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Purifying Challenging Proteins

Principles and Methods

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Purifying Challenging Proteins

Principles and Methods

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Introduction

This handbook is intended for students and experienced researchers with an interest in the isolation of integral membrane proteins, multiprotein complexes, or in refolding proteins from inclusion bodies. The aim is to present tools, strategies, and solutions available to meet the purification challenges associated with these three classes of proteins.

For a background on techniques for protein purification, in general and handling recombinant proteins, we recommend the *Recombinant Protein Purification Handbook* and other handbooks in this series (see "Related literature" on page 100).

Challenging proteins

Our knowledge and understanding of the structural and functional biology of soluble proteins has increased dramatically over the last decade. Much of the technology for the production, purification, and analysis of soluble proteins is now at a stage where generic purification protocols allow relatively high success rates.

The situation is different for the areas that this handbook covers; integral membrane proteins, multiprotein complexes, and inclusion bodies. The need to handle and study these more difficult groups of proteins is clear, given that:

- about 30% of a typical cell's proteins are membrane proteins, and more than 50% of the current drugs on the market exert their actions via membrane proteins
- while carrying out their enzymatic, structural, transporting, or regulatory functions, proteins most often interact with each other, forming multiprotein complexes
- a large proportion of normally soluble proteins that are overexpressed in *E. coli* end up as incorrectly folded and insoluble protein in inclusion bodies

Outline

After a general introduction to each area, high-level consensus workflows are presented to summarize current best practices in each area. Rather than providing a number of detailed protocols that have been optimized for individual proteins, this handbook provides general advice or generic protocols in a step-by-step format. The generic protocols are intended as starting points for development of separation protocols. Details will typically have to be changed to tailor the protocols for individual proteins. Furthermore, the required variations to the generic protocols cannot be predicted and unless appropriate changes are made, the protocols will only work poorly, if at all—this is one of the major challenges for the researcher involved with these groups of proteins. To address this issue, the generic protocols are presented with critical parameters identified, together with ranges of values to test for the parameters. The handbook also provides guidance, hints, and tips when using protocols other than those described here.

Common acronyms and abbreviations

A_{280}	absorbance at specified wavelength (in this example, 280 nanometers)
AC	affinity chromatography
BCA	bicinchoninic acid
CBP	calmodulin binding peptide
CDNB	1-chloro-2-4-dinitrobenzene
C. elegans	*Caenorhabditis elegans*
CF	chromatofocusing
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CMC	critical micellar concentration
CV	column volume
DAB	3,3'-diaminobenzidine
DDM	dodecyl maltoside
DNase	deoxyribonuclease
DS	desalting (sometimes referred to as buffer exchange)
DTT	dithiothreitol
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
FF	Fast Flow
FW	formula weight
GF	gel filtration (sometimes referred to as SEC: size exclusion chromatography)
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione-S-transferase
Gua-HCl	guanidine-HCl
HIC	hydrophobic interaction chromatography
HMW	high molecular weight
HP	High Performance
HRP	horseradish peroxidase
IEX	ion exchange chromatography (also seen as IEC in the literature)
IMAC	immobilized metal ion affinity chromatography
IPTG	isopropyl β-D-thiogalactoside
LDAO	lauryldimethylamine oxide
LMW	low molecular weight
LTAB	lauryl trimethylammonium bromide
MBP	maltose binding protein
MPa	megaPascal
M_r	relative molecular weight
MS	mass spectrometry
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
PMSF	phenylmethylsulfonyl fluoride
psi	pounds per square inch
PVDF	polyvinylidene fluoride
r	recombinant, as in rGST
RNase	ribonuclease
RPC	reversed phase chromatography
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAP	Tandem affinity purification
TCEP	Tris (2-carboxyethyl) phosphine hydrochloride
TEV	Tobacco etch virus
u	units (e.g., of an enzyme)
Y2H	Yeast-two-hybrid

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.

Yellow highlights indicate chemicals, buffers, and equipment

Blue highlights indicate an experimental protocol

Chapter 1
Membrane proteins

Introduction

Membrane proteins play key roles in fundamental biological processes, such as transport of molecules, signaling, energy utilization, and maintenance of cell and tissue structures. About 30% of the genes determined by genome sequencing encode membrane proteins, and these proteins comprise more than 50% of the current drug targets. Despite their importance, our knowledge of the structure and function of membrane proteins at the molecular level lags far behind that for soluble proteins. For instance, at the time of the publication of this handbook, membrane proteins only represent around 1% of the 3-D atomic resolution structures that have been deposited in the Protein Data Bank (http://www.pdb.org/).

Integral membrane proteins exist in a lipid environment of biological membranes (biomembrane), but the available techniques for purifying, handling, and analyzing them were optimized for water-soluble proteins in an aqueous environment. To be able to handle and study membrane proteins they must be dispersed in an aqueous solution. This is usually accomplished by adding a detergent that solubilizes the biomembrane and forms a soluble complex with the lipids and membrane proteins (Fig 1.1). Solubilization is a harsh treatment that has to be carefully optimized to avoid protein loss and inactivation. Protein denaturation and/or aggregation are frequently encountered. Solubilization is one of the most critical aspects in handling membrane proteins.

Other difficulties contribute to our lack of detailed structural and functional understanding of membrane proteins. These include:

- Low abundance: The quantity of membrane proteins is often very low in their natural setting. This makes their natural source impractical as a starting material for their preparation.
- Difficult production: Heterologous overexpression often results in low expression levels and inactive protein due to insufficient membrane insertion and folding or lack of post-translational modifications. Over-expression of membrane proteins can be toxic to the cell.

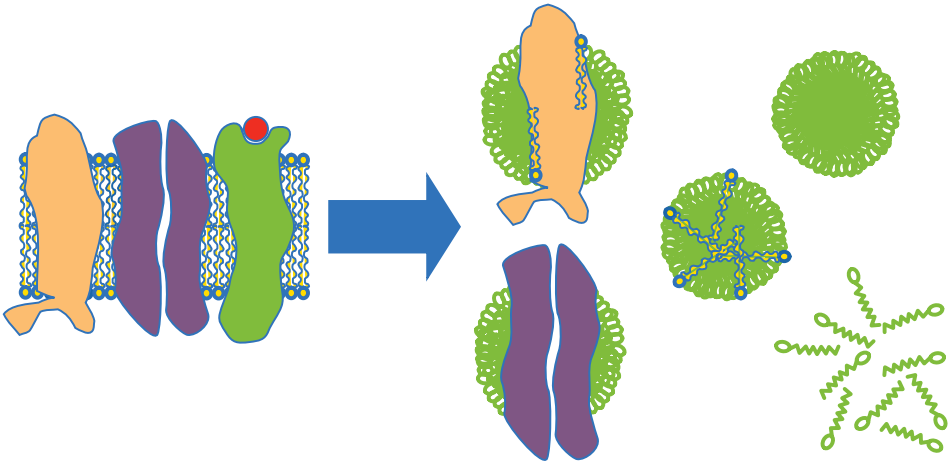


Fig 1.1. Schematic drawing of detergent solubilization of membrane proteins. Membrane proteins are transferred from the natural lipid bilayer (blue and yellow) to complexes with detergent (green) and, in some cases, lipids. A lipid-detergent micelle, a detergent micelle, and free detergent are also shown.

Membrane protein expression, purification, and analysis present considerable challenges. Nevertheless, a substantial number of membrane proteins, especially from bacterial origin, have been over-produced, isolated, and characterized in molecular detail. Also, several studies aiming at mapping the membrane proteome in different organisms have been published. Due to great efforts in a number of membrane protein labs, generic protocols for membrane protein work have begun to emerge. These protocols are extremely useful as a starting point in the lab. In the main part of this chapter, such protocols are provided together with optimization advice and references for further reading.

Classification of membrane proteins

Membrane proteins are classified as peripheral or integral. Peripheral membrane proteins are loosely associated with the membrane and are usually water soluble after being released from the membrane. Peripheral membrane proteins generally present limited methodological challenges when compared with integral membrane proteins. Throughout this handbook, the term "membrane protein" refers to integral membrane protein unless otherwise indicated. Integral membrane proteins are insoluble in water. They have one or more transmembrane segments comprising polypeptide stretches that span the membrane. The transmembrane moiety can be constituted of a single or a bundle of α-helices or of β-barrel-like structures composed of multiple polypeptide stretches. These proteins are called α-helical membrane proteins (Fig 1.2, left) and β-barrel membrane proteins (Fig 1.2, right), respectively. The β-barrel membrane proteins are predominant in the outer membrane of Gram-negative bacteria and mitochondria. Some proteins display both structures.

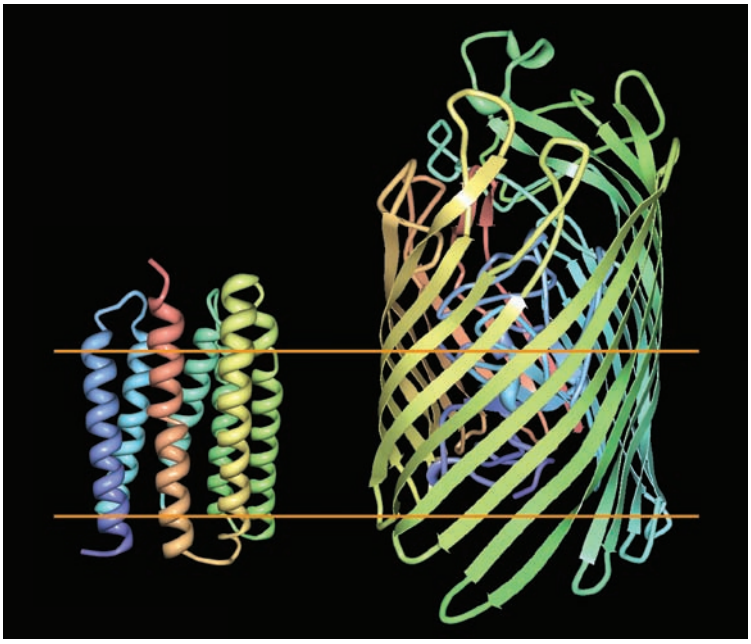


Fig 1.2. Three dimensional structure representations of an α-helical membrane protein (left; *Anabaena* sensory rhodopsin; PDB ID: 1XIO; [1]) and a β-barrel membrane protein (right; ferric hydroxamate uptake receptor (fhua) from *E. coli;* PDB ID: 1FCP; [2]). The structures are oriented such that the externally exposed area of each protein is at the top. The yellow lines show the approximate locations of the lipid bilayer membrane. The yellow, horizontal lines are for illustration purposes only and are not based on crystallographic data. Structures from The Protein Data Bank (http://www.pdb.org/).

Purification of integral membrane proteins for structural and functional studies

The high level workflow for the production and purification of integral membrane proteins for structural and functional studies is shown in Figure 1.3. Each of the different stages in the workflow is described in detail, with relevant protocols. The protocols are intended as starting points in the lab. Hints, tips, useful variations, and troubleshooting advice are also given. The focus is on protocols for bacterial membrane production and purification since this is most common. Protocols for eukaryotic membrane proteins are less well developed. However, much of the general advice is also valid for work with eukaryotic membrane proteins.

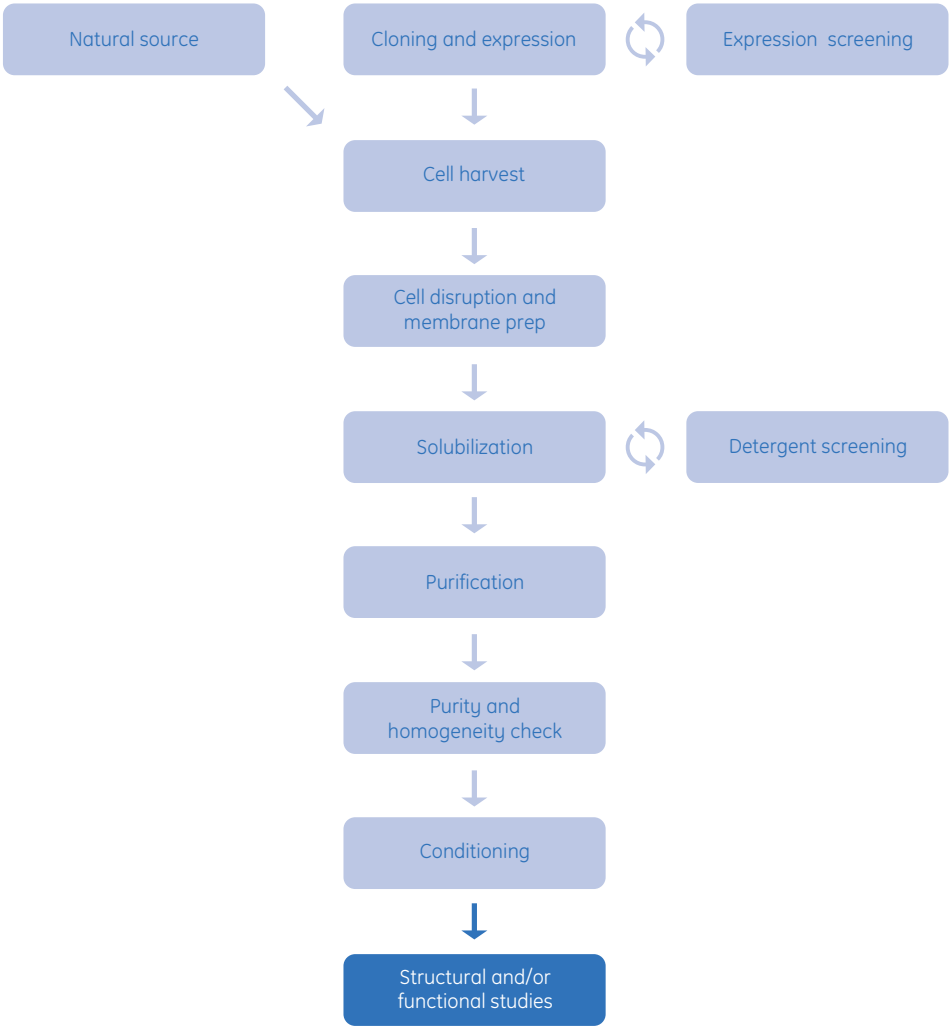


Fig 1.3. Workflow overview for membrane protein isolation and purification for structural and functional studies.

Starting material

Membrane proteins from natural sources

The natural source of a membrane protein can be considered as a starting material for purification. The only three-dimensional structure in molecular detail that has been reported to date for a eukaryotic G-protein coupled receptor (GPCR), bovine rhodopsin, was obtained with protein purified from bovine retina, where the protein is highly abundant (8). In many cases, however, low abundance of the target protein precludes the use of the natural source as starting material.

Examples of purifications from natural sources are presented later in this chapter.

Cloning

Vectors used for the expression of soluble proteins are also commonly used for the production of membrane proteins. It is useful to design a number (10 to 50) of different constructs, including different homologues, to increase the chance that a particular membrane protein can be produced in an active form.

In addition to the general considerations for choosing a vector (see *Recombinant Protein Purification Handbook*, in "Related literature" on page 100), a number of other aspects relate more specifically to choosing a vector for expressing membrane proteins.

- Affinity tagging greatly facilitates expression screening based on chromatographic enrichment, as well as optimization and use of protocols for purification of membrane proteins. Polyhistidine tags are commonly used for membrane proteins, but the GST-tag and others have also been used successfully. The insertion of a protease cleavage site between the affinity tag and the target protein enables removal of the tag before further analyses.
- While a hexahistidine tag (His_6) is the standard option for water-soluble proteins, longer histidine tags (with 8 or 10 histidine residues) are often used for membrane proteins to increase the binding strength and thus improve yields in IMAC purification. Drawbacks with longer (> 6 histidine residues) histidine tags are that expression levels have been reported to be decreased in some cases and that a higher imidazole concentration is required for elution.
- Tags should generally be placed on the C-terminal end of the protein to reduce risk of affecting the membrane insertion process based on the N-terminal signal peptide.
- Fusion of the target membrane protein to a fluorescent protein tag such as GFP in combination with a histidine tag allows direct and convenient visualization of the target during expression, solubilization, and purification and can speed up the optimization of these processes (6).

Expression and screening

To correctly decide which conditions and constructs will be best suited for producing the protein for the intended study, an efficient screening protocol is essential. Because of the relatively low concentrations of overexpressed membrane proteins, it is useful to apply affinity tags combined with separation methods that allow enrichment of the target protein.

Overexpression is a major bottleneck in the overall workflow for membrane protein production. Several host systems are available and the final choice will depend both on protein-specific requirements (e.g., for post-translational modifications) and practical aspects (e.g., available equipment in the lab and expertise). It is often useful to try different hosts or host strains in parallel for a particular target protein to increase the likelihood of success. In addition, homologous membrane proteins from several sources can be cloned in parallel to be able to select those that express well.

E. coli strain BL21 (DE3) is the most commonly used host for overexpression of membrane proteins, in combination with a pET vector. "High" expression levels for functional membrane proteins are usually more than an order of magnitude lower than for overexpression of water-soluble proteins in *E. coli*. One inherent issue is that membrane proteins need to be inserted into membranes, and the availability of membrane structures in most cells is limited.

The issue with limited membrane availability can be addressed by using a host with large amounts of internal membranes (e.g., *Rhodobacter spp.*; (3)). Another way of avoiding the limitations set by available membranes is to produce the membrane protein as inclusion bodies. This is usually not desired but may allow preparation of active protein through solubilization of the inclusion bodies using denaturants followed by refolding. Successful refolding of β-barrel membrane proteins from inclusion bodies has been achieved (4) but refolding of α-helical membrane proteins is an even greater challenge. For a separate discussion on inclusion bodies, see Chapter 3.

A modest growth and expression rate is beneficial to avoid the formation of inclusion bodies when using *E. coli* as a host. This can be achieved by the use of a weak promoter, a low concentration of inducer and/or lowering the growth temperature after induction.

An overview of different expression systems for membrane proteins is provided in Table 1.1. For a review on important considerations for membrane protein expression, see reference 7.

