC GCA TCG
BamH I · EcoR I · Sma I · Sal I · Xho I · Not I

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

PreScission Protease

Leu Glu Val Leu Phe Gln↓Gly Pro Leu Gly Ser Pro Asn Ser Arg Val Asp Ser Ser Gly Arg
CTG GAA GTT CTG TTC CAG GGG CCC CTG GGA TCC CCG AAT TCC CGG GTC GAC TCG AGC GGC CGC
BamH I · EcoR I · Sma I · Sal I · Xho I · Not I

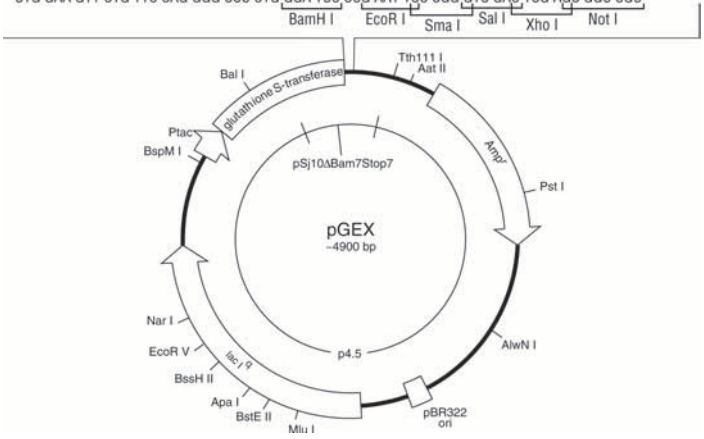
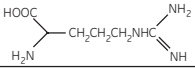
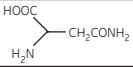
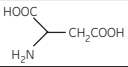
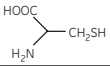
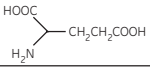
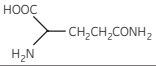
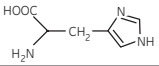
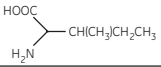
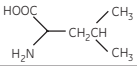
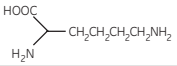
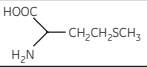
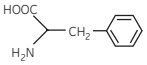
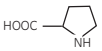
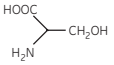
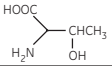
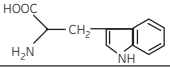
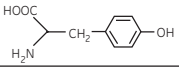
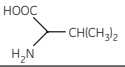


Fig 97. Map of the GST vectors showing the reading frames and main features.

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

Complete DNA sequences and restriction site data are available with each individual vector's product information, at the GE Healthcare Web site (www.gehealthcare.com/lifesciences).

Appendix 8
Amino acids table

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

Formula	M_r	Middle unit residue ($-H_2O$) Formula	M_r	Charge at pH 6.0–7.0	Hydrophobic (nonpolar)	Uncharged (polar)	Hydrophilic (polar)
$C_3H_7NO_2$	89.1	C_3H_5NO	71.1	Neutral	■		
$C_6H_{14}N_4O_2$	174.2	$C_6H_{12}N_4O$	156.2	Basic (+ve)			■
$C_4H_8N_2O_3$	132.1	$C_4H_6N_2O_2$	114.1	Neutral		■	
$C_4H_7NO_4$	133.1	$C_4H_5NO_3$	115.1	Acidic(-ve)			■
$C_3H_7NO_2S$	121.2	C_3H_5NOS	103.2	Neutral		■	
$C_5H_9NO_4$	147.1	$C_5H_7NO_3$	129.1	Acidic (-ve)			■
$C_5H_{10}N_2O_3$	146.1	$C_5H_8N_2O_2$	128.1	Neutral		■	
$C_2H_5NO_2$	75.1	C_2H_3NO	57.1	Neutral		■	
$C_6H_9N_3O_2$	155.2	$C6H_7N_3O$	137.2	Basic (+ve)			■
$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	■		
$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	■		
$C_6H_{14}N_2O_2$	146.2	$C_6H_{12}N_2O$	128.2	Basic(+ve)			■
$C_5H_{11}NO_2S$	149.2	C_5H_9NOS	131.2	Neutral	■		
$C_9H_{11}NO_2$	165.2	C_9H_9NO	147.2	Neutral	■		
$C_5H_9NO_2$	115.1	C_5H_7NO	97.1	Neutral	■		
$C_3H_7NO_3$	105.1	$C_3H_5NO_2$	87.1	Neutral		■	
$C_4H_9NO_3$	119.1	$C_4H_7NO_2$	101.1	Neutral		■	
$C_{11}H_{12}N_2O_2$	204.2	$C_{11}H_{10}N_2O$	186.2	Neutral	■		
$C_9H_{11}NO_3$	181.2	$C_9H_9NO_2$	163.2	Neutral		■	
$C_5H_{11}NO_2$	117.1	C_5H_9NO	99.1	Neutral	■		