Table 1.1. Overexpression systems used for prokaryotic and eukaryotic membrane protein production

Expression system	Advantages	Disadvantages
E. coli	The most widely used overexpression system for (prokaryotic) membrane protein production.	Often not suitable for overexpression of eukaryotic membrane proteins No glycosylation and limited post-translational modifications
Yeast	Can perform some post-translational modifications Several different yeast systems have been used for membrane protein production (5)	Does not produce high cell densities (*S. cerevisiae*) Hyperglycosylation can occur (*S. cerevisiae*) Different lipids (compared with mammalian cells)
Insect cells	Less complex growth conditions compared with mammalian cells Relatively high expression levels Glycosylation	More costly and complex than *E. coli* or yeast; different lipids (compared with mammalian cells)
Mammalian cells	CHO, BHK and other cell lines are often used for functional studies of receptors Authentic (mammalian) protein is produced	High cost and complex work
Rhodobacter spp.	High expression levels through coordinated synthesis of foreign membrane proteins with synthesis of new internal membranes (3)	Different lipids (compared with mammalian cells)
Cell free	Allows expression of toxic proteins and proteins that are easily degraded *in vivo* Allows incorporation of labeled and non-natural amino acids.	High cost Membrane protein insertion in membrane or detergent micelle has not been fully developed

Disposable 96-well filter plates, from GE Healthcare, prepacked with affinity purification media for histidine- or GST-tagged proteins can be used for reproducible, high-throughput screening of protein expression. Typical applications include expression screening of different constructs, screening for suitable detergents and solubility of proteins, and optimization of the conditions for small-scale parallel purification. Plates are available prepacked with Ni Sepharose™ High Performance or Ni Sepharose 6 Fast Flow for working with histidine-tagged proteins (His MultiTrap™ HP or His MultiTrap FF, respectively); and Glutathione Sepharose 4 Fast Flow or Glutathione Sepharose 4B for working with GST-tagged proteins (GST MultiTrap FF or GST MultiTrap 4B, respectively).

Small-scale expression screening of histidine-tagged membrane proteins from E. coli *lysates*

Cell lysis and solubilization

Buffer preparation

Lysis buffer: 20 mM sodium phosphate, 100 mM NaCl, 2 mM $MgCl_2$, 20 mM imidazole, 0.5 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), 5 u/ml benzonase, 1 mg/ml lysozyme, EDTA-free protease inhibitor cocktail, (concentration according to manufacturer's recommendation), 1-2% of a selection of detergents, pH 7.4

Procedure

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. Suspend the bacterial pellet by adding 5 to 10 ml of lysis buffer for each gram of wet cells. 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.
4. Leave for 2 h with mild agitation at room temperature or 4°C, depending on the sensitivity of the target protein.
5. Measure and adjust pH if needed.

Expression screening procedure

Materials

His MultiTrap HP or His MultiTrap FF

Centrifuge that handles 96-well plates

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 500 mM NaCl, 20 to 40 mM imidazole, 0.5 mM TCEP, 1 to 2% detergent, pH 7.4. (The optimal imidazole concentration is protein dependent; 20 to 40 mM is suitable for many proteins.)

Wash buffer: 20 mM sodium phosphate, 500 mM NaCl, 20 to 40 mM imidazole, 0.5 mM TCEP, 0.03% dodecyl maltoside (DDM), 1 to 2% detergent, pH 7.4,

Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, 0.5 mM TCEP, 0.03% DDM, 1 to 2% detergent, 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.

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 200 × 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 200 × g.
7. Add 500 µl of binding buffer to each well to equilibrate the medium. Centrifuge for 2 min at 200 × g. Repeat once. The filter plate is now ready for use.

Blank run: Reducing agents may be used in sample and buffers. If this is the case, replace step 7 with the following steps:

7. Add 500 µl of elution buffer/well. **No reducing agent should be used in the elution buffer during this blank run.** Centrifuge the plate for 2 min at 200 × g
8. Add 500 µl of binding buffer **including** reducing agent to each well to equilibrate the medium. Centrifuge for 2 min at 200 × g. Repeat once. The filter plate is now ready for use with reducing agent. Do not store His MultiTrap plates with buffers containing reducing agents.

Centrifugation procedure

Check that all wells are drained after centrifugation. If not, then increase the centrifugation force slightly.

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

1. Apply 100 µl of lysate to each well of the filter plate and incubate for 3 min.

   Note: If the yield of protein is very low, increase the incubation time and/or gently agitate the filter plate to mix. The lysate volume can also be increased, and several aliquots of lysate can be added successively to each well.
2. Centrifuge the plate at 100 × g for 4 min or until all the wells are empty. Discard the flowthrough.
3. Add 50 µl of binding buffer per well. Centrifuge at 200 × g for 2 min.
4. Add 200 µl of wash buffer per well. Centrifuge at 200 × g for 2 min. Repeat twice.
5. Add 50 µl of elution buffer per well and mix for 1 min.

   Note: The volume of elution buffer can be varied (50 to 600 µl per well), depending on the concentration of target protein required.
6. Change the collection plate and centrifuge at 200 × g for 2 min to collect the eluted protein. Repeat twice or until all the target protein has been eluted (A_{280} should be < 0.1, indicating that all protein has been eluted).

   Note: If necessary, change the collection plate between each elution to prevent unnecessary dilution of the target protein.

The following detergents have been used with this protocol: 1% FOS-Choline™ 12, 1% undecyl maltoside, 1% dodecyl maltoside, 1% Cymal™-5, 1% Cymal-6, 2% octyl glucoside, 1% Triton™ X-100, 1% lauryl dimethylamine oxide (LDAO).

To optimize the protocol, vary the concentration of imidazole in the sample and in the binding and wash buffers. A common variation range with His MultiTrap plates is 20 to 40 mM imidazole. If binding of the target protein is too low with these concentrations, try 5 to 20 mM imidazole. In general, too low of an imidazole concentration in the binding and wash buffers can cause adsorption of unwanted host proteins (and hence a lower purity). Too high of an imidazole concentration can lead to a reduced yield of the target protein.

Analysis

Samples can by analyzed by SDS-PAGE with Coomassie™ Blue staining (see "Purity and homogeneity check" on page 40), or by dot-blot analysis on nitrocellulose membrane. Histidine-tagged proteins can be detected using Anti-His Antibody.

Cell harvest

Methods for cell harvest are host dependent and the same protocols are used for the recovery of membrane proteins as for intracellular water-soluble proteins. Cell harvest of suspension cultures is done by low speed centrifugation.

Cell harvest of *E. coli* cultures

Buffer preparation

PBS: 10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4

Centrifugation procedure

1. Centrifuge at 6000 to 9000 × g at 4°C for 15 min to collect the cells. Discard the supernatant. Resuspend the cells in 500 ml ice-cold PBS.
2. Centrifuge at 6000 to 9000 × g at 4°C for 15 min. Discard the supernatant. Resuspend the cell pellet in 10 ml ice-cold PBS, or another volume as required.
3. The resuspended cell pellet can be stored at –80°C.

Cell disruption and membrane preparation

Methods for cell disruption are host dependent and essentially the same protocols are used for recovery of membrane proteins as for water-soluble proteins. Cell disruption yields a suspension of membrane fragments/vesicles that contains the membrane proteins. The suspension also contains soluble proteins, remaining intact cells, various cell debris, and other material as contaminants. These contaminants may need to be removed, depending on the purification procedure used. Differential centrifugation is the standard approach for the isolation of membrane fragments/vesicles after cell disruption.

The pellet from cell harvest is resuspended in a suitable buffer for cell disruption (e.g., PBS). DNase is added to reduce viscosity. It is useful to add a protease inhibitor cocktail to reduce possible protein degradation. A selection of commonly used techniques for cell disruption are summarized in Table 1.2.

Table 1.2. Overview of techniques for cell disruption to yield a suspension of membrane vesicles

Technique	Principle	Advantages (+) / Disadvanatges (-)
Liquid shear pressure (e.g., French press)	Rapid pressure drop by transferring the sample from a chamber at high pressure through an orifice into a chamber at low pressure	+ Fast and efficient, also for large volumes - Causes heating of the sample (cooling is required)
Ultrasonication	Cells disrupted by high frequency sound	+ Simple - Causes heating of the sample, which can be difficult to control by cooling - Proteins may be destroyed by shearing - Noisy - Not for large volumes
Glass bead milling	Agitation of the cells with fine glass beads	+ Useful for cells that are more difficult to disrupt (e.g., yeast) - Somewhat slow and noisy
Osmotic shock	Change from high to low osmotic medium	+ Simple, inexpensive - Only useful for disruption of cells with less robust walls (e.g., animal cells)
Repeated freezing and thawing	Cells disrupted by repeated formation of ice crystals; usually combined with enzymatic lysis	+ Simple, inexpensive + Yields large membrane fragments - Slow - May damage sensitive proteins and dissociate membrane protein complexes - Low yield
Enzymatic lysis	Often used in combination with other techniques, e.g., freeze-thawing or osmotic shock; lysozyme is commonly used to break cell walls of bacteria	+ Gentle + Yields large membrane fragments - Slow - Low yield

Cell disruption from frozen *E. coli* cell paste with overexpressed membrane protein

Solutions

PBS:	10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4
$MgCl_2$:	1 M
Pefabloc™:	100 mM
DNase:	20 mg/ml
Lysozyme:	10 mg/ml

Cell disruption

1. For each gram of cell paste combine, from the above solutions, 5 ml PBS, 5 µl $MgCl_2$, 50 µl Pefabloc, 5 µl DNase, and 80 µl lysozyme. Mix until the suspension is homogenous.
2. Sonicate on ice. Use the manufacturer's recommended settings for amplitude and time for the probe being used (e.g., 5 min of accumulated time; 9 s on, 5 s off).
3. Continue immediately with the membrane preparation.

This procedure was modified from reference 6.

Membrane preparation should be performed immediately after cell disruption.

Membrane preparation from E. coli.

All steps are carried out at 4°C or on ice.

1. Centrifuge at 24 000 × g for 12 min. Collect the supernatant.
2. Centrifuge the supernatant at 150 000 × g for 45 min. Remove the supernatant and resuspend the pellet in 10 ml PBS. The pellet contains the membrane fraction.
3. Centrifuge the resuspended pellet at 150 000 × g for 45 min. Remove the supernatant and resuspend the pellet in 5 ml PBS.
4. Determine protein concentration using standard methods for soluble proteins such as the Biuret method or the bicinchoninic acid (BCA) method.
5. For storage, rapidly freeze the membrane suspension dropwise using liquid nitrogen and store at –80°C.

This procedure was taken from reference 6.

Different cell disruption protocols may give rise to different size fragments; the centrifugation speed needs to be optimized accordingly.

Water crystals formed upon slow freezing may harm membrane proteins. Fast freezing by submersion of the membrane suspension in liquid nitrogen forms amorphous ice structures thus reducing the negative effects of freezing (some researchers avoid freezing completely and always perform membrane protein preparation from cell to pure protein as fast as possible, without interruptions; see next point).

For unstable membrane proteins, it may be beneficial to proceed directly with purification after the preparation of membranes, and thus avoid freezing and storing the membranes.

For small scale (0.5 to 50 ml) membrane preparations from *E. coli*, a procedure with lysozyme treatment followed by osmotic shock and centrifugation is often efficient.

To facilitate protein purification, it can be useful to separate the inner and outer membranes from *E. coli* membrane preparations. This can be particularly helpful for large (1 to >10 l) preparations. The inner membrane can be selectively solubilized with 2% N-lauroylsarcosine (9). The outer membranes can then be recovered in the pellet after a 1 h centrifugation. An alternative is to separate the inner and outer membranes by a long (~10 h) sucrose gradient centrifugation, following cell disruption.

It is sometimes possible to omit the fairly lengthy and cumbersome membrane preparation step. The alternative is to first disrupt the cells and then directly solubilize membrane proteins by the addition of detergent to the cell lysate, with no prior isolation of membranes. The resulting "solubilisate" can be used for chromatography directly. By using chromatography columns that accept direct loading of unclarified homogenized cell lysate and detergent-treated unclarified lysate (e.g., HisTrap™ FF crude columns), histidine-tagged membrane proteins can be purified directly from the cell lysate (see "Purification of histidine-tagged membrane protein directly from crude, solubilized *E. coli* lysate", page 33).

Solubilization

This is one of the most critical stages during the preparation of membrane proteins. During the solubilization stage, membrane proteins are extracted from their natural environment, the lipid membrane, to an aqueous environment by the use of detergents. Detergents act by disintegrating the lipid bilayer while incorporating lipids and proteins in detergent micelles.

The hydrophobic surface areas of the membrane proteins and the lipid "tails" are buried in the hydrophobic interior of the detergent micellar structures, while hydrophilic parts of the proteins are in contact with the aqueous environment (Fig 1.1). An efficient solubilization dissociates most lipid-protein and protein-protein interactions, thereby allowing the separation of proteins.

The target protein can be purified in the presence of detergent by applying essentially any of the existing protein purification techniques available for soluble proteins. A successful solubilization protocol extracts the membrane protein at a high yield and results in stable protein-detergent complexes (or protein-lipid-detergent complexes) where the protein retains its active conformation.

Some membrane proteins require the interaction with native lipids from the lipid bilayer or added exogenous lipids to remain in their active conformation. In such cases, it is essential that the solubilization protocol enables the formation of a stable protein-lipid-detergent complex and that it does not remove the required native lipid(s) associated with the target protein. Harsh solubilization and purification procedures may lead to the removal of such essential lipids, and hence inactivation of the protein.

Detergents and CMC

Detergents are amphipathic substances with a polar (hydrophilic) head group and a nonpolar, (hydrophobic) tail. The polar part can be nonionic, anionic, cationic, or zwitterionic and detergents are often classified accordingly (i.e., a detergent with an anionic polar part is referred to as an anionic detergent). Advantages and disadvantages of the different classes of detergents are listed in Table 1.3.

Table 1.3. Advantages and disadvantages with the different classes of detergents

Detergent class	Advantages	Disadvantages
Nonionic (e.g., dodecyl maltoside)	Generally mild and non-denaturing Widely used	May give low solubilization yields
Ionic (anionic or cationic) (e.g., SDS, LTAB)	Can be extremely efficient in solubilization	Often denaturing Interfere with ion exchange separations
Zwitterionic (e.g., FOS-Choline 12)	Often used in membrane protein crystallization. Combines the advantages of ionic and non-ionic detergents	More denaturing than nonionic detergents

Above a certain concentration in an aqueous environment, detergent molecules associate to form multimolecular complexes, micelles, with hydrophobic interiors and hydrophilic surfaces. This concentration is referred to as the critical micellar concentration (CMC). The CMC is different for different detergents and it also varies with pH, temperature and ionic strength.

In general, all buffers and solutions used for membrane protein preparations (for solubilization, purification, storage, etc.) should have a detergent concentration above the CMC.

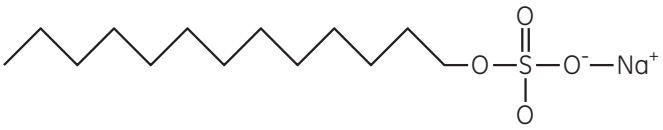
Several detergents that are recommended for solubilization of membrane proteins are listed in Table 1.4. Chemical structures of a few example detergents from the different classes are shown in Figure 1.4.

Table 1.4. Some recommended detergents for solubilization of membrane proteins

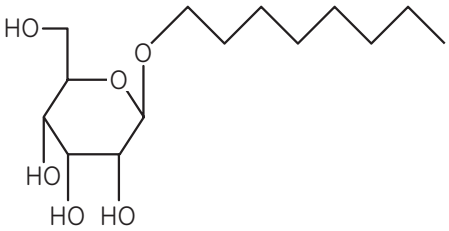
Detergent	Class[1]	FW	CMC[2] (mM)
Brij™ 35	N	1200	0.07
C12E8	N	539	0.11
CHAPS (3[(3-Cholamidopropyl) dimethylammonio] propanesulfonic acid)	Z	615	8
Cymal 7 (Cyclohexyl-*n*-heptyl-β-D-maltoside)	N	522	0.19
Decyl maltoside	N	483	1.8
Digitonin	N	1229	< 0.5
DDM (dodecyl maltoside)	N	511	0.17
FOS-Choline 12	Z	352	0.12
Hecameg (6-O-(N-Heptylcarbamoyl)methyl-α-D-gluco pyranoside)	N	335	19.5
LDAO (lauryldimethylamine oxide)	Z	229	1
Nonidet P40	N	615	0.25
Nonyl glucoside	N	306	6.5
Octyl glucoside	N	292	18
Tween™ 20	N	1228	0.06
Triton X-100	N	647	0.23

[1] N = nonionic; Z = zwitterionic
[2] At 20°C to 25°C and ~50 mM Na^+

Ionic: Sodium dodecyl sulfate



Non-ionic: Octyl glucoside



Zwitterionic: LDAO (Lauryl dimethylamine oxide)

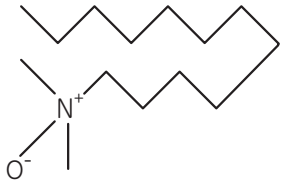


Fig 1.4. Chemical structures of selected detergents from the different classes.

Detergent screening

Several different detergents and conditions should be screened to establish the best conditions for each membrane protein. Solubilization yield is monitored by assaying for the target membrane protein in the solubilized fraction. In the protocol below, non-solubilized material is removed by ultracentrifugation.

It is also possible to apply non-clarified solubilisate directly to specially designed chromatography columns or multiwell plates that can handle cellular debris (see "Expression screening of histidine-tagged membrane proteins from *E. coli* lysates"). Filtration can also be used for clarification.

Protein detection may be performed by Western blot after SDS-PAGE. For low-expressed proteins, a chromatographic enrichment step may be required before protein detection is possible (see "Conditioning" on page 43). For highly expressed proteins, solubilization yield can be estimated by SDS-PAGE with Coomassie Blue staining.

In addition to determination of yield and homogeneity, it is often necessary to monitor protein activity, which is the best indication of intact protein structure (and function). For some membrane proteins, functional assays can be applied to the detergent-solubilized protein. In other cases, reinsertion of the membrane protein into an artificial lipid bilayer (membrane protein reconstitution) is necessary to perform a functional assay.