Appendix 9
Principles and standard conditions for different 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 well-suited 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]. Affinity chromatography is frequently used as the first step (capture step) of a two-step purification protocol, followed by a second chromatographic step (polishing step) to remove remaining impurities.

The target protein(s) is specifically and reversibly bound by a complementary binding substance (ligand). The sample is applied under conditions that favor specific binding to the ligand. Unbound material is washed away, and the bound target protein is recovered by changing conditions to those favoring desorption. Desorption is performed specifically, using a competitive ligand, or nonspecifically, by changing the pH, ionic strength, or polarity. Samples are concentrated during binding, and protein is collected in purified and concentrated form. The key stages in an affinity chromatographic separation are shown in Figure 98. Affinity chromatography is also used to remove specific contaminants; for example, Benzamidine Sepharose 4 Fast Flow can remove serine proteases.

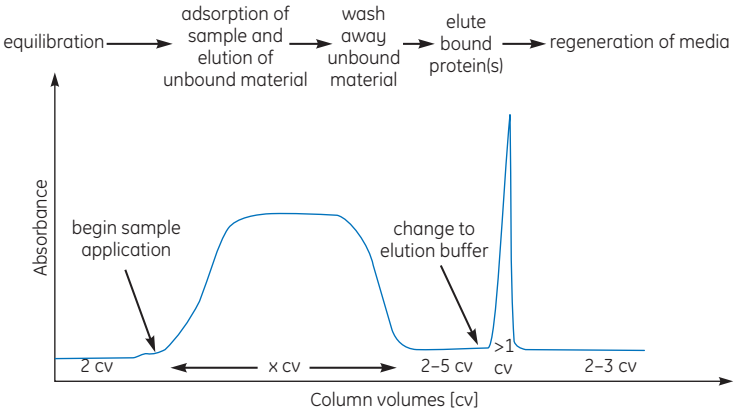


Fig 98. Typical affinity purification.

Further information

Protein Purification Handbook (Code No. 18-1132-29)
Affinity Chromatography Handbook: Principles and Methods (Code No. 18-1022-29)
Chapters 3 and 5 in this handbook for the purification of histidine- and GST-tagged proteins, respectively.

Ion exchange chromatography (IEX)

IEX separates proteins with differences in surface 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 (Fig 99). Target proteins are concentrated during binding and collected in a purified, concentrated form.

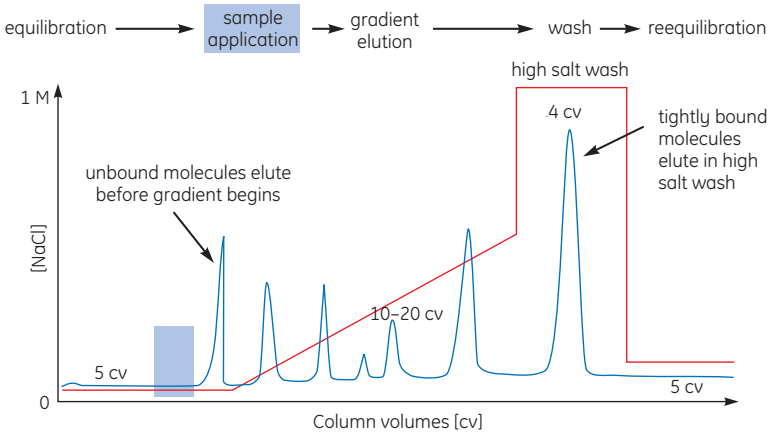


Fig 99. Typical IEX gradient elution.

The net surface charge of proteins varies according to the surrounding pH. Typically, 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. However, it should be noted that binding depends on charge and that surface charges may thus be sufficient for binding even on the other side of the pI. 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 100.

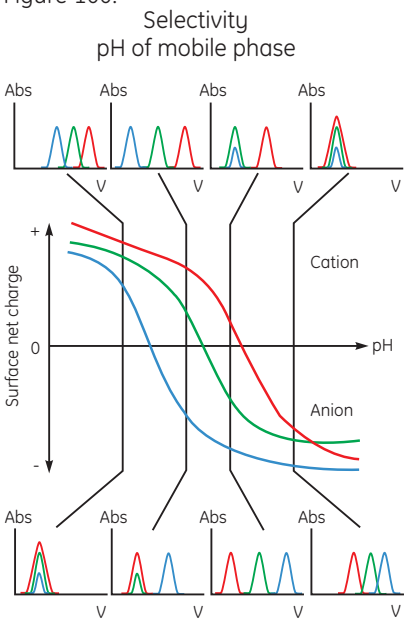


Fig 100. Effect of pH on protein elution patterns.

Method development (in priority order)

1. Select optimal ion exchanger using small columns as in the HiTrap IEX Selection Kit to save time and sample.
2. Scout for optimal pH to maximize capacity and resolution. Begin 0.5 to 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 101. It is often possible to increase sample loading when using step elution.

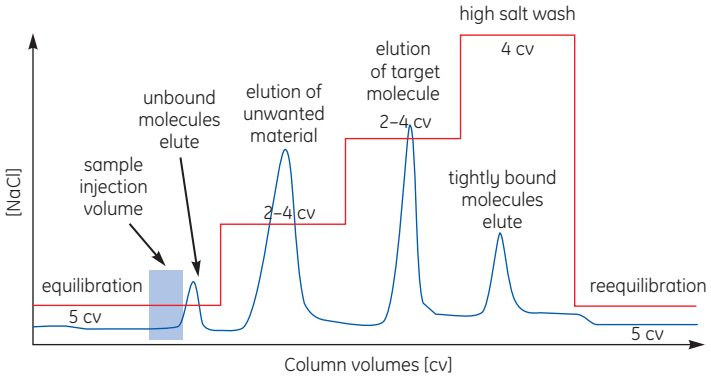


Fig 101. Step elution.

Further information

Protein Purification Handbook (Code No. 18-1132-29)
Ion Exchange Chromatography and Chromatofocusing Handbook: Principles and Methods (Code No. 11-0004-21)

Hydrophobic interaction chromatography (HIC)

HIC separates proteins with differences in hydrophobicity. The technique is well-suited for the capture or intermediate steps in a purification protocol. 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 excellent "next step" after precipitation with ammonium sulfate or elution in high salt during IEX. Samples in high ionic strength solution (e.g., 1.5 M ammonium sulfate) 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 (Fig 102). Changes are made stepwise or with a continuous decreasing salt gradient. Most commonly, samples are eluted with a decreasing gradient of ammonium sulfate. Target proteins are concentrated during binding and collected in a purified and 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.

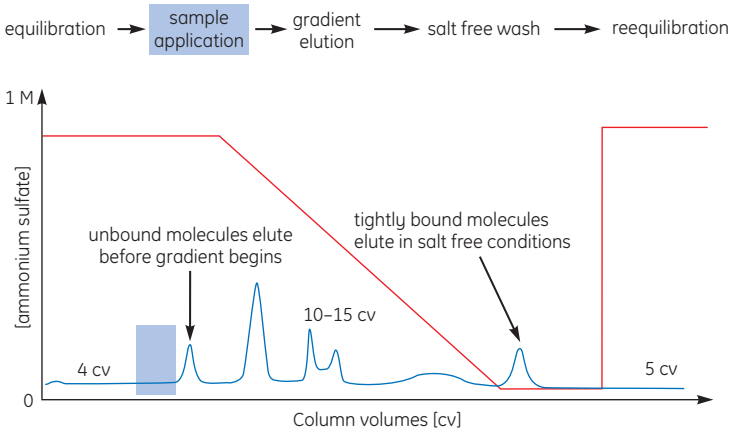


Fig 102. Typical HIC gradient elution.