General detergent screening procedure

Buffer preparation

PBS: 10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4

Tris buffer: 20 mM at neutral pH

Table 1.5. Additives to PBS or Tris buffer for detergent screening

	Membrane protein concentration (mg/ml)	Detergent concentration (%)	NaCl (mM)	Temp (°C)	Time
Start condition	5	1 (always above CMC)	100	4	2 h
Variation range	1 to 10	0.4 to 2 (always above CMC)	100 to 500	4 to 37	5 min –5 h

For a list of recommended detergents, see Table 1.4.

Solubilization

1. Starting with the membrane preparation of known protein concentration, combine materials as shown in Table 1.5 to give a total volume of 1 ml. Incubate with gentle mixing at the desired temperature for the time indicated.
2. Centrifuge at 100 000 × g at 4°C for 45 min.
3. Assay the supernatant for target protein. Generic assays that can be used are SDS-PAGE (see "Purity and homogeneity check" on page 40), rapid affinity purification followed by gel filtration (see "Purity and homogeneity check" on page 41) and affinity tag specific assays (e.g., a dot blot using tag-specific antibodies). Also, assays for membrane protein activity should be considered.

The expression screening protocol on page 15, "Small-scale expression screening of histidine-tagged membrane proteins from *E. coli* lysates" can be adapted to a combined solubilization screening and binding screening protocol.

The results from a preliminary solubilization screening of EM29, an M_r 29 000 histidine-tagged membrane protein from *E. coli*, is shown in Fig 1.5. A strong band at the expected position for the target protein indicates high expression and/or efficient solubilization. FC12, Triton X-100 and LDAO gave weaker bands than the other tested detergents. It was thus concluded that these detergents were less suitable for solubilization of this protein. It should be stated also that high or reasonable yields must be combined with preservation of activity and stability in detergent solution after solubilization—which is the next screening analysis that should be considered.

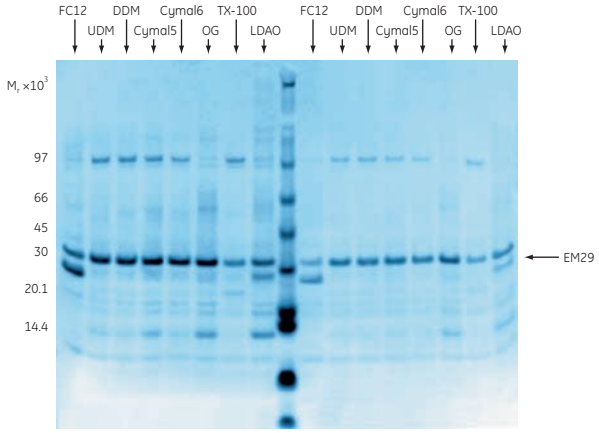


Fig 1.5. Analysis by SDS-PAGE and Coomassie Blue staining of a combined expression screening and preliminary solubilization screening of EM29. Eight different detergents were tested and elution was done in two steps (left and right half of the gel, respectively). FC12 = FOS-Choline 12; UDM = undecyl maltoside; DDM = dodceyl maltoside; OG = octyl glucoside; TX-100 = Triton X-100; LDAO = lauryl dimethylamine oxide. Data kindly provided by Dr. Said Eshaghi, Karolinska Insitute, Stockholm, Sweden.

- Specific lipids may be associated with the protein in the native membrane, and their presence can be essential for protein activity. Retaining activity requires that these lipids are still present after solubilization and purification. Harsh solubilization and purification conditions can lead to removal of such lipids, resulting in inactivation of the membrane protein.
- DDM is often a good detergent to try in initial solubilization tests.
- CHAPS and digitonin have been reported to work particularly well for solubilization of membrane proteins from *Pichia pastoris*.

Solubilization screening for GST-tagged membrane proteins produced in *E. coli*

The method described below (Fig 1.6) provides a simple and rapid method to select for optimal solubilization conditions to obtain the highest yield of GST-tagged membrane protein. This screening procedure is based on the affinity of GST for Glutathione Sepharose 4B media. The detergents selected for the screening must not affect the GST-binding activity

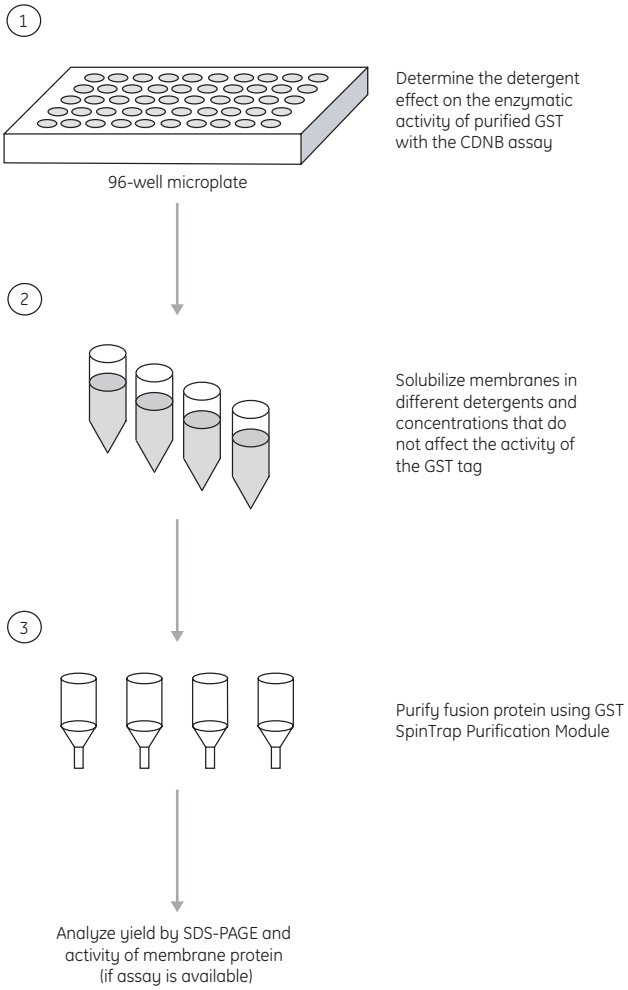


Fig 1.6. Detergent screening assay for a GST-tagged membrane protein.

Materials

GST Detection Module	
GST SpinTrap™ Purification Module	
GST MultiTrap 4B	
Binding buffer:	10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4 (PBS)
Elution buffer:	Binding buffer supplemented with 0.2% (w/v) detergent and 10 mM reduced glutathione

Solubilization screening

If the enzymatic activity of GST (as an indicator of GST binding activity) in the presence of the selected detergents is unknown, evaluate GST activity in the detergents using the CDNB assay provided in the GST Detection Module. Compare GST activity in the presence and absence of each detergent. GST is known to be fully active in DDM, CHAPS, octyl glucoside, Tween 20, Triton X-100, Brij35 and NP 40 at detergent concentrations between 0.3 to 10 times CMC for each detergent.

1. Disrupt cells by lysozyme treatment combined with freeze-thawing and isolate membranes by centrifugation at ~50,000 × g for 30 min at 4°C (see "Membrane preparation from *E. coli*" on page 19 for additional information). Higher speed and longer centrifugation times (e.g., 100 000 × g for 60 min) may be required if more harsh cell disruption methods are used.
2. Solubilize the membrane pellet (1:10 w/v) in 5% (w/v) detergent solutions for 2 h on ice with mild agitation (see "Detergent screening" on page 23 for additional information).
3. Clarify by centrifugation at ~50 000 × g for 30 min at 4°C (or at 18 000 × g for 60 min at 4°C).
4. Decant 500 µl of the supernatant and purify with GST SpinTrap Purification Module or GST MultiTrap 4B according to the supplied instructions. Elute with 500 µl elution buffer. Assay for GST activity with GST Detection Module.
5. Analyze by SDS-PAGE (see "Purity and homogeneity check" on page 40).

This procedure was taken from reference 10.

Optimization of solubilization conditions

After establishing the initial conditions for solubilization, the conditions can be optimized by further screening with one or a limited number of detergents. Useful screening parameters to investigate are:

- ratio of protein concentration to detergent concentration. Protein concentrations in the range of 1 to 10 mg/ml are typical
- solubilization time and mixing conditions (e.g., mild agitation, end-over-end rotation, or vigorous stirring)
- pH
- ionic strength

Size homogeneity can be used as an indicator of stability (and therefore optimum solubilization conditions) because membrane proteins often oligomerize or aggregate rapidly when destabilized. Size homogeneity can be rapidly evaluated using Superdex™ 200 5/150 GL (see "Size homogeneity characterization" on page 40). Figure 1.7 demonstrates how Superdex 200 5/150 GL (column volume 3 ml) can be used to screen for homogeneity under various pH and salt conditions.

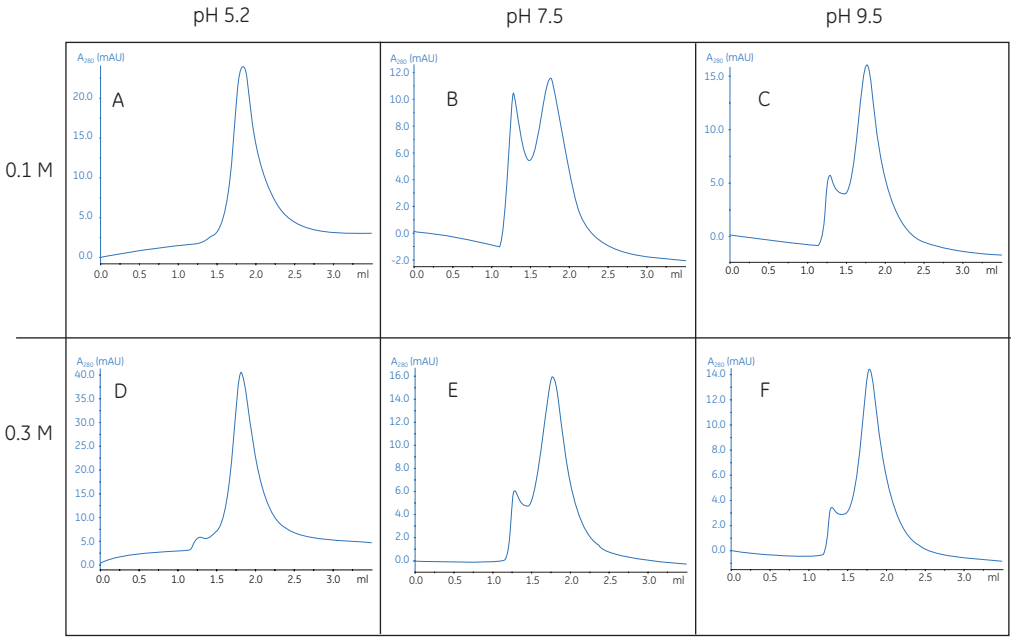


Fig 1.7. Screening of pH and ion strength conditions for optimal homogeneity and stability of a detergent-protein complex. Rapid gel filtration with Superdex 200 5/150 GL showed a symmetrical peak when the separation was performed at pH 5.2 in 0.1 M NaCl (A), indicating a homogenous protein under these conditions. At somewhat higher salt concentration (D) a small peak appeared close to the void volume, indicating that oligomerization or aggregation appeared to a limited extent. At both pH 7.5 and pH 9.5 significant peaks were obtained close to the void volume, indicating severe oligomerization or aggregation. The complete screening procedure was achieved in only a few hours, including the time for column equilibration. Sample consumption was 6 × 10 µl for the complete screen. Data kindly provided by Dr. Said Eshagi, Karolinska Institute, Stockholm, Sweden.

Mixtures of detergents could also be considered.

Additives such as lipids and small amphiphiles (e.g., 1,2,3-heptanetriol) or known ligands for the target protein may be useful to test. Small amphiphiles may affect crystallization behavior by changing the size of detergent micelles. Ligands are believed to reduce the dynamics of the protein structure and thus stabilize the protein in detergent solution.

Solubilization for purification

After optimization, the solubilization protocol is performed at a scale that is appropriate for the amount of membrane protein that needs to be obtained.

It is recommended to proceed with purification immediately after solubilization, to minimize the loss of membrane protein activity due to aggregation, loss of structure, or proteolytic degradation.

A detergent that works well for the solubilization of a particular membrane protein may be less suited for other operations with the same protein (e.g., crystallization). Procedures for exchanging detergents are described in Conditioning (see page 43).

For reviews on the use of detergents in membrane protein purification, see references 11 and 12.

Solubilization with organic solvents

Small and stable membrane proteins are sometimes possible to extract from the lipid bilayer in their active form by the use of organic solvents. For instance, a 110 amino acid membrane protein was extracted from *E. coli* with a chloroform:methanol mixture, and initial purification of the extract was done in this environment using an organic solvent resistant chromatography medium and column (Fig 1.8; 13). For this membrane protein, a 1:1 ratio of chloroform:methanol gave the best separation.

Column: Sephadex™ LH-20 (40 ml bed volume) in 10 mm diameter column.
Sample: Organic extract of *E. coli* membranes in which EmrE (a multidrug resistance transporter protein) was overexpressed
Eluent: Chloroform (C):methanol (M) 3:1 (upper) and C:M 1:1 (lower).
Flow rate: 2 ml/min
System: ÄKTApurifier™

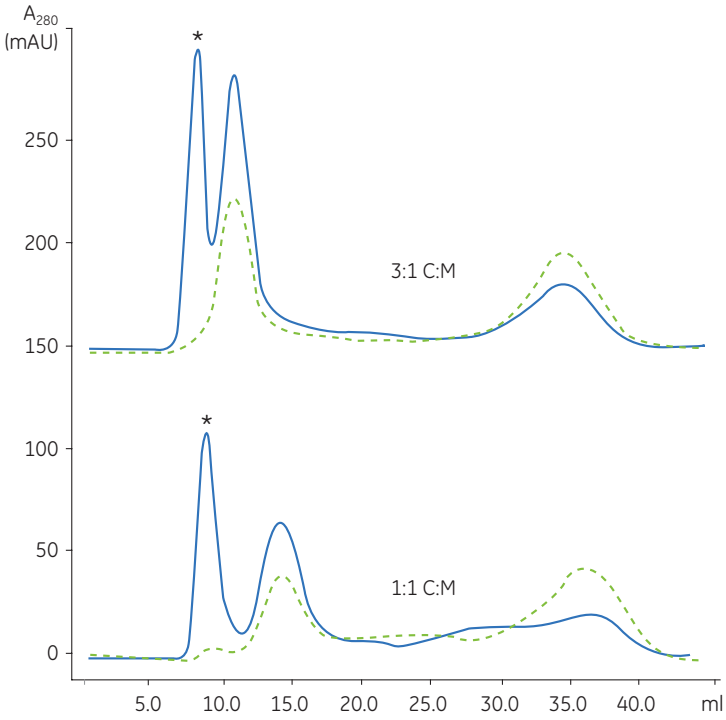


Fig 1.8. Gel filtration separation of solvent-extracted membrane proteins. Solid line = organic extract of *E. coli* membranes in which EmrE (a multidrug resistance transporter protein) was overexpressed; broken line = organic extract of "blank" *E. coli* membranes. Two ratios of chloroform (C) and methanol (M) were used. The asterisk marks EmrE containing peaks. From reference 13. Used with permission. Copyright 2002 Elsevier Science (USA) Publication.

Purification

Membrane proteins are usually purified as protein-lipid-detergent complexes. The solubility of the complexes in an aqueous environment allow the application of essentially the same separation techniques as used for water-soluble proteins. The main difference is that the purification of membrane proteins is carried out with detergent present in all solutions. This is necessary because protein-detergent complexes are dynamic and would immediately lose detergent molecules in the absence of free detergent. Detergent concentrations should be above the CMC but can be kept about 10 times lower than what was used during solubilization (typically in the 0.1% range).

- High detergent concentrations can reduce the stability of the protein. However, detergent concentrations need to be high during solubilization if the concentration of membrane components is high. Once solubilization is completed, detergent concentrations can be reduced. Detergents are often expensive, and it is also useful to limit consumption for cost reasons.
- Over-purification can lead to the removal of essential lipids from the protein-lipid-detergent complex with concomitant loss of protein activity.
- Membrane protein stability can often be improved by having 5% glycerol in all buffers throughout the purification.

Purification of histidine-tagged membrane proteins

Histidine-tagged proteins have 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 most other cellular proteins will not bind or bind weakly. Elution is achieved by increasing the concentration of imidazole. This chromatographic technique is often termed immobilized metal ion affinity chromatography (IMAC).

Water-soluble, $(histidine)_6$-tagged proteins are usually straightforward to purify following standard protocols. Histidine-tagged membrane proteins are sometimes more problematic, and weak binding to IMAC media is often reported. It has been speculated that this is due to restricted accessibility of the histidine-tag for the IMAC ligand due to the binding of detergent to the protein. To address the issue, longer histidine tags are routinely used for overexpression of membrane proteins. Also, the presence of a linker, such as green fluorescent protein (GFP), between the histidine tag and the target membrane protein has been suggested to improve binding to IMAC media.

Historically, batch-wise purification has often been employed for the purification of histidine-tagged membrane proteins. Batch-wise purification involves mixing the sample with the chromatography media in an open vessel for a designated time, often overnight. The suspension is then packed into a column for washing and elution of the bound protein. Batch-wise purification can sometimes improve yields since adsorption times are longer than for column separations. On the other hand, since a batch-wise procedure is longer it also leaves the protein more exposed to proteolytic degradation or inactivation, and may thus compromise the quality of the purified protein.

Purification of histidine-tagged membrane proteins can also be performed using column-based methods (see protocols on following pages).

Depending on the level of purity required for the final application, additional purification steps can be performed after IMAC. For this purpose, gel filtration is possibly the most common, and has the advantage that optimization is usually not needed. Anion exchange chromatography can also be used and is often included between IMAC and gel filtration steps (see the section “Additional purification steps” later in this chapter).

Purification of histidine-tagged membrane protein from the solubilized, isolated membrane fraction

Materials

Column:	HisTrap HP, 1 ml
Binding buffer:	PBS, 20 mM imidazole, 0.1 to 1 % detergent (e.g., DDM), pH 7.4
Elution buffer:	PBS, 0.5 M imidazole, 0.1 to 1% detergent (e.g., DDM), pH 7.4

Sample preparation

To 5 ml of solubilized membrane protein, add 0.20 ml of elution buffer to give a final imidazole concentration of 20 mM.