Method development (in priority order)

1. The hydrophobic behavior 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 optimal binding and elution over the required range of salt concentration. For proteins with unknown hydrophobic properties begin with 0–100%B (0%B, e.g., 1M ammonium sulfate). Knowledge about the solubility of the protein in the binding buffer is important because high concentrations of, for example, ammonium sulfate may precipitate proteins.
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 103. It is often possible to increase sample loading when using step elution.

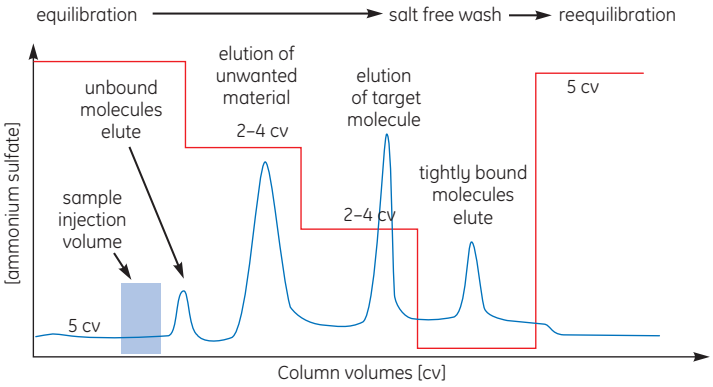


Fig 103. Step elution.

Further information

Protein Purification Handbook (Code No. 18-1132-29)
Hydrophobic Interaction Chromatography and Reversed Phase Handbook: Principles and Methods (Code No. 11-0012-69)

Gel filtration (GF) chromatography

GF separates proteins with differences in molecular size. The technique is well-suited 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, Fig 104). Buffer conditions are varied to suit the sample type or the requirements for further purification, analysis, or storage, because buffer composition usually does not have major effects on resolution. Proteins are collected in purified form in the chosen buffer.

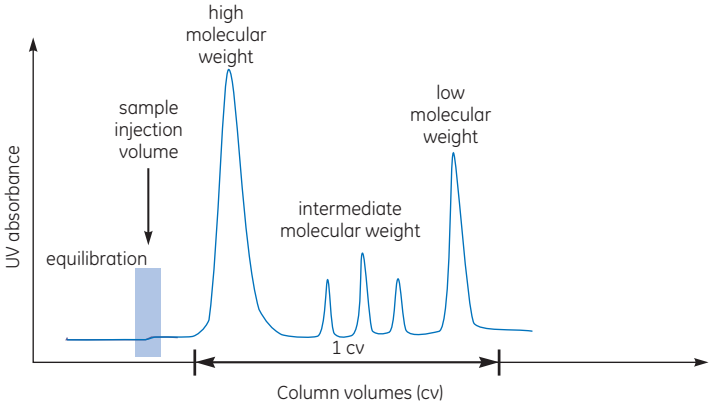


Fig 104. Typical GF elution.

Further information

Protein Purification Handbook (Code No. 18-1132-29)
Gel Filtration Handbook: Principles and Methods (Code No. 18-1022-18)

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. Binding may be modulated by 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 105.

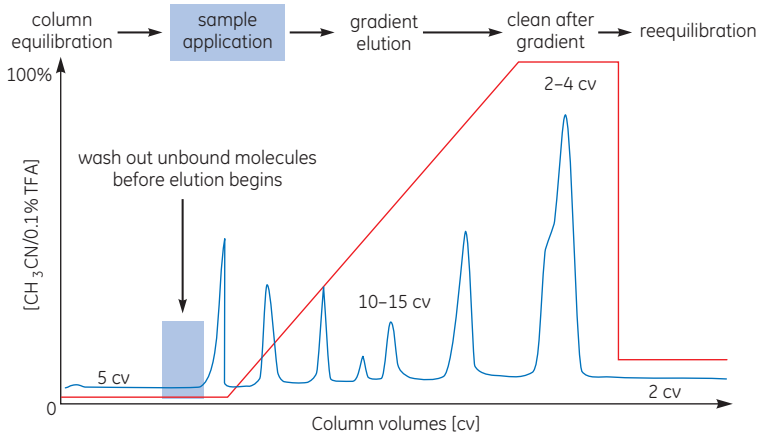


Fig 105. Typical RPC gradient elution.