Purification

This purification procedure should be performed at 4°C.

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 (CV) of distilled water.
4. Equilibrate the column with 10 CV of binding buffer at a flow rate of 1 ml/min*
5. Apply the sample (using a syringe fitted to the Luer connector or by pumping it onto the column). Use a flow rate of 1 ml/min.
6. Wash with 10 CV of binding buffer at a flow rate of 1 ml/min.
7. Elute with a gradient of 0% to 75% elution buffer in 20 CV at a flow rate of 1 ml/min. When a syringe is used, elute stepwise with successively higher concentrations of imidazole.
8. After elution, wash the column with 5 CV 100% elution buffer followed by 5 CV binding buffer.

**One ml/min corresponds to approximately 30 drops/min when using a syringe with a HiTrap™ 1-ml column. When using a larger column, a higher flow rate can be used. See column instructions.*

The procedure can be scaled up by connecting two or three columns in series or by using HisTrap HP 5 ml columns.

A relatively low NaCl concentration (e.g., PBS is 150 mM NaCl) is recommended because membrane proteins tend to be less soluble at higher ionic strengths. Higher concentrations (e.g., 300 to 500 mM NaCl) are often recommended for IMAC of water-soluble proteins to reduce ionic interactions of contaminants with the chromatographic medium. For some cases, even lower NaCl concentrations (e.g., < 150 mM) should be applied for a membrane protein. Alternatively, the NaCl concentration can be reduced directly after the IMAC step by desalting the material using a HiTrap Desalting column.

It has been reported that by using gradient elution (with increasing concentrations of imidazole) from an IMAC column, as in the protocol above, protein-lipid-detergent complexes that differ only in lipid content can be separated.

Re-application of the flowthrough material, after step 5 above, to allow the sample to pass through the IMAC column several times can be useful to maximize yield (14).

The yield can also be increased by decreasing the flow rate during sample loading.

For further purification, ion exchange chromatography and/or gel filtration is often suitable (see "Additional purification steps" on page 35).

Figure 1.9 shows the purification of YedZ-TEV-GFP-$(His)_8$ using the protocol above. YedZ is a transporter membrane protein from *E. coli* that was overexpressed in *E. coli* as a fusion protein with GFP, a C-terminal $(histidine)_8$ tag and a tobacco etch virus (TEV) protease cleavage sequence.

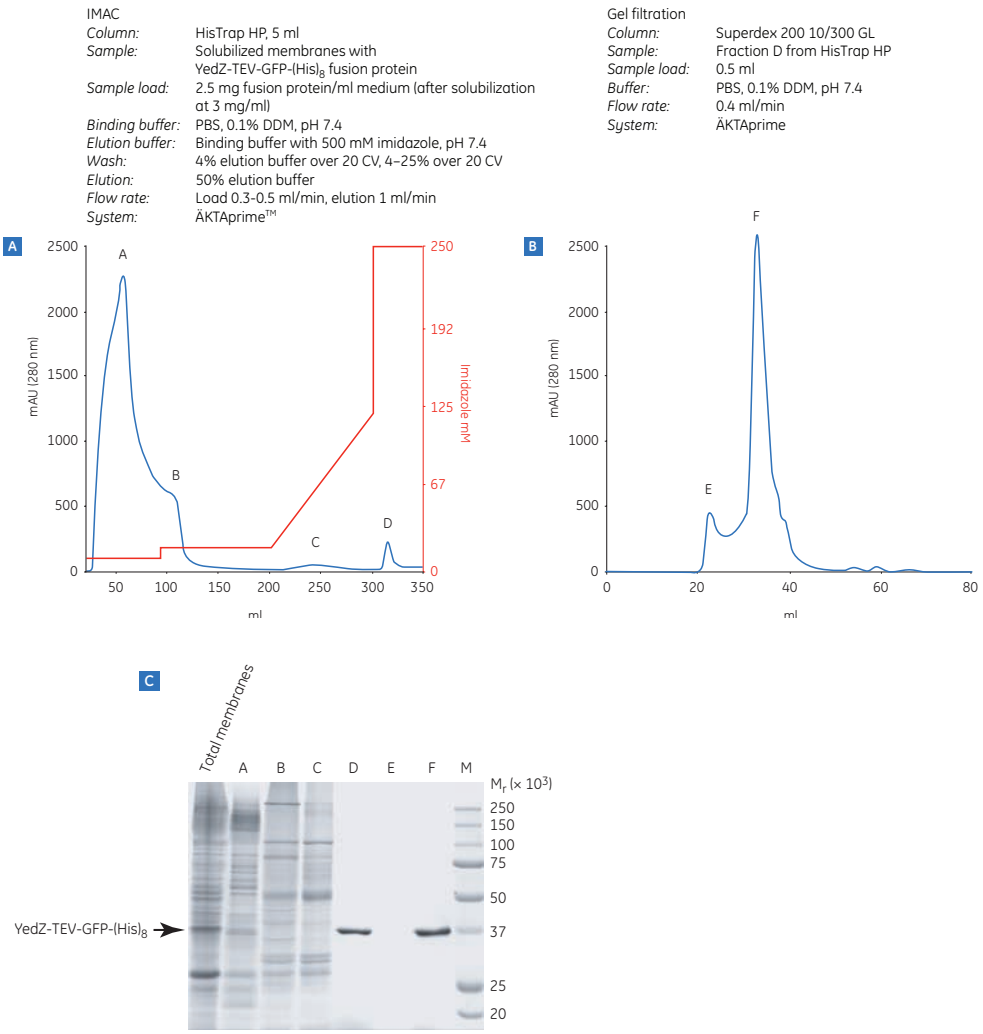


Fig 1.9. Two-step purification of *E. coli* YedZ-TEV-GFP-$(His)_8$ from solubilized membranes prepared from *E. coli* cell culture. Fractions from (A) IMAC were further purified by (B) gel filtration. (C) SDS-PAGE analysis of selected fractions shows the purity of the target protein. Fraction D from the HisTrap HP column was essentially homogeneous. M = molecular weight marker. Peak E most likely contained light-scattering detergent-lipid aggregates. Data kindly provided by Dr. David Drew, Center for Biomembrane Research, Stockholm University, Stockholm, Sweden.

Purification of histidine-tagged membrane protein directly from crude, solubilized *E. coli* lysate

This protocol circumvents the need for membrane preparation and centrifugations. It uses a chromatography column that was designed for the application of unclarified crude cell lysate.

Material

Column:	HisTrap FF crude, 1 ml
Binding buffer:	PBS, 40 mM imidazole, 0.1% detergent (e.g., DDM), pH 7.4
Elution buffer:	PBS, 1 M imidazole, 0.1% detergent (e.g., DDM), pH 7.4

Sample preparation

Suspend *E. coli* cell paste by addition of 5 ml PBS with 40 mM imidazole for each gram of paste. Perform cell lysis by lysozyme treatment and sonication (see Table 1.2). Add detergent to the unclarified lysate from a concentrated stock (e.g., DDM to a final concentration of 0.8%). Stir on ice for 1.5 hours.

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 CV of distilled water.
4. Equilibrate the column with 10 CV of binding buffer at a flow rate of 1 ml/min*
5. Apply the detergent-treated, unclarified lysate with a pump (0.5 ml/min) or syringe. Loading volumes of unclarified lysate are highly dependent on each specific sample.

   **One ml/min corresponds to approximately 30 drops/min when using a syringe with a HiTrap 1-ml column. When using a larger column, a higher flow rate can be used. See column instructions.*

Continuous, gentle 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. Depending on the sample volume (larger sample volumes require larger wash volumes), wash with 10 to 30 CV of binding buffer at a flow rate of 1 ml/min.
7. Elute with a gradient of 0% to 12% elution buffer in 10 CV followed by 12% to 100% elution buffer in 5 to10 CV, all at a flow rate of 1 ml/min. When a syringe is used, elute stepwise with successively higher concentrations of imidazole.
8. After elution, wash the column with 5 CV elution buffer followed by 5 CV binding buffer.

Figure 1.10 shows the purification of YedZ-TEV-GFP-$(His)_8$ using the protocol above.

For further purification, ion exchange chromatography and gel filtration is often suitable (see “Additional purification steps” on page 35).

The procedure can be scaled up by connecting two or three columns in series or by using HisTrap FF crude 5 ml columns.

Column: HisTrap FF crude, 1 ml
Sample: *E. coli* cell lysate, with overexpressed YedZ-TEV-GFP-$(His)_8$ solubilized in 0.8% dodecyl maltoside for 100 min on ice.
Sample load: 50 ml
Binding buffer: PBS, 40 mM imidazole, 0.1% DDM, pH 7.4
Elution buffer: PBS, 1 M imidazole, 0.1% DDM, pH 7.4
Flow rate: 1 ml/min
Gradient: 4% to 12% elution buffer in 10 ml; 12% to 100% elution buffer in 5 ml
System: ÄKTAexplorer™

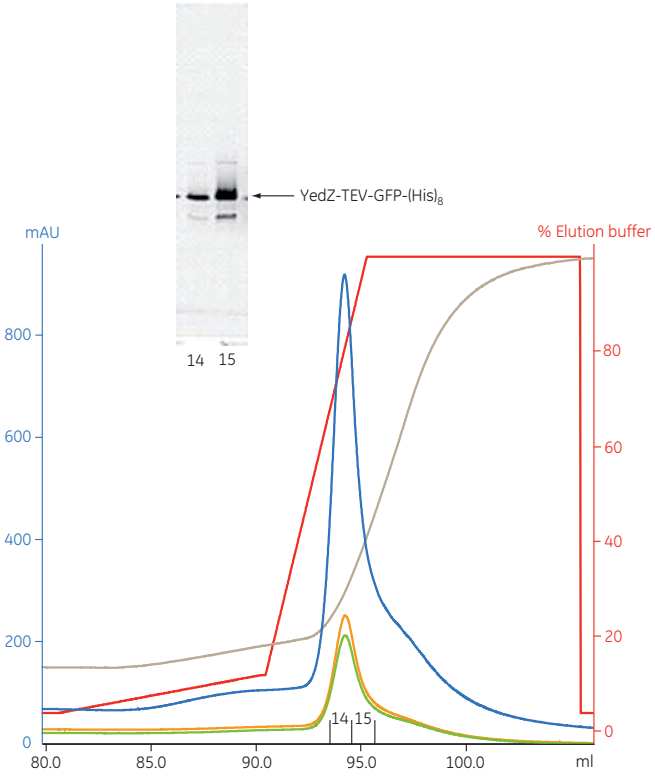


Fig 1.10. Purification of a $(histidine)_8$-tagged membrane protein, YedZ-TEV-GFP-$(His)_8$ directly from crude, solubilized *E. coli* lysate using HisTrap FF crude, 1 ml. Peak fractions 14 and 15 were analyzed by SDS-PAGE. The gel was scanned to detect the GFP portion of the fusion protein. An arrow indicates the band corresponding to YedZ-TEV-GFP-$(His)_8$. In the chromatogram, blue =A_{280}; orange = A_{425} (to detect YedZ); green = A_{485} (to detect GFP); gray = conductivity; red = % elution buffer. The overexpression vector was kindly provided by Dr J.-W. deGier, Centre for Biomembrane Research, Stockholm, Sweden.

Additional purification steps

Further purification of the IMAC-purified, histidine-tagged membrane protein is usually necessary for applications that require highly pure homogeneous material (e.g., for structural characterizations). One or two additional chromatographic steps are usually sufficient. For maximum efficiency, the purification scheme should be designed so that different separation principles are utilized in the different steps.

A highly efficient purification scheme may consist of IMAC (separation according to specific affinity) followed by desalting and ion exchange (separation according to charge differences) and finally by gel filtration (separation according to size differences). Gel filtration is most often used as the last step for the final removal of aggregates and for transfer of the sample to a buffer suitable for further functional and structural studies. The separations are carried out in the presence of detergent above the CMC (typically about 0.1%). Other conditions are the same as for water-soluble proteins. Some suitable columns are listed in Table 1.6. ÄKTAxpress™ chromatography system can be used to automate multistep purifications to produce highly pure proteins with minimum hands-on time. ÄKTAdesign™ systems are presented in Appendix 2.

High-resolution anion exchange can be used for both purification and characterization of the charge homogeneity of purified membrane proteins. In most cases the ionic strength of samples from IMAC must be reduced before application to ion exchange chromatography. Fig 1.14 shows charge characterization of an IMAC-purified membrane protein using Mono Q™.

Table 1.6. Prepacked ion exchange and gel filtration columns for the additional purification of membrane proteins after initial IMAC purification

Technique	Column	Column volume (ml)	Comment
Anion exchange	Mono Q 5/50 GL	1	For the highest resolution and purity in anion exchange chromatography at mg scale
	Mini Q	0.24 or 0.8	For the highest resolution and purity in anion exchange chromatography at µg scale
	RESOURCE™ Q	1 or 6	A strong anion exchange medium; for high throughput and easy scale up
	HiTrap Q HP	1 or 5	A strong anion exchange medium; for fast separations with high resolution and sharp peaks
	HiLoad™ 16/10 Q Sepharose HP	20	Same as previous, but for larger sample amounts
	HiLoad 26/10 Q Sepharose HP	53	Same as previous, but for larger sample amounts
	HiTrap Q FF	1 or 5	A strong anion exchange medium; for fast separations with good resolution
	HiPrep™ 16/10 Q FF	20	Same as the previous, but for larger sample amounts
	HiTrap DEAE FF	1 or 5	Weak anion exchange medium; an alternative to the strong "Q" anion exchangers
	HiPrep 16/10 DEAE FF	20	Same as the previous, but for larger sample amounts
Cation exchange	Mono S™ 5/50 GL	1	For the highest resolution and purity in cation exchange chromatography at mg scale
	Mini S	0.24 or 0.8	For the highest resolution and purity in cation exchange chromatography at µg scale
	RESOURCE S	1 or 6	A strong cation exchange medium; for high throughput and easy scale up
	HiTrap SP HP	1 or 5	A cation exchange medium; for fast separations with high resolution and sharp peaks
	HiLoad SP Sepharose HP	20	Same as previous, but for larger sample amounts
	HiLoad SP Sepharose HP	53	Same as previous, but for larger sample amounts
	HiTrap SP FF	1 or 5	For fast separations with good resolution
	HiPrep 16/10 SP FF	20	Same as the previous, but for larger sample amounts

Table continued next page.

Table 1.6. continued

Technique	Column	Column volume (ml)	Comment
Gel filtration	Superdex 200 10/300 GL	24	For highest resolution size separations of membrane protein-detergent complexes in the molecular weight range 10 000 to 600 000* in sample volumes up to 250 µl
	HiLoad 16/60 Superdex 200 pg	120	For size separations of proteins in the molecular weight range 10 000 to 600 000* in sample volumes up to 5 ml (120 ml column) or 12 ml (320 ml column)
	HiLoad 26/60 Superdex 200 pg	320	
	Superdex 75 10/300 GL	24	For highest resolution size separations of proteins in the molecular weight range 3000 -70 000* in sample volumes up to 250 µl
	HiLoad 16/60 Superdex 75 pg	120	For size separations of membrane protein-detergent complexes in the molecular weight range 3000 to 70 000* in sample volumes up to 5 ml (120 ml column) or 12 ml (320 ml column)
	HiLoad 26/60 Superdex 75 pg	320	

* Determined for soluble, globular proteins.

The solubility of membrane proteins can be very sensitive to ionic strength. When this is the case, fractions eluted in a salt gradient from an ion exchange chromatography column should be immediately diluted or run on a PD-10 Desalting, HiTrap Desalting, or a HiPrep 26/10 Desalting column to reduce the salt concentration (e.g., to < 50 mM).

Gel filtration is excellent for the final purification step as it both removes aggregates and simultaneously achieves buffer exchange, if required.

Tag cleavage

The affinity tag can be removed after purification if a protease cleavage site has been inserted between the tag and the target protein. If using an ÄKTAxpress system, on-column cleavage protocols can be performed automatically. Protease activity can be affected by the presence of detergents. Comprehensive data on the activity of different proteases in detergents is largely lacking, but both PreScission™ Protease and thrombin have been used successfully with detergents. It is recommended to check for the extent of cleavage under different conditions by SDS-PAGE (see "Purity and homogeneity" check on page 40).

PreScission protease exhibits excellent cleavage properties at 4°C and is a useful alternative for cleavage of sensitive proteins (15).

TEV protease is fully active in 9 mM decyl maltoside, but is partly inactive in several other detergents (16).

See "Ordering information" on page 103 for proteases available from GE Healthcare.

Purification of non-tagged membrane proteins

For abundant membrane proteins, the natural source, rather than a heterologous host, is often the best starting material for the purification. Due to the lack of an affinity tag, and hence a highly specific initial purification step, a combination of several classical protein purification techniques usually has to be employed to obtain sufficient purity. The columns listed in Table 1.6 are also commonly used in purification schemes for non-tagged membrane proteins, in combination with columns for other chromatography techniques. For an overview, see the *Protein Purification Handbook* in this series.

Ion exchange chromatography is often used as the main purification step for the purification of non-tagged membrane proteins. Figure 1.11 shows the anion exchange purification step for a decyl maltoside solubilized glucose transporter membrane protein. The purity after this step was above 90%.

Column:	HiTrap Q HP, 1 ml
Sample:	Solubilized human erythrocyte membranes
Start buffer:	10 mM Bis–Tris, 0.5 mM EDTA, 0.2% DM, pH 6.0
Gradient:	0 to 500 mM NaCl in 15 CV
Flow rate:	Load: 0.5 ml/min; elution: 0.25 ml/min

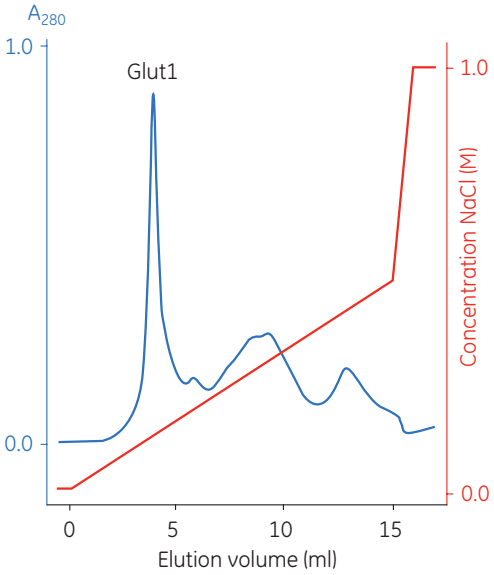


Fig 1.11. Purification of the glucose transporter from human erythrocyte membranes (Glut1) by anion exchange chromatography. Erythrocyte membranes were solubilized with decyl maltoside. Glut1 eluted as a sharp peak at 100 mM NaCl with a purity greater than 90%. From reference 17. Used with permission. Copyright 2001 Elsevier Publication.

Avoid using anionic detergents with anion exchange columns, and cationic detergents with cation exchange columns.

As indicated earlier, gel filtration is the ideal final purification step for membrane proteins. It is excellent for polishing as it removes aggregates and other impurities that are of a different size than the target protein, while simultaneously performing buffer exchange.

Figure 1.12 shows the use of repeated gel filtration for final purification of the bovine creatine transporter from HEK293 cells (18). Membranes were solubilized with decyl maltoside and initial purification was performed on a lectin column (wheat germ agglutinin immobilized on Sepharose). Initial gel filtration on a Superdex 200 column gave a broad, nonsymmetrical peak (Fig 1.12, upper). Additional gel filtration of a narrow fraction on the same column gave a symmetrical peak, indicating homogeneity. Purity was confirmed by SDS-PAGE.

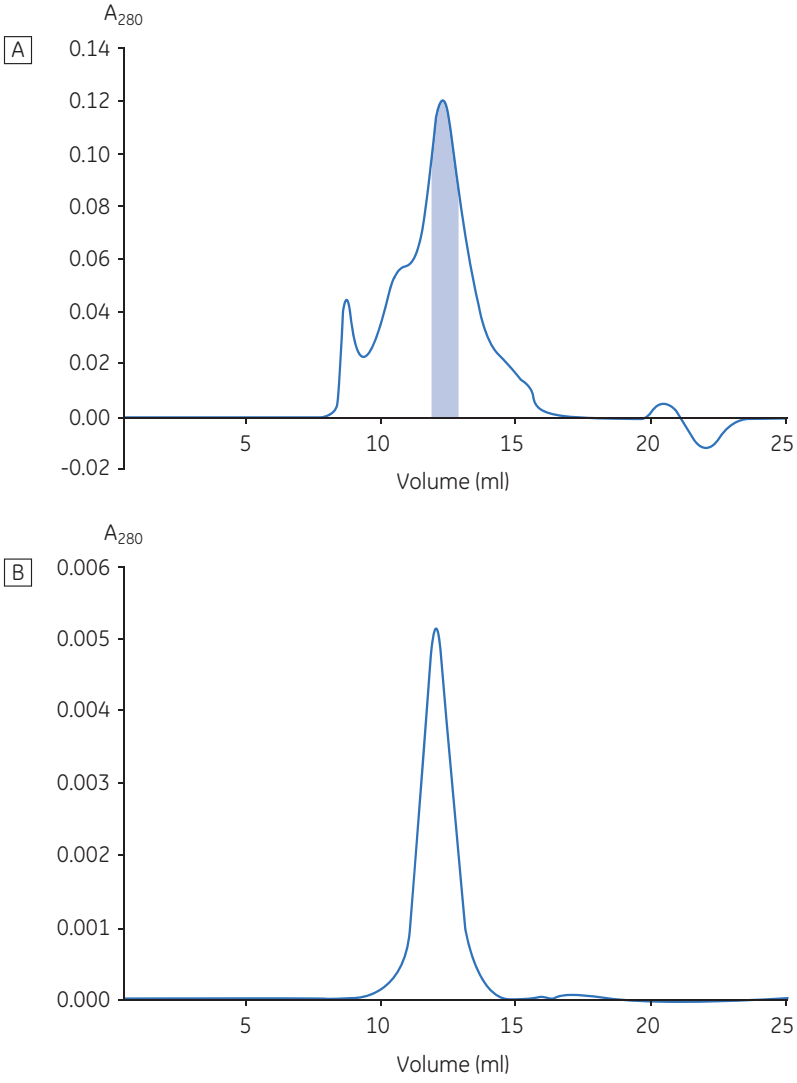


Fig 1.12. Purification of the bovine creatine transporter from HEK293 cells. Membranes were solubilized with decyl maltoside and initial purification was done on immobilized wheat germ agglutinin (WGA, not shown). (A) Material eluted from the WGA–Sepharose column was concentrated and run on a Superdex 200 HR 10/30 column, which yielded multiple peaks. (B) Material in the peak fractions (shown by the shaded bar) was concentrated. Re-chromatography of pooled fractions from this central region gave a symmetrical peak, indicating homogeneity. From reference 18. Used with permission. Copyright 2005 Elsevier Scientific Publication.

Purity and homogeneity check

Purity check

As with water-soluble proteins, SDS-PAGE is the most widespread method for assessing the purity of membrane proteins. Coomassie Blue, silver staining, or Deep Purple™ (for fluorescence) can be used for detection. The Laemmli system (19) is commonly used. Some modifications may be necessary for membrane proteins, as outlined below. Suitable products are listed in the ordering information section.

Boiling of the sample with SDS can cause aggregation of membrane proteins. As an alternative to boiling, incubation at 60°C for 30 min or at 37°C for 60 min are useful starting points for preparing the sample for SDS-PAGE. On the other hand, some membrane proteins are fully compatible with boiling and boiling may be required for complete solubilization with SDS.

Membrane proteins frequently do not move according to the expected molecular weight in SDS-PAGE. They often move faster (*i.e.,* appear smaller) possibly due to incomplete unfolding or due to binding more SDS per mass unit protein as compared with a water-soluble protein.

SDS-PAGE Clean-Up kit has successfully been used to treat samples containing interfering detergent or that are too dilute for SDS-PAGE. With this kit, proteins are quantitatively precipitated while interfering substances remain in solution.

Size homogeneity characterization

Protein aggregation is a common issue with membrane proteins. Aggregation often appears to be irreversible and it may occur slowly over time but also rapidly and unexpectedly with modest changes in ionic strength, pH, protein:detergent ratio, and other factors. For membrane proteins, it is as important to keep track of aggregation as it is to monitor protein activity.

Aggregation may not always be detected by SDS-PAGE since SDS solubilizes most aggregates. Gel filtration is the method of choice for rapid detection of aggregation and it can be applied under a wide variety of conditions. It is widely used as an efficient assay to assess the size homogeneity in purified membrane protein samples.

Gel filtration allows detection of relatively small changes in the size of detergent-protein complexes when different detergents are compared. Gel filtration is often used to give an indication of the suitability of different detergents for a particular protein. Separation with gel filtration is thus an important tool for qualifying the membrane protein preparation for further analysis. As an example, gel filtration is very often used for assessing the suitability of different detergents for membrane protein crystallization. In some cases, however, membrane proteins have been found to crystallize under conditions where the protein preparation did not look homogenous.

Rapid size homogeneity characterization of membrane proteins by gel filtration with Superdex 200 5/150 GL

Material

Column:	Superdex 200 5/150 GL (CV = 3 ml)
Buffer:	Buffers in the pH range 3 to 12 can be used. An ionic strength above 15 mS/cm is recommended. Include the selected detergent(s) at a concentration above the CMC.

Procedure

1. Equilibrate the column with 2 CV of buffer.
2. Allow the protein to equilibrate in the buffer to avoid aggregation or disaggregation of the detergent-membrane protein complex during the gel filtration run. Check that the sample is not turbid. If it is, centrifuge at 10 000 × g for 10 min.
3. Apply the sample (4 to 50 µl) and perform the separation at a flow rate of 0.6 ml/min. Back pressure must not exceed 1.5 MPa. The run will take approximately 6 min. (Fig 1.7)

Size homogeneity characterization of membrane proteins by gel filtration with Superdex 200 10/300 GL

Material

Column:	Superdex 200 10/300 GL (CV = 24 ml)
Buffer:	Buffers in the pH range 3 to 12 can be used. An ionic strength above 15 mS/cm is recommended. Include the selected detergent(s) at a concentration above the CMC.

Procedure

1. Equilibrate the column with 2 CV of buffer.
2. Allow the protein to equilibrate in the buffer to avoid aggregation or disaggregation of the detergent-membrane protein complex during the gel filtration run. Check that the sample is not turbid. If it is, centrifuge at 10 000 × g for 10 min..
3. Apply the sample (25 to 250 µl) and perform the separation at a flow rate of 0.75 ml/min. Back pressure must not exceed 1.5 MPa. The run will take approximately 40 min.

It is possible to achieve the same information at a slightly lower resolution in only 6 min using a Superdex 200 5/150 GL column (Fig 1.7).

Size homogeneity analysis of purified membrane proteins using Superdex 200 10/300 GL is shown in Figure 1.13.

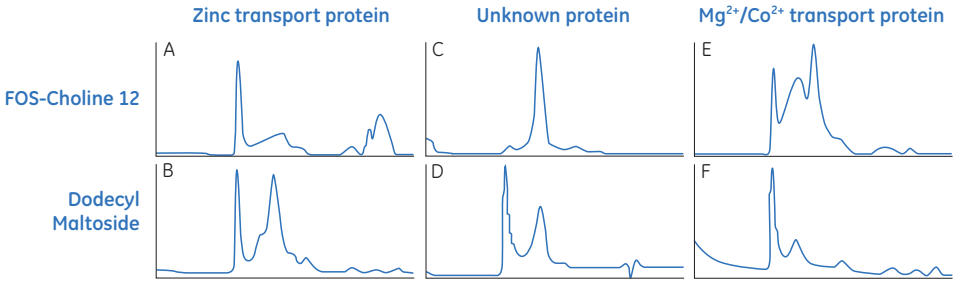


Fig 1.13. Size homogeneity characterization of membrane proteins by gel filtration. IMAC purifications of three *E. coli* membrane proteins were analyzed by gel filtration using Superdex 200. Each protein was purified separately by IMAC in the presence of two different detergents. Gel filtration analysis was done with the same detergent as used for purification. A symmetric peak (which is not at the void volume), as in chromatogram C, shows that the protein did not aggregate, indicating that purification was successful. Blue line = A_{280} trace. From reference 20. Used with permission. Copyright 2005 The Protein Society.

Charge homogeneity characterization

High-resolution anion exchange chromatography is widely used for purification of membrane proteins, often as a second chromatography step after IMAC. It is also an excellent technique for charge homogeneity characterization of purified membrane proteins. Figure 1.14 shows charge homogeneity characterization of fractions obtained from the IMAC purification of histidine-tagged cytochrome *bo*$_3$ ubiquinol oxidase, a membrane protein complex, overexpressed in *E. coli*. The first IMAC fraction gave a shouldered peak on Mono Q (left). Heterogeneity was confirmed by SDS-PAGE. The second IMAC fraction gave a single sharp, symmetrical peak on Mono Q. Homogeneity was confirmed by SDS-PAGE which showed four bands corresponding to the expected subunit molecular weights of 58 000, 33 000, 22 000, and 17 000.

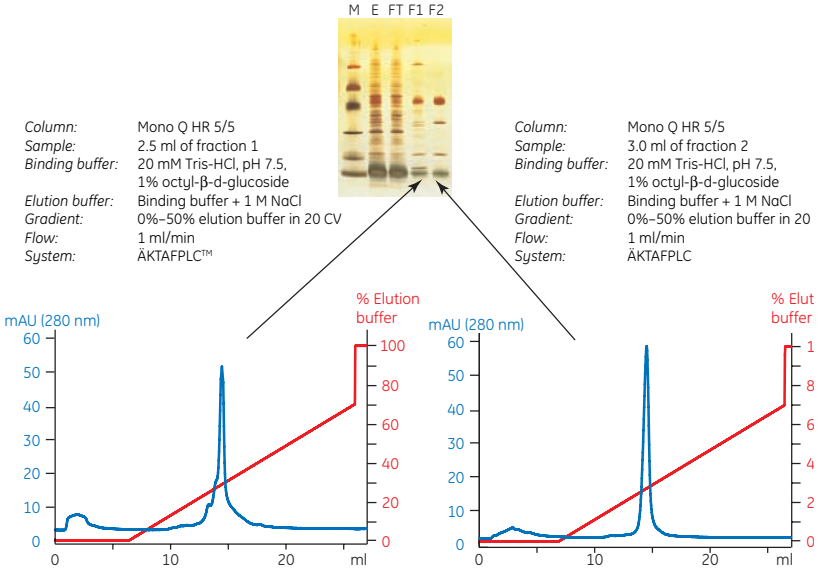


Fig 1.14. Charge homogeneity characterization of histidine-tagged cytochrome bo$_3$ ubiquinol oxidase using anion exchange chromatography with Mono Q. Two fractions obtained from IMAC purification were analyzed. Electrophoretic analysis was done with PhastSystem™ using PhastGel™ 8-25% and silver staining. M = Low Molecular Weight Calibration Kit; E = detergent extract of *E. coli* membranes; FT = flowthrough material; F1 = fraction 1 from HiTrap Chelating HP 1 ml; F2 = fraction 2 from HiTrap Chelating HP 1 ml. See application note 18-1128-92 "Purification and chromatographic characterization of an integral membrane protein."

Conditioning

The purified membrane protein often has to be concentrated or transferred to a suitable environment for further analysis. Common manipulations include concentration, desalting, buffer exchange, detergent exchange, and detergent removal. Protocols for these operations are given below.

Concentration

Ultrafiltration-based concentration techniques are commonly used for membrane proteins. They often also concentrate the detergent micelles and hence change the detergent:protein ratio, which may lead to precipitation. Another potential problem is an increased protein concentration at the filter surface which may lead to aggregation and precipitation. Column chromatography is an alternative, milder concentration technique. IMAC is excellent for concentration of histidine-tagged proteins, and ion exchange chromatography is used for both tagged and non-tagged proteins. Small diameter (10 to 35 µm) and monodisperse chromatography beads are advantageous to use because they minimize band broadening at elution. Desalting may be needed to remove imidazole or salt after column concentration.

HisTrap columns can be used for concentration of purified histidine-tagged membrane proteins under mild conditions. The purified protein is adsorbed at neutral pH in a buffer containing 0 to 20 mM imidazole. Several mg of protein can usually be adsorbed, but the maximum column capacity is protein dependent. Elution is achieved in a single step by elution with 0.5 to 1 M imidazole, as required.

Ion exchange columns (e.g., HiTrap Q HP, RESOURCE Q, or Mono Q 5/50 GL) can be used for concentration of membrane proteins under mild conditions. Elution is achieved in a single step with high salt (e.g., 0.5 to 1 M NaCl) or a change in pH. Protocol details will depend on the specific protein.

Desalting or buffer exchange

Desalting protocols for membrane proteins use detergents in buffers, but are otherwise identical to those for soluble proteins.

Material

Sample volume per run (ml)	Product	Operation
0.01 to 0.1	illustra™ MicroSpin™ G-25 Columns	Centrifuge (spin column)
1.5 to 2.5	Disposable PD-10 Desalting Columns	Manual (gravity feed)
0.25 to 1.5	HiTrap Desalting	Chromatography system, a peristaltic pump or a syringe (sample volume can be increased to 3 or 4.5 ml when using two or three columns, respectively, connected in series)
2.5 to 15	HiPrep 26/10 Desalting	Chromatography system (sample volume can be increased to 30, 45, or 60 ml when using two, three, or four columns, respectively, connected in series)

Procedure

Follow the recommended procedure supplied with each product. The general outline is described below.

1. Equilibrate the column with the desired buffer.
2. Apply the sample. The maximum recommended sample volume should not be exceeded.
3. Apply elution buffer and collect the protein material in recommended fraction volumes.

Detergent exchange

Detergent exchange may be needed for a number of reasons. Both for cost reasons and performance aspects, it is common that the detergent used for solubilization and/or purification is exchanged for a detergent that is more suitable for further analyses.

Dialysis is often used for this purpose but can be very inefficient. Detergents with a low CMC are dialyzed very poorly since micelles pass slowly or not at all through the dialysis membrane. Ultrafiltration-based approaches can be inefficient for the same reason. In addition to problems with permeability, the protein concentration may become very high close to the dialysis/ultrafiltration membrane, which may lead to extensive aggregation.

Column-based approaches for detergent exchange are often mild and efficient. A potential drawback with column-based methods is that the elution buffer may contain unwanted components that need to be removed from the eluted membrane protein. This problem can be solved by desalting and buffer exchange as described in the previous section.

Detergent exchange of purified membrane proteins with Q Sepharose Fast Flow or RESOURCE Q

Material

Column choices:

HiTrap Q FF, 1 ml

HiTrap Q FF, 5 ml

RESOURCE Q, 1 ml

RESOURCE Q, 6 ml

Procedure

Use the flow rate recommended in the column instructions.

The buffer conditions of the sample and binding buffer (ionic strength and pH) that allow binding need to be determined for each protein.

1. Equilibrate the column with the 2 CV of a low ionic strength buffer, containing the same detergent and detergent concentration as the protein sample. A suitable buffer is 50 mM Tris-HCl, 50 mM NaCl, pH 8.0, plus detergent.
2. Apply the sample.
3. Wash the column with 5 to 12 CV of a low ionic strength buffer containing the new detergent. A suitable buffer is 50 mM Tris-HCl, 50 mM NaCl, pH 8.0, plus the new detergent.
4. Elute in one step with the same buffer containing 0.6 M NaCl. A higher salt concentration can be used if the protein is not quantitatively eluted.

This procedure was taken from references 21 and 22.

Avoid using anionic detergents with this protocol.

HisTrap HP, 1 ml can be used for detergent exchange of purified histidine-tagged membrane proteins following a similar protocol as for Q Sepharose Fast Flow above. Elution is achieved in a single step by elution with 0.5 to 1 M imidazole.

Detergent exchange with Phenyl Sepharose

Detergent saturated Phenyl Sepharose is used to exchange detergents for one another in the presence of membrane proteins. Protein binding to the medium is minimized by using a high pH. The original reference (23) used another Sepharose base matrix than those of the current products listed below.