RPC is often used in the final polishing of oligonucleotides and peptides and is well-suited 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, because many proteins are denatured in the presence of organic solvents.

Method development

1. Select medium from screening results.
2. Select optimal gradient to give acceptable resolution. For unknown samples begin with 0–100% elution buffer.
3. Select highest flow rate that 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 (Code No. 18-1132-29)
Hydrophobic Interaction and Reversed Phase Chromatography Handbook: Principles and Methods (Code No. 11-0012-69)

Product index

Related literature

	Code No.
Handbooks	
GST Gene Fusion System	18-1157-58
Affinity Chromatography: Principles and Methods	18-1022-29
Antibody Purification	18-1037-46
Gel Filtration: Principles and Methods	18-1022-18
Hydrophobic Interaction and Reversed Phase Chromatography: Principles and Methods	11-0012-69
Ion Exchange Chromatography and Chromatofocusing: Principles and Methods	11-0004-21
Protein Purification	18-1132-29
Challenging Protein Purification	28-9095-31
2-D Electrophoresis	80-6429-60
Selection guides/brochures	
Ni Sepharose and IMAC Sepharose, selection guide	28-4070-92
Affinity Columns and Media, selection guide	18-1121-86
Convenient Protein Purification, HiTrap column guide	18-1129-81
Gel Filtration Columns and Media, selection guide	18-1124-19
Ion Exchange Columns and Media, selection guide	18-1127-31
Prepacked chromatography columns with ÄKTAdesign systems, selection guide	18-1173-49
Protein purification—applications that meet your needs, application brochure	11-0027-81
CD	
Column Packing CD—The Movie	18-1165-33
The Protein Purifier—Software-based learning aid for purification strategies	18-1155-49
Data files and application notes	
Ni Sepharose 6 Fast Flow, HisTrap FF, and HisPrep FF 16/10 columns	11-0008-86
Ni Sepharose High Performance and HisTrap HP columns	18-1174-40
HisTrap FF crude columns and HisTrap FF crude Kit	11-0012-37
His GraviTrap	11-0036-90
His MultiTrap FF and His MultiTrap HP	11-0036-63
His SpinTrap	28-4046-59
IMAC Sepharose 6 Fast Flow, HiTrap IMAC FF, and HiPrep IMAC FF 16/10 columns	28-4041-06
IMAC Sepharose High Performance and HiTrap IMAC HP columns	28-4041-05
Glutathione Sepharose High Performance and GSTrap HP columns	18-1174-32
Glutathione Sepharose 4 Fast Flow, GSTPrep FF 16/10, and GSTrap FF	18-1136-89
GSTrap 4B columns	28-4048-14
GST MultiTrap FF, GST MultiTrap 4B	28-4081-57
Addition of imidazole during binding improves purity of histidine-tagged proteins	28-4067-41

Ordering information

Product	Quantity	Code No.
Histidine-tagged proteins		
Purification		
Ni Sepharose High Performance	25 ml	17-5268-01
	100 ml*	17-5268-02
HisTrap HP	5 × 1 ml	17-5247-01
	100 × 1 ml†	17-5247-05
	1 × 5 ml	17-5248-01
	5 × 5 ml	17-5248-02
	100 × 5 ml†	17-5248-05
His MultiTrap HP	4 × 96-well filter plates	28-4009-89
His SpinTrap	50 × 100 µl	28-4013-53
Ni Sepharose 6 Fast Flow	5 ml	17-5318-06
	25 ml	17-5318-01
	100 ml	17-5318-02
	500 ml*	17-5318-03
HisTrap FF	5 × 1 ml	17-5319-01
	100 × 1 ml†	17-5319-02
	5 × 5 ml	17-5255-01
	100 × 5 ml†	17-5255-02
HisTrap FF crude	5 × 1 ml	11-0004-58
	100 × 1 ml†	11-0004-59
	5 × 5 ml	17-5286-01
	100 × 5 ml†	17-5286-02
HisTrap FF crude Kit	3 × 1 ml, buffers	28-4014-77
HisPrep FF 16/10	1 × 20 ml	17-5256-01
His MultiTrap FF	4 × 96-well filter plates	28-4009-90
His GraviTrap	10 × 1 ml	11-0033-99
His GraviTrap Kit	20 × 1 ml, buffers	28-4015-51
IMAC Sepharose High Performance	25 ml	17-0920-06
	100 ml*	17-0920-07
HiTrap IMAC HP	5 × 1 ml	17-0920-03
	5 × 5 ml	17-0920-05
IMAC Sepharose 6 Fast Flow	25 ml	17-0921-07
	100 ml*	17-0921-08
HiTrap IMAC FF	5 × 1 ml	17-0921-02
	5 × 5 ml	17-0921-04
HiPrep IMAC FF 16/10	1 × 20 ml	17-0921-06
His Buffer Kit		11-0034-00
HiTrap Chelating HP	5 × 1 ml	17-0408-01
	1 × 5 ml	17-0409-01
	5 × 5 ml	17-0409-03
	100 × 5 ml†	17-0409-05
Chelating Sepharose Fast Flow	50 ml	17-0575-01
	500 ml*	17-0575-02
Detection		
Anti-His antibody	170 µl	27-4710-01