Material

Column choices:

HiTrap Phenyl FF (high sub), 1 ml

HiTrap Phenyl FF (low sub), 1 ml

HiTrap Phenyl HP, 1 ml

Buffer: 20 mM Tris-HCl, 1 mM EDTA, appropriate detergent at or slightly above the CMC, pH 8 to 9

Sample: 1 to 4 mg membrane protein/ml, in 0.5 to 1 ml of buffer and the detergent to be removed.

Procedure

1. Saturate the column with the new detergent in buffer. Detergent concentration should be at or slightly above the CMC.
2. Apply the sample to the column and elute with buffer (isocratic elution). The detergent in the sample displaces detergent that had been adsorbed to the column.

For membrane proteins that tolerate organic solvents, detergent can be removed using polymer-based reversed phase chromatography (RPC) media (e.g., RESOURCE RPC, 1 ml). The purified, detergent-solubilized membrane protein is adsorbed to the column, washed with a weak phosphate buffer (without detergent) and eluted with acetonitrile. The eluted protein can be lyophilized and reconstituted.

Proteomic analysis of membrane proteins

A common objective of proteomic analysis is to identify and quantitate all proteins expressed in a cell or a tissue at a particular point in time. The standard approach towards this goal is to separate proteins by 2-D gel electrophoresis followed by trypsin digestion and analysis by mass spectrometry (MS). However, cell surface proteins, and in particular integral membrane proteins, can be difficult to detect in a 2-D gel without prefractionation by chromatography or some other type of enrichment. This is due to their low abundance and the difficulty of solubilizing membrane proteins and of maintaining their solubility in the first-dimension separation (isoelectric focusing).

Prefractionation using an aqueous two-phase system

In one example of prefractionation (24), mitochondrial proteins from *Saccharomyces cerevisiae* were fractionated in an aqueous two-phase system (Fig 1.15) consisting of a polymer and a non-ionic detergent. Membrane proteins were enriched in the detergent phase, which was then used for ion exchange chromatography. Eluted fractions were further separated by SDS-PAGE and gel bands were analyzed by MS. More membrane proteins were identified by this method than by using 2-D gel electrophoresis alone.

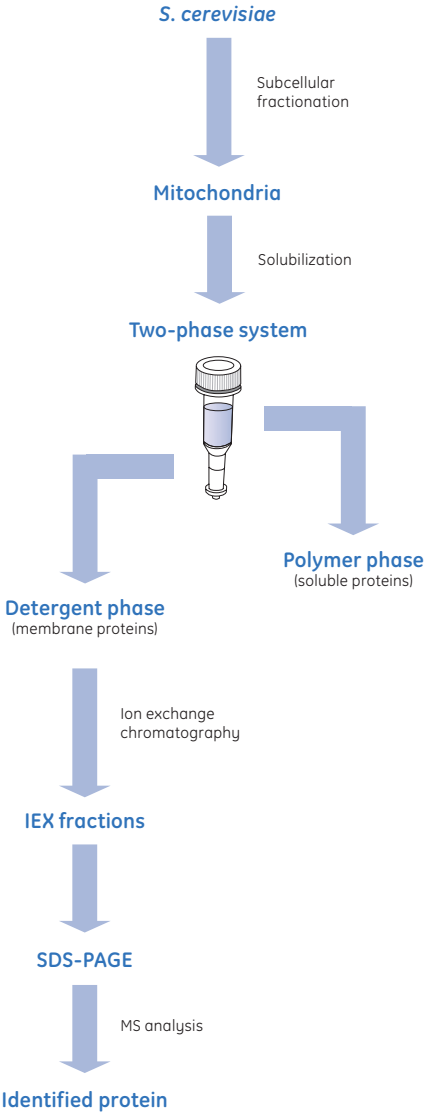


Fig 1.15. Mitochondria from S. *cerevisae* were isolated by differential centrifugation. The isolated mitochondria were fractionated in a detergent–polymer aqueous two-phase system formed by addition of polymer and salt to the detergent solubilized mitochondrial membranes. Membrane proteins partition to the detergent (bottom) phase and the bulk of soluble proteins to the polymer (top) phase. The detergent phase was further fractionated with ion exchange chomatography and fractions were analyzed with one-dimension gel electrophoresis. Bands were identified with MS analysis. From reference 24. Used with permission. Copyright 2004. Elsevier BV.

Selective labeling of the extracellular portion of membrane proteins

CyDye™ DIGE Fluor minimal dyes can be used to visually enrich for, and detect the extracellular portion of membrane proteins. The method is fast, simple to use, and pre-fractionation is not required. All CyDye DIGE Fluor minimal dyes can be used to label cell surface proteins.

These features allow for multiplexing using Ettan™ DIGE technology and analysis of protein expression using DeCyder™ 2-D software. In this way, small changes in abundance can be detected with high accuracy, and results are supported by defined statistical methods. This protocol is schematically shown in Figure 1.16.

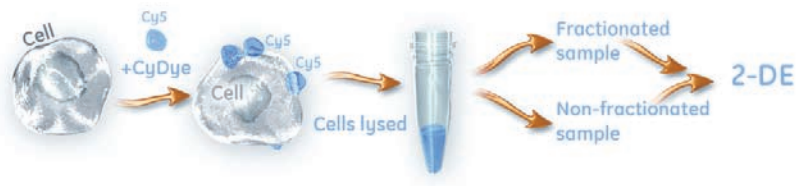


Fig 1.16. Outline of a procedure for selective labeling of cell-surface proteins using CyDye DIGE Fluor minimal dyes.

Buffers

Hanks Buffered Salt Solution (HBSS)
137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$, pH 8.5

Labeling buffer: HBSS, 1 M urea, pH 8.5

Lysis buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, 5 mM magnesium acetate, pH 8.5

2× Sample buffer for CyDye DIGE Fluor minimal dyes:
7 M urea, 2 M thiourea, 2% CHAPS, 2% IPG buffer pH 3–10, 130 mM dithiothreitol

Material

CyDye DIGE Fluor Cy™2 minimal dye

CyDye DIGE Fluor Cy3 minimal dye

CyDye DIGE Fluor Cy5 minimal dye

Procedure

1. Grow the cells of interest until a minimal cell number of 5-10 × 10^6 cells (confluent or cells in suspension) is reached. For cells growing in suspension, proceed to step 3.
2. Carefully detach adherent cells non-enzymatically (cell dissociation media, enzyme free PBS-based).
3. Count the cells and pellet them by centrifugation. For the following steps, keep the cells on ice.
4. Wash the cells by resuspending the pellet in 1 ml cold HBSS buffer, pH 8.5, and transfer to a 1.5 ml microcentrifuge tube. Centrifuge the suspension at 800 × g and 4°C for 2 min.
5. Remove the supernatant and resuspend the cell pellet (containing 5-10 × 10^6 cells) in 200 µl cold labeling buffer. Add 1.5 µl CyDye working solution (600 pmoles). Mix briefly by vortexing at slow speed.
6. Label the cell surface for 20 min on ice in the dark. To quench the reaction, add 20 µl of 10 mM lysine and incubate another 10 min.
7. After labeling and quenching, wash the cells twice in 500 µl cold HBSS pH 7.4 by centrifugation at 800 × g and 4°C for 2 min.
8. Resuspend the pellets directly in 150 µl cold lysis buffer and leave on ice for at least 1 h with occasional vortexing. Centrifuge the lysate at 10 000 × g and 4°C for 5 min. Transfer the supernatant to a new tube.

   Note: Fractionation of the sample may be desired. It is not necessary for improved detection of cell surface proteins but could be used to verify lack of labeling of cytosolic proteins. After fractionation the sample can be directly applied on 2-D electrophoresis.
9. Add 2× sample buffer to the lysed cell surface labeled sample. Now the sample is ready for 2-D electrophoresis. Proceed with the recommended protocols for 2-D DIGE electrophoresis.

Table 1.7 provides a summary of measures that can be taken to overcome the challenges associated with 2-D gel electrophoresis of membrane proteins. For further information on 2-D gel electrophoresis, see *2-D Electrophoresis - Principles and Methods*.

Table 1.7. Challenges and proposed remedies for proteomic analysis of membrane proteins with 2-D gel electrophoresis

Challenge	Proposed remedy
Poor representation of membrane proteins in the stained protein spots	Selectively label surface proteins with CyDye dyes to enhance detection. (Application Note 11-0033-92).
Maintaining the solubility of membrane proteins during isoelectric focusing	Use an isothiourea/urea mixture instead of only urea.
Streaking, unclear bands	Maintain stable reducing conditions (DeStreak Reagent)
Poor results in standard 2-D gel electrophoresis	Run 2-D detergent electrophoresis

For information on using SDS-PAGE for purity checking of membrane proteins, see “Purity and homogeneity check” on page 40.

An alternative to the 2-D gel electrophoresis approach is "shotgun proteomics" involving solution proteolysis of proteins followed by peptide separation by liquid chromatography and sequencing by MS/MS. To specifically study membrane proteins with this approach, the membrane fraction is typically obtained by cell disruption (for methods, see Table 1.2) and differential centrifugation to prepare organelles and cell membranes. The detailed centrifugation protocol will depend on the cell type studied. Some alternative methodologies for the subsequent combined solubilization and proteolysis step are listed in Table 1.8.

Table 1.8. Examples of solubilization and proteolysis approaches for "shotgun proteomics" of membrane proteins from enriched membranes

Cell type	Solubilization	Proteolysis	Reference
Yeast	90% formic acid	Cyanogen bromide	25
Human myeloid leukemia (HL-60)	0.5% SDS	Trypsin	26
Deinococcus radiodurans	60% methanol	Trypsin	27, 28
Rat brain	8 M urea, pH 11	Proteinase K	29*

* This procedure involves cleavage (and identification) of the exposed hydrophilic domains of membrane proteins, but not global solubilization.

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Chapter 2
Multiprotein complexes

Introduction

Proteins do not carry out their functions in isolation but mainly through interactions with other proteins. Global protein-protein interaction maps ("the interactome") have been published for some organisms. Such maps reveal extensive networks of multiprotein complexes and intercomplex connections. Genome analyses have suggested that the complexity of higher organisms is not mediated by a larger number of genes but rather it is obtained, to a large extent, by post-translational modifications and by protein functions (e.g., regulation of gene expression and alternative splicing, enzyme action, transport, signaling, structure, etc.). It has been found that most processes require protein-protein interactions and that these interactions constitute the mechanistic basis for much of the physiology of all organisms. Clearly, protein-protein interaction networks are extremely complex. For instance, more than 5000 interactions have been mapped in the *C. elegans* network (Fig 2.1). Also, the fundamental importance of protein-protein interactions has lead to the realization that protein complexes are potentially important drug targets.

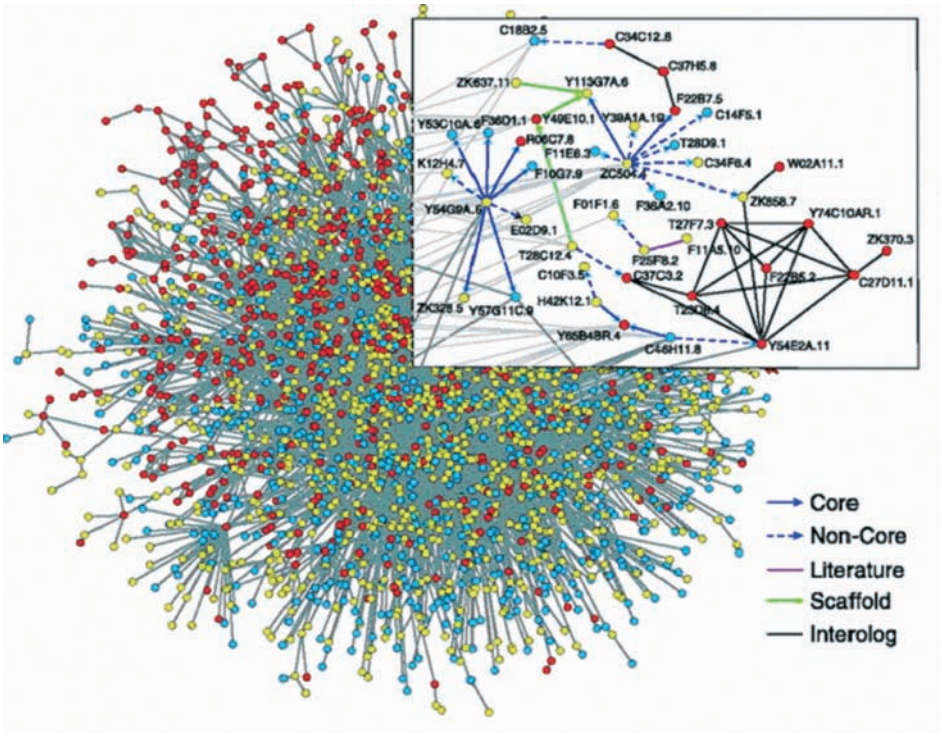


Fig 2.1. Analysis of the *C. elegans* network. Nodes (representing proteins) are colored according to their phylogenic class: ancient (red), multicellular (yellow), and worm (blue). Edges represent protein-protein interactions. The inset highlights a small part of the network. From Li, S. *et al.* A map of the interactome network of the metazoan *C. elegans*. *Science* **303**, 540–543 (2004). Reprinted with permission from AAAS.

Many protein-protein interactions result in the formation of stable complexes that can be isolated and characterized in molecular detail. For example, stable binary complexes such as simple antigen-antibody complexes and protease-protease inhibitor complexes can be readily isolated and studied. Protein complexes can be very large with above 10 and even above 100 different subunits (Fig 2.2). The experimental challenges increase with size and number of different subunits.

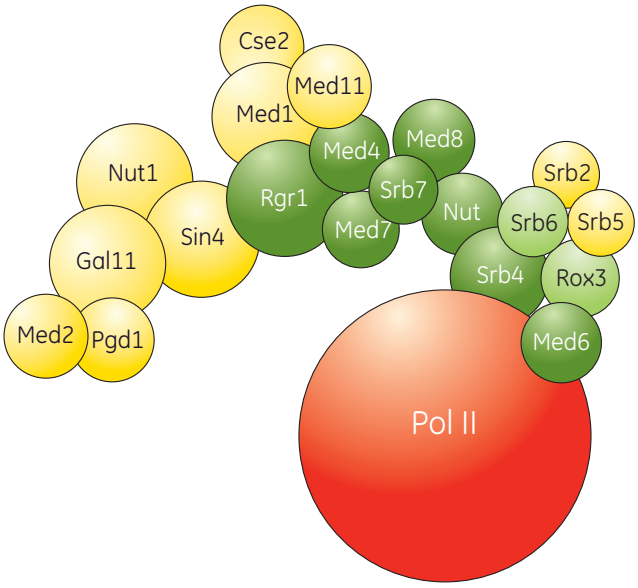


Fig 2.2. An example of a multiprotein complex. A model of the *Saccharomyces cerevisiae* (transcription) mediator complex consisting of 20 subunits. Obtained from H. Spahr, Karolinska Institute, Stockholm. Used with permission.

Key challenges related to multiprotein complex research include:

- **Stability:** Subunits can be loosely associated with a particular complex, and may be lost when standard biochemical methods are used for complex isolation.
- **False positives:** Irrelevant components may co-purify with the complex and appear as false positives if isolation conditions are not appropriate. Also, artifactual interactions may occur after the disruption of subcellular compartmentalization. Artifactual interactions may also occur when components of a complex are overexpressed. Stringent control experiments are essential to avoid pitfalls.
- **Dynamics:** Some components are transiently associated with a particular complex. Such components may easily be overlooked in studies.
- **Size:** Multiprotein complexes can be very large and therefore difficult to manage by conventional biochemical techniques. They may, for instance, not be able to pass through standard filters for particle removal or enter pores of chromatographic media.
- **Production:** Over-expression of multiprotein complexes consisting of a large number of different subunits is not feasible. Large amounts of start material are required for biochemical studies of low abundance complexes.
- **Analytical techniques:** Mass spectrometry can be limited by very high mass charge ratios (*m/z*) and by large ions deviating from ideal trajectories.

This chapter focuses on technologies that involve the isolation of multiprotein complexes for the purpose of identifying their constituents and for structural and functional characterization. The different areas that are covered in this handbook are schematically shown in Fig 2.3. Each of these three areas will be covered in this chapter.

In a wider perspective, the field of protein-protein interaction analysis is vast and technologies for characterizing them are plentiful. An overview is outside the scope of this handbook. The reader is referred to reference 1 for a more comprehensive overview.

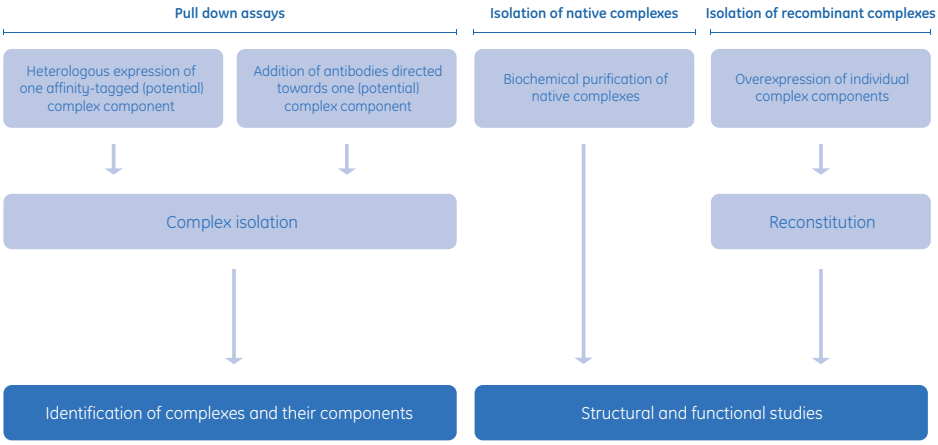


Fig 2.3. Workflows for the production and analysis of multiprotein complexes.

Emerging workflows

Co-expression of proteins in prokaryotic and eukaryotic hosts is becoming an alternative for the production of multiprotein complexes in sufficient amounts for biochemical studies. This approach has earlier been associated with decreasing yields as the number of expressed genes increase. Although still an issue, this has successfully been addressed by the use of high cell density fermentation technology. For an overview, see reference 2.