Product	Quantity	Code No.
GST-tagged proteins		
Protein expression		
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 All vectors include *E. coli* B21	25 µg	27-4599-01
Purification		
Glutathione Sepharose High Performance	25 ml 100 ml*	17-0579-01 17-0579-02
GSTrap HP	5 × 1 ml 100 × 1 ml† 1 × 5 ml 5 × 5 ml 100 × 5 ml†	17-5281-01 17-5281-05 17-5282-01 17-5282-02 17-5282-05
Glutathione Sepharose 4 Fast Flow	25 ml 100 ml 500 ml*	17-5132-01 17-5132-02 17-5132-03
GSTrap FF	2 × 1 ml 5 × 1 ml 100 × 1 ml† 1 × 5 ml 5 × 5 ml 100 × 5 ml†	17-5130-02 17-5130-01 17-5130-05 17-5131-01 17-5131-02 17-5131-05
GSTPrep FF 16/10	1 × 20 ml	17-5234-01
GST MultiTrap FF	4 × 96-well filter plates	28-4055-01
Glutathione Sepharose 4B	10 ml 100 ml (function tested) 300 ml*	17-0756-01 27-4574-01 17-0756-04
GSTrap 4B	5 × 1 ml 100 × 1 ml† 1 × 5 ml 5 × 5 ml 100 × 5 ml†	28-4017-45 28-4017-46 28-4017-47 28-4017-48 28-4017-49
GST SpinTrap Purification Module	50 × 50 µl	27-4570-03
GST MultiTrap 4B	4 × 96-well filter plates	28-4055-00
Detection		
GST Detection Module	50 detections	27-4590-01
GST 96-Well Detection Module	5 plates	27-4592-01
Anti-GST Antibody	0.5 ml, 50 detections	27-4577-01
Anti-GST HRP Conjugate	75 µl	RPN1236

Product	Quantity	Code No.
Tag cleavage		
Enzymes		
Thrombin	500 units	27-0846-01
Factor Xa	400 units	27-0849-01
PreScission Protease	500 units	27-0843-01
Removal of thrombin and Factor Xa		
HiTrap Benzamidine FF (high sub)	2 × 1 ml 5 × 1 ml 1 × 5 ml	17-5143-02 17-5143-01 17-5144-01
Benzamidine Sepharose 4 Fast Flow (high sub)	25 ml*	17-5123-10
Companion products		
E. coli B21	1 vial	27-1542-01
Isopropyl β-D-thiogalactoside (IPTG)	1 g 5 g 10 g	27-3054-03 27-3054-04 27-3054-05
Western blotting		
Hybond-P	10 sheets	RPN2020F
Hybond-ECL	10 sheets	RPN2020D
ECL Western Blotting		
Anti-GST HRP Conjugate	75 µl	RPN1236
ECL GST Western Blotting Detection Kit	1 kit	RPN1237
Detection Reagents	for 1000 cm^2	RPN2109
ECL Plus Western Blotting		
Detection System	for 1000 cm^2	RPN2132
Desalting and buffer exchange		
PD-10 Desalting Columns	30	17-0851-01
HiTrap Desalting	5 × 5 ml 100 × 5 ml†	17-1408-01 17-1408-05
HiPrep 26/10 Desalting	1 × 53 ml 4 × 53 ml	17-5087-01 17-5087-02
Gel filtration		
HiLoad 16/60 Superdex 30 pg	1 x 120 ml 1 x 320 ml	17-1139-01 17-1140-01
HiLoad 16/60 Superdex 75 pg	1 x 120 ml 1 x 320 ml	17-1068-01 17-1070-01
HiLoad 16/60 Superdex 200 pg	1 x 120 ml 1 x 320 ml	17-1069-01 17-1071-01
HiPrep 16/60 Sephacryl S-100 HR	1 x 120 ml 1 x 320 ml	17-1165-01 17-1194-01
HiPrep 16/60 Sephacryl S-200 HR	1 x 120 ml 1 x 320 ml	17-1166-01 17-1195-01
HiPrep 16/60 Sephacryl S-300 HR	1 x 120 ml 1 x 320 ml	17-1167-01 17-1196-01

* Larger quantities are available; please contact GE Healthcare.

† Special pack size delivered on specific customer order

Product	Quantity	Code No.
Empty columns		
Complete information on the range of Tricorn columns is available at ***www.gehealthcare.com/protein-purification-labresearch***		
Tricorn 5/100 column	1	18-1163-10
Tricorn 5/150 column	1	18-1163-11
Tricorn 5/200 column	1	18-1163-12
Tricorn 10/100 column	1	18-1163-15
Tricorn 10/150 column	1	18-1163-16
Tricorn 10/200 column *Tricorn columns are delivered with a column tube, adaptor unit, end cap, a filter kit containing adaptor and bottom filters and O-rings, two stop plugs, two fingertight fittings, adaptor lock and filter holder, and two M6 connectors for connection to FPLC™ System, if required.*	1	18-1163-17
XK 16/20 column	1	18-8773-01
XK 26/20 column	1	18-1000-72
XK 50/20 column *XK columns are delivered with one AK adaptor, TEFZEL tubing (0.8 mm i.d. for XK 16 and XK 26 columns, 1.2 mm i.d. for XK 50 columns, with M6 connectors, thermostatic jacket, support snap-on net rings, dismantling tool (XK 16 and XK 26 only), and instructions.*	1	18-1000-71
HR 16/5 column	1	18-1000-98
HR 16/10 column	1	19-7403-01
HR 16/50 column *HR columns are delivered with a column tube, adaptor unit, end cap, a filter kit containing adaptor and bottom filters and O-rings and M6 male fittings for connection to FPLC System.*	1	18-1460-01
Empty disposable PD-10 Desalting columns	50	17-0435-01
Accessories and spare parts		
For a complete listing refer to GE Healthcare BioDirectory or ***www.gehealthcare.com/protein-purification***		
LabMate PD-10 Buffer Reservoir	10	18-3216-03
Packing Connector XK 16	1	18-1153-44
Packing Connector XK 26	1	18-1153-45
Tricorn packing equipment 10/100 *Tricorn packing equipment 10/100 includes Tricorn packing connector 10-10, Tricorn 10/100 glass tube, bottom unit and stop plug.*	1	18-1153-25
Tricorn packing connector 10-10‡ *Connects extra glass column to a Tricorn 10 column to act as a packing reservoir for efficient packing.*	1	18-1153-23
1/16" male/Luer female‡	2	18-1112-51
Tubing connector flangeless/M6 female‡	2	18-1003-68
Tubing connector flangeless/M6 male‡	2	18-1017-98
Union 1/16" female/M6 male‡	6	18-1112-57
Union M6 female /1/16" male	5	18-3858-01
Union Luerlock female/M6 female	2	18-1027-12
HiTrap/HiPrep, 1/16" male connector for ÄKTAdesign	8	28-4010-81
Stop plug female, 1/16"§	5	11-0004-64
Fingertight stop plug, 1/16"¶	5	11-0003-55

‡ One connector included in each HiTrap package

§ Two, five, or seven female stop plugs included in HiTrap packages, depending on products

¶ One fingertight stop plug is connected to the top of each HiTrap column

www.gehealthcare.com/protein-purification
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