Technology for directly studying large protein complexes by mass spectrometry is also emerging. Limitations due to very high mass to charge (m/z) ratios, and challenges with large ions deviating from ideal trajectories in the mass spectrometer have been addressed by modifying existing instruments (3).

Pull-down assays

Pull-down assays involve isolation of a protein complex by adsorbing the complex onto beads. Immobilized ligands on the beads bind specifically to a component of the complex, either via an affinity tag (e.g., GST, histidine, maltose binding protein, etc.) or an antibody. Pull-down assays are often used for the isolation of low µg amounts of complexes, mainly for the purpose of identifying individual subunits. They have also been used to isolate material for limited functional studies, but other methods appear more suitable for the production of larger (mg) amounts. The components of the captured complex are often eventually analyzed by mass spectrometry to identify the subunits.

Pull-down assays are important tools for mapping protein-protein interaction networks. They have been successfully used on a global scale to map protein-protein interactions in a number of organisms (e.g., yeast, *E. coli*, *C. elegans*). Genetic approaches involving the yeast-two-hybrid (Y2H) system and similar technologies are useful complements to multiprotein complex isolation. False positives are a major concern associated with mapping protein-protein interactions on a global scale. Therefore, in addition to the relevant control experiments, it is useful to perform both complex isolation and Y2H experiments independently to validate data sets (4). This can be followed by verification in a more *in vivo*-like milieu.

This chapter covers three different pull-down methods for isolating small amounts of complexes for the purpose of identifying components of the complex. Two of the methods involve the affinity tagging of one (known or supposed) component of the complex. The third method, co-immunoprecipitation, involves the use of an antibody directed towards one (known or suspected) component. All three methods use beads with appropriate ligands to pull down the complex from a crude biological sample. An overview of the pros and cons of the different methods is shown in Table 2.1.

Table 2.1. Advantages and disadvantages with different pull-down assays for the retrieval of protein complexes

Method	Advantages	Disadvantages
Affinity pull-down	Generic	The presence of a protein tag may influence results
	Ability to purify low-abundant protein complexes	Competition with the endogenous complex
Tandem affinity purification (TAP)	Generic	The presence of a protein tag may influence results
	Ability to purify low-abundant protein complexes	Competition with the endogenous complex
	Mild conditions used throughout	
Co-immunoprecipitation	Does not require cloning and heterologous expression.	Not generic - requires access to specific antibodies
	Rapid if antibody is available	

Affinity pull-down assay

Affinity pull-down assays are well established for the isolation and subsequent identification of protein complexes (for an overview, see reference 5). GST pull-down assays involve affinity purifications of one or several unknown proteins from a biological sample using a GST-tagged bait protein. The basic principle is that the GST-tagged bait protein binds to its partners, and the resulting complex is captured on beads with immobilized glutathione. A control is included to identify false positives that bind to GST in the absence of a bait protein. The control can either be lysate from separately transformed cells that express GST (not the bait fusion protein) or lysate from non-transformed cells to which GST is added. Design of the appropriate controls for the experiment is essential.

A common assay format is to spin down the beads and the bound proteins with a centrifuge, hence the term "pull-down". The principle is shown in Fig 2.4.

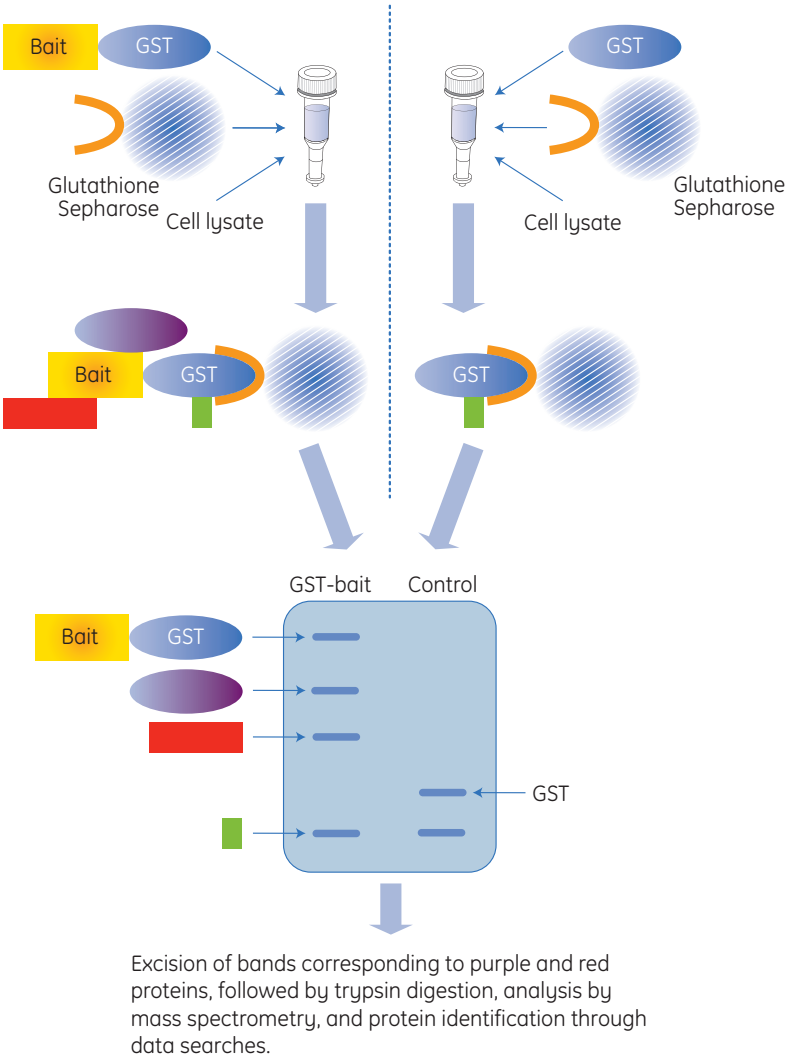


Fig 2.4. Outline of a GST pull-down experiment. The right-hand procedure shows the control experiment. In this example, two proteins (purple and red) were identified by SDS-PAGE as interaction partners with the bait protein (left lane). One additional protein (green) was pulled down but identified as a false positive (GST binder) by the control (right lane).

For cloning and expression of GST-fusion proteins for use as bait proteins, see the *GST Gene Fusion System Handbook* (see "Related literature", page 100). For the pull-down assay procedure, see below.

GST pull-down assay using GST SpinTrap Purification Module

Materials

GST SpinTrap Purification Module containing 10× PBS, MicroSpin columns, dilution buffer, and reduced glutathione

Buffer preparation

1× PBS:	To prepare 1× PBS, dilute the supplied 10× PBS ten-fold with sterile water. Store at 4°C. Final concentration will be 10 mM phosphate, 2.7 mM KCl, 140 mM NaCl, pH 7.3.
Dilution buffer:	as supplied, 50 mM Tris-HCl, pH 8.0
Elution buffer:	10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0 To prepare elution buffer, pour the entire 50 ml volume of dilution buffer supplied with the module into the bottle containing the reduced glutathione (0.154 g). Shake until completely dissolved. Store as 1 to 20 ml aliquots at -20°C.

Procedure

The amount of lysate required to enable the detection of protein complex components varies. A useful starting point is a lysate volume corresponding to 10^6-10^7 tissue culture cells. Use a second column to perform a control experiment using a cell lysate with GST only (no bait fusion protein).

1. Resuspend Glutathione Sepharose 4B in each SpinTrap 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. Centrifuge at 735 × g for 1 min.
4. Discard the buffer from each centrifuge tube and replace the bottom closures.
5. Apply up to 600 µl of lysate containing the GST-fusion (bait) protein to a column. For a control, apply the same volume of lysate with GST (no bait protein).
6. Recap each column securely and mix by gentle, repeated inversion. Incubate at 4°C for 2 h.
7. Remove (and save) the top caps and bottom closures. Place each column into a clean, pre-labelled 1.5 or 2 ml microcentrifuge tube.
8. Centrifuge at 735 × g for 1 min to collect the flowthrough. Save the flowthrough for analysis by SDS-PAGE.
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 centrifugation procedure. Additional 600 µl washes with 1× PBS can be performed if desired. It is important to optimize this step for good results.
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. It is important to optimize this step for good results.
12. Remove and discard top caps and bottom closures and place each column into a clean 1.5 or 2 ml microcentrifuge tube.
13. Centrifuge all columns and collect the eluates. Analyze by SDS-PAGE.

This procedure was adapted from reference 5.

The selection of buffer conditions could affect protein-protein interactions.

It is necessary to optimize wash conditions for each experiment.

Poor recovery of a complex may be due to that the affinity tag is not accessible for binding to the affinity ligand when the complex has been formed. In such cases, it is recommended to change the tagging site (e.g., from N- to C-terminal) or to tag another complex component.

Depending on the scale and the required throughput, a number of options are available for GST pull-down assays (see "Ordering information" section)

Tandem affinity purification, TAP

The TAP method (7), with or without modifications, has been used to purify and identify complexes from yeast, trypanosomes, fruit flies, humans, and plants (see reference 8 for a review).

TAP differs from the approach described earlier by employing two successive affinity chromatography steps to isolate protein complexes. The rationale behind the use of two affinity steps is to enhance the specificity of the purification procedure and hence reduce the number of false positives. Furthermore, the conditions used throughout this method are mild in order to preserve complex integrity and to maximize yield. The method has been used successfully in many cases.

The basic principle behind TAP is shown in Fig 2.5. Briefly, a bait protein (a known or suspected complex component) is tagged with both protein A and calmodulin binding peptide (CBP) with a tobacco etch virus (TEV) protease cleavage site between the two tags. The double-tagged bait protein is expressed at near natural levels, since overexpression may trigger non-physiological interactions. The complex is first adsorbed to IgG Sepharose beads, via the protein A tag, and eluted by cleavage with TEV. The complex is then adsorbed to calmodulin Sepharose beads (via the CBP tag) and eluted with EGTA. The complex components are separated by SDS-PAGE and analyzed by mass spectrometry.

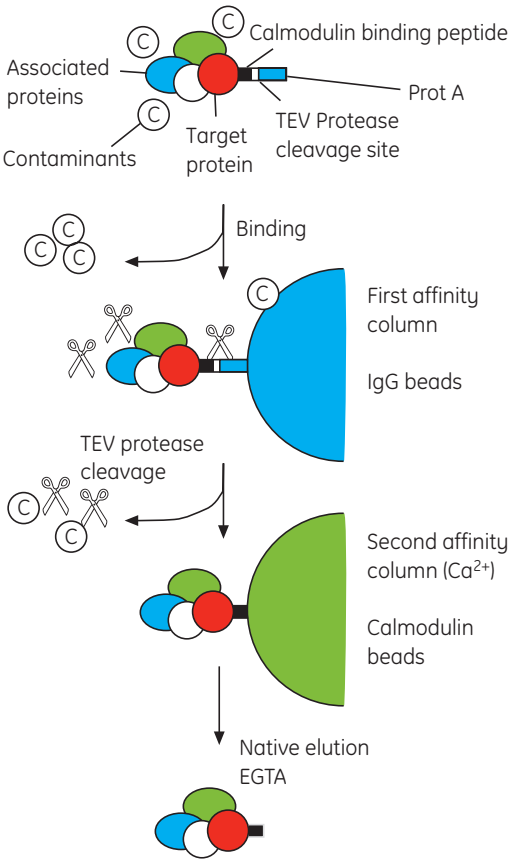


Fig 2.5. Overview of the tandem affinity purification (TAP) method (7). The TAP tag comprises CBP (calmodulin binding peptide) linked to a Protein A domain separated by a TEV cleavage site. Permission to reprint this figure was granted by Elsevier Global Rights Department.

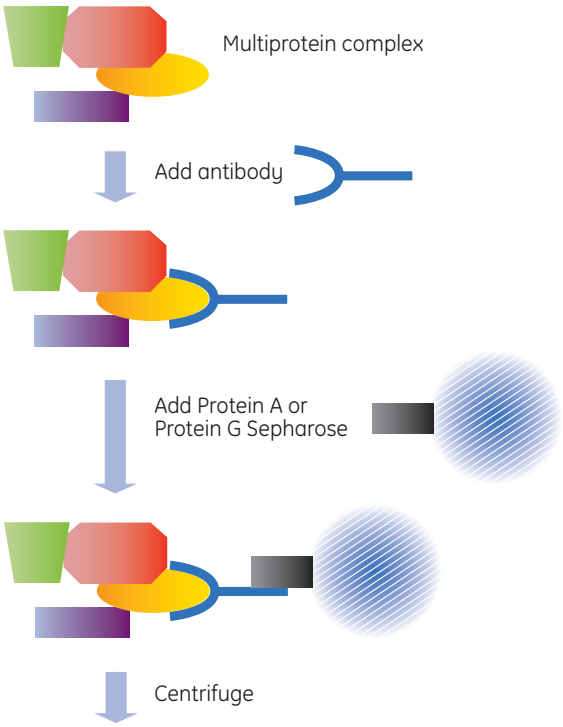
For detailed protocols, see the TAP method home page (http://www-db.embl-heidelberg.de/jss/servlet/de.embl.bk.wwwTools.GroupLeftEMBL/ExternalInfo/seraphin/TAP.html).

In higher organisms it may be necessary to supress the corresponding endogenous gene for the target protein by the use of RNAi technology (iTAP, 9).

Co-immunoprecipitation

Immunoprecipitation is a well-established technique that uses antibodies to isolate antigens from crude biological samples. The name is historical and originates from analysis methods based on the precipitation reaction obtained when mixing antibody and antigen at the correct ratio. The method described here is a pull-down assay rather than a precipitation reaction. Co-immunoprecipitation uses antibodies directed towards one (known or supposed) component of a complex. The antibody binds to its antigen, which is part of a multiprotein complex. The antibody-protein complex assembly is then captured through the addition of protein A or protein G Sepharose beads to the mixture. The principle is shown in Figure 2.6.

Alternatively, an appropriate antibody can be immobilized on an activated matrix, such as NHS-activated Sepharose. Similarly, an antibody can be cross-linked to a Protein A or Protein G medium and used to co-immunoprecipitate components of protein complexes.



Elution and separation with SDS-PAGE followed by trypsin digestion, analysis by mass spectrometry and protein identification through database searches.

Fig 2.6. Outline of a co-immunoprecipitation experiment.

Co-immunoprecipitation with protein A or protein G

Materials

Immunoprecipitation Starter Pack (contains nProtein A Sepharose 4 Fast Flow and Protein G Sepharose 4 Fast Flow)

Lysis buffers

Table 2.3 Common lysis buffers for mammalian tissue culture cells

Name	Description	Stringency
Low salt	1% Igepal™ CA-630, 50 mM Tris, 1 mM PMSF, pH 8.0	+
Nonidet™ P-40	150 mM NaCl, 1% Igepal CA-630, 50 mM Tris, 1 mM PMSF, pH 8.0	++
RIPA	150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 50 mM Tris, 1 mM PMSF, pH 8.0	+++
High salt	500 mM NaCl, 1% Igepal CA-630, 50 mM Tris, 1 mM PMSF, pH 8.0	++++

Other buffers

PBS:	1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4
Wash buffer:	50 mM Tris, pH 8
Sample buffer: (reducing)	1% SDS, 100 mM DTT, 50 mM Tris, pH 7.5

Media preparation

nProtein A Sepharose 4 Fast Flow and Protein G Sepharose 4 Fast Flow are supplied preswollen in 20% ethanol. Each medium must be thoroughly washed prior to use to remove the 20% ethanol storage solution. Residual ethanol may interfere with subsequent procedures.

You will need 50 µl of nProtein A Sepharose 4 Fast Flow or Protein G Sepharose 4 Fast Flow 50% suspension per sample. Make sure that the beads are properly suspended before transferring.

1. Gently shake the bottle of nProtein A Sepharose 4 Fast Flow or Protein G Sepharose 4 Fast Flow to resuspend the medium.
2. Use a pipette with a wide-bore tip to remove sufficient slurry, and transfer the slurry to an appropriate container/tube. If needed, cut the pipette tip to create a larger opening.
3. Sediment the medium by centrifuging at 12 000 × g for 20 s. Carefully decant the supernatant.
4. Wash the medium by adding 10 volumes of lysis buffer (see below). Invert to mix.
5. Sediment the medium by centrifuging at 12 000 × g for 20 s. Decant the supernatant.
6. Repeat steps 4 and 5 for a total of three washes.
7. For each ml of the original slurry of medium dispensed in step 2, add 0.5 ml of lysis buffer. This results in a 50% slurry. Store at 4° C and mix well before use.

Cell lysis

Cell lysis must be harsh enough to release the target antigen, but mild enough to maintain the protein complex and the immunoreactivity of the antigen. Lysis conditions are therefore very critical and need to be optimized for each protein complex.

Some commonly used lysis buffers are listed in Table 2.3. Nonidet P-40 (Igepal CA-630) and RIPA buffer release most soluble cytoplasmic or nuclear proteins without releasing chromosomal DNA and are a good choice for initial experiments. Some parameters that may affect the extraction of antigens include salt concentration (0–1 M NaCl), non-ionic detergents (0.1–2%), ionic detergents (0.01–0.5%), and pH (6–9). For more detailed information about optimization of lysis procedures, see reference 6.

1. Collect cells
   a) Adherent cells: Remove all culture medium and wash twice with ice-cold PBS. Discard the wash solutions and drain well.

   b) Cells in suspension: Collect cells by centrifugation at 1000 × g for 5 minutes and discard the culture medium supernatant. Resuspend the pellet in ice-cold PBS, centrifuge and discard the supernatant. Repeat the wash once.
2. Resuspend cells in lysis buffer
   a) Adherent cells**:** Place the tissue culture dish on ice. Add ice-cold lysis buffer to a concentration of 10^6–10^7 cells/ml (1 ml to a 10 cm diameter cell culture plate). Incubate on ice for 10 to 15 min with occasional rocking.

   b) Cells in suspension: Suspend the washed pellet in ice-cold lysis buffer at a concentration of 10^6–10^7 cells/ml (a volume of lysis buffer corresponding to 10 times the volume of the cell pellet). Incubate on ice for 10 to 15 minutes with gentle mixing.
3. Transfer the cells to a suitable homogenization tube.
4. Further disrupt the cells by sonication or homogenization. Keep the cells on an ice bath to prevent the temperature from rising. See section "Cell disruption and membrane prep" in Chapter 1 for additional information.
6. Centrifuge at 12 000 × g for 10 min at 4°C to remove particulate matter.
7. Transfer the lysate (the supernatant) to a fresh tube. Keep on ice.

Binding of the antigen

1. Aliquot samples (500 µl) into new microcentrifuge tubes.
2. Add polyclonal serum (0.5 to 5 µl), hybridoma tissue culture supernatant (5 to 100 µl), ascites fluid (0.1 to 1 µl), or purified monoclonal or polyclonal antibodies (add the volume corresponding to 1 to 5 µg). For controls, use non-immune antibodies that are as close to the specific antibody as possible (for example, polyclonal serum should be compared with normal serum from the same species).
3. Gently mix for 1 h at 4°C.

Precipitation of the immune complexes

1. Add 50 µl nProtein A Sepharose 4 Fast Flow or Protein G Sepharose 4 Fast Flow suspension (50% slurry).
2. Gently mix for 1 h at 4ºC.
3. Centrifuge at 12 000 × g for 20 s and save the pellet (the beads).
4. Wash the pellet three times with 1 ml lysis buffer and once with wash buffer. Centrifuge at 12 000 × g for 20 s between each wash and carefully remove the supernatants.

Dissociation and analysis

1. Suspend the final pellet in 30 µl sample buffer.
2. Heat to 95ºC and incubate for 3 min.
3. Centrifuge at 12 000 × g for 20 s to remove the beads. Carefully transfer the supernatant to a clean tube.
4. Add 1 µl 0.1% bromphenol blue to the supernatant.
5. Analyze the supernatant by SDS-PAGE, followed by protein staining, trypsin digestion, and analysis by mass spectrometry.

Isolation of native complexes

Natural multiprotein complexes (non-recombinant, without affinity tags) can be purified for structural and functional studies using classical biochemical methods. The natural source of the target complex is used as starting material for the purification and a combination of chromatography and other techniques is used to isolate the complex. (See *The Protein Purification Handbook* and other handbooks in this series for a thorough description of techniques). This approach, in principle, is identical to that which has been used successfully for the isolation of individual proteins since the 1960s.

Some points to consider with this approach are listed in Table 2.4.

Table 2.4. Points to consider for the isolation of native complexes using a classical biochemical approach

Pros	Cons
Use of the relevant biological source limits the risk for artifacts	Large amounts of starting material are required for the isolation of naturally low abundant complexes
No manipulation (e.g., affinity tagging) of the subunits limits the risk for artifacts	A new purification procedure has to be empirically developed for every complex. Purification schemes tend to have multiple chromatographic steps and be more tedious than for tagged proteins
No re-assembly of the complex required – limits the risk for artifacts	

A purification scheme is best designed by combining orthogonal techniques in sequence, for example, combining affinity chromatography (separation according to biospecificity) with ion exchange (separation according to charge properties) and gel filtration (separation according to size). Different techniques should be combined in a fashion that minimizes the requirements for sample treatment between the purification steps (see Appendix 1). Useful chromatography techniques are shown in Table 2.5.

Table 2.5. Chromatography techniques and considerations for multiprotein complex purification

Technique	Separation principle	Pros (+) and cons (-) for multiprotein complex purification
Gel filtration	Size and shape	+ Mild separation conditions; separation can be achieved over a broad pH and ionic strength range + Additives can be used
Affinity chromatography	Biospecific affinity	+ Excellent specificity - Affinity ligands (for the chromatography beads) cannot be found for all proteins - Elution may require conditions (pH, ionic strength) that may disrupt intracomplex interactions
Ion exchange chromatography	Charge	+ High resolution can often be achieved - A salt gradient is often used for elution This may disrupt intracomplex interactions
Hydrophobic interaction chromatography	Hydrophobicity	+ A useful complement to the other techniques - High salt concentrations (e.g., 1–2 M ammonium sulfate) are often used during sample application. This may disrupt intracomplex interactions

It is important to keep the number of steps to a minimum to maximize purification yields. However, for challenging purifications, such as those for multiprotein complexes, a larger number of purification steps may have to be employed to achieve the desired purity. For instance, the human (20S) proteasome (containing 14 subunits, M_r ~700 000) and precursor complexes were isolated from a human cell line using a combination of anion exchange chromatography, sucrose gradient centrifugation, affinity chromatography, hydrophobic interaction chromatography, and gel filtration (10).

Harsh separation conditions involving extremes of pH or ionic strength should be avoided in complex purification in order to minimize the risk for dissociation of subunits during the purification process.

Gel filtration separates according to size and is an ideal technique for isolation of multiprotein complexes because it is mild, and also because many complexes are much larger than the main contaminants. Useful gel filtration media for protein complexes and large molecules are listed in Table 2.6.

Table 2.6. Gel filtration media with high porosity and thus particularly suitable for multiprotein complex purification

Product	Useful separation range*	Comment
Superdex 200	10 000 – 600 000	Available as prepacked columns
Superose™ 6	5 000-5 000 000	Available as prepacked columns
Sephacryl™ S-300 HR	10 000 – 1 500 000	Available as prepacked columns
Sephacryl S-400 HR	20 000 – 8 000 000	
Sephacryl S-500 HR	No data (higher porosity than Sephacryl S-400 HR)	Exclusion limit ~200 nm
Sephacryl S-1000 SF	No data (higher porosity than Sephacryl S-500 HR)	Exclusion limit ~400 nm

* M_r for globular proteins

Isolation of recombinant protein complexes

For structural studies and for comprehensive functional studies of multiprotein complexes it is often necessary to isolate protein amounts in the mg range. As mentioned above, pull-down methods are often not well suited for the isolation of such large protein quantities. An alternative approach is *in vitro* reconstitution. This can be used to assemble complexes that are subsequently isolated. The examples in this section cover *in vitro* assembly of complexes by two different recombinant approaches.

Purification of a 145-subunit complex by affinity chromatography

The human spliceosome, a ~145 subunit complex containing both RNA and protein, was purified using affinity chromatography as the main step in an approach that used a recombinant bait protein as a bridge between the complex and the affinity medium (11).

Spliceosomes were assembled by *in vitro* reconstitution using a nuclear extract from HeLa cells and a ^{32}P-labeled pre-mRNA. To facilitate the purification, an affinity tagged protein that binds to the labeled pre-mRNA (but is not a component of the spliceosome) was added. The spliceosome was then captured via the tagged protein by affinity chromatography. Final purification was performed using gel filtration (Fig 2.7).

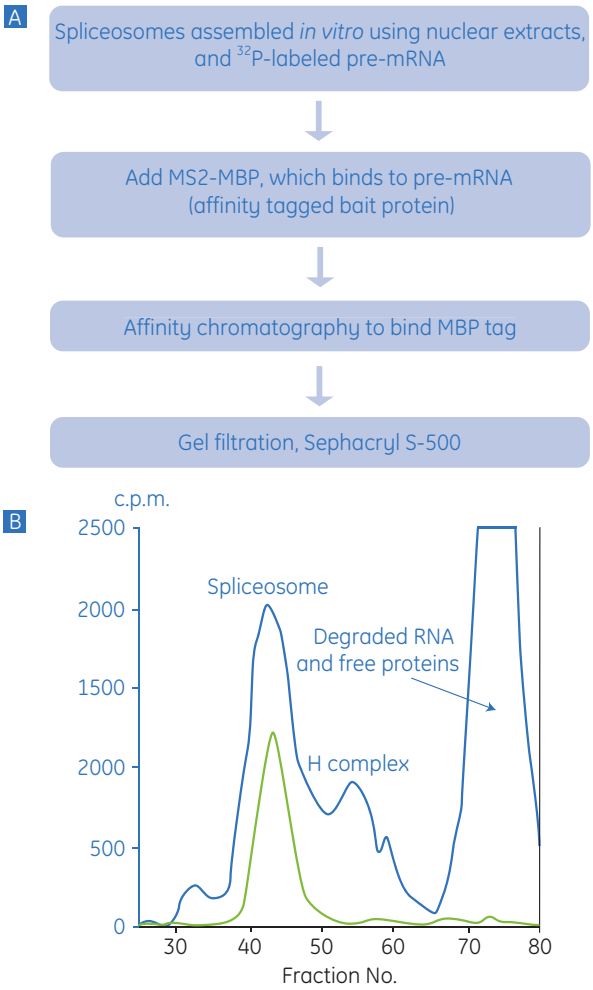


Fig 2.7. Purification of human spliceosomes, a multiprotein complex with more than 140 subunits. From reference 11. A) Purification scheme for human spliceosomes. B) Gel filtration analysis monitored by scintillation counting. Spliceosomes were separated on Sephacryl S-500 before (blue) and after (green) affinity purification c.p.m. = counts per minute. From reference 11. Used with permission. Copyright 2002 Nature Publishing.

Purification of individual overexpressed proteins and *in vitro* reconstitution of a multiprotein complex

Complexes with a limited number of subunits have been reconstituted *in vitro*. One example is shown in Figure 2.8. The nucleosome (the nucleoprotein complex of eukaryotic chromatin) consists of an octamer formed by four histone proteins (H2A, H2B, H3 and H4), and 146 base pairs of DNA.

Each of the four different histones were individually overexpressed in *E. coli* and obtained in inclusion bodies. The inclusion bodies were solubilized in guanidinium HCl and purified under denaturing conditions. The purified, denatured histones were then mixed and refolding and reconstitution were performed by dialysis. The histones spontaneously folded into soluble octamers. Remaining aggregates were removed by gel filtration (Superose 12). The 146-bp DNA was added, and the multiprotein complex (the nucleosome core particle) was spontaneously assembled. The complex was then crystallized for 3-D structure determination (12).

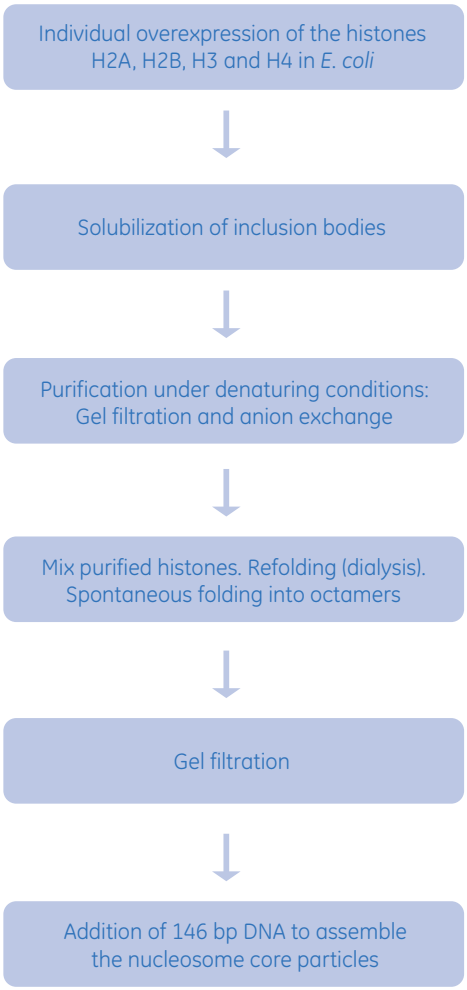


Fig 2.8. Purification of the individual subunits and *in vitro* reconstitution of the nucleosome. From reference 12. Used with permission. Copyright 1997 Nature Publishing.

Another example (13) involves the study of regulatory factor complex (RFX complex), a subcomplex of the major histocompatibility complex class II (MHCII) enhanceosome. The DNA binding RFX complex is required for activation of MHCII gene expression. The RFX complex is composed of three proteins, RFXB, RFXAP and RFX5. The RFX5 protein is identified to have five domains and the N-terminal domain (1-90) is responsible for oligomerization.

In this study, RFXB and constructs of different lengths of RFX5 were expressed as histidine-tagged proteins and purified as show in Figure 2.9. For RFXAP, as it formed a stable complex with the N terminal domain of RFX5, it was copurified with RFX5(1-90). Cells containing these two overexpressed proteins were lysed together, and purification of the complex of two proteins was performed using IMAC. The complex of two proteins was subjected to a second IMAC step under denaturing conditions. RFXAP was recovered in the flowthrough, refolded, concentrated and further purified by gel filtration.

The purified proteins and protein fragments were used in oligomerization studies to develop a DNA binding model for the interaction between the RFX complex and the MHCII promoter.

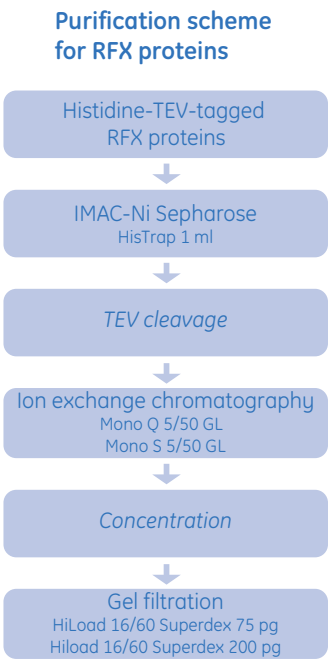


Fig 2.9. Purification of the RFX complex proteins of the MHCII enhanceosome (13). The RFX complex is composed of three proteins RFXB, RFXAP, and RFX5 that associate in a 1:1:2 or 1:1:4 ratio, respectively. Full-length RFXB and RFXAP, and protein fragments of RFX5 were overexpressed and purified using IMAC, ion exchange chromatography and gel filtration.

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Chapter 3
Inclusion bodies

Recombinant protein that accumulates intracellularly in *E. coli* is frequently deposited in the form of inclusion bodies. Inclusion bodies are insoluble aggregates of misfolded protein, lacking biological activity, which can be observed by phase microscopy as dark intracellular particles. Inclusion bodies range in size from 0.2 to 0.6 µm (1).

The (denatured) recombinant protein is the major component of inclusion bodies, and the initial isolation of inclusion bodies with centrifugation is thus an efficient protein purification step. Native recombinant protein is recovered from inclusion bodies through a process known as refolding. While refolding can be straightforward for smaller proteins, and for proteins that do not contain disulfide bridges, it is challenging in many cases. Optimal conditions for refolding are protein-dependent and cannot be predicted. However, much refolding data is available through the literature and has been compiled in databases (e.g., REFOLD database, http://refold.med.monash.edu.au/). Table 3.1 summarizes the advantages and disadvantages of working with inclusion bodies.

Table 3.1. Advantages and disadvantages of inclusion bodies

Advantages	Disadvantages
High expression levels	Refolding is often cumbersome and optimal conditions cannot be predicted
Inclusion bodies can be isolated to high purity	
Inclusion bodies can offer protection from proteolytic enzymes	
Allows expression of toxic proteins	

If your overexpressed protein is accumulated in inclusion bodies there are two major options to consider: either take a step back and optimize for soluble expression, or accept the formation of inclusion bodies and develop strategies to solubilize and refold the protein. In some cases where inclusion bodies form, a small percentage of the overexpressed protein may remain soluble (and presumably correctly folded). The soluble amount may be sufficient for some stuides.

Optimizing for soluble expression

The reasons for inclusion body formation are not well understood. However, it is well known that a reduced growth rate usually leads to more soluble expression and hence reduces the tendency to form inclusion bodies.

A few straightforward modifications to culture conditions, aimed at reducing the growth rate and/or the rate of expression, are thus worthwhile to consider for optimizing soluble expression. A drawback is that the overall yield of recombinant protein is also likely to decrease as a result.

A reduced growth rate can be achieved by lowering the growth temperature to between 20°C and 30°C.

For proteins that are expressed under the control of an inducible promoter, the rate of expression can also be reduced by altering the induction conditions:

- induce at lower cell densities (A_{600} = 0.5)
- induce for a shorter period of time
- induce using a lower concentration of the inducing agent (e.g., 0.1 mM IPTG).

Should these modifications prove insufficient, more comprehensive changes can be considered. These include the use of fusion tags, such as GST and maltose binding protein (MBP), which have been reported to enhance solubility (2). Other options include co-expression with chaperonins or other folding-machinery components, and the use of an alternative host organism. A comprehensive description of procedures that increase soluble expression is outside the scope of this handbook.

Strategies for handling inclusion bodies

A general workflow for handling inclusion bodies in shown in Figure 3.1. The unique aspects are isolation of inclusion bodies, solubilization, and refolding. These three operations are described in more detail below.

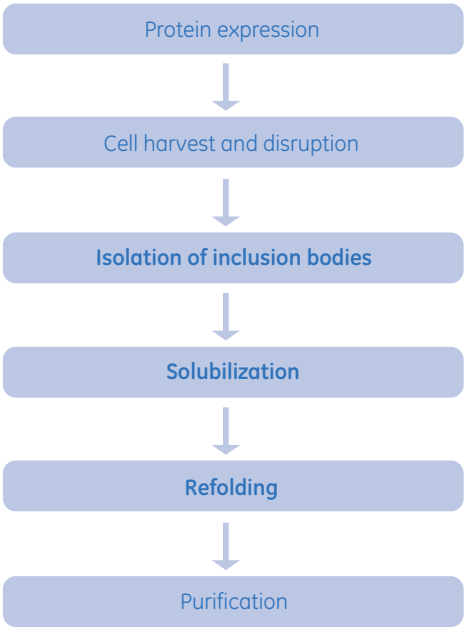


Fig. 3.1. Workflow for handling inclusion bodies. The operations unique to working with inclusion bodies are shown in bold.

It is sometimes easier to refold a more purified protein. IMAC, ion exchange chromatography, and gel filtration can all be performed using, for example, 8 M urea.

Isolation of inclusion bodies

Following cell disruption, inclusion bodies are isolated using centrifugation. This is a highly efficient purification step since the target protein is often the major component of the inclusion bodies.

DNA, membrane vesicles, and cell wall fragments are in the same size range as inclusion bodies and they may co-sediment with inclusion bodies during centrifugation. The method used for cell disruption determines the size of cell debris that is generated. High-pressure homogenization or sonication is often used.

High-pressure homogenization (e.g., French press at 20 000 psi for three rounds) is recommended to reduce the cell debris size so it is more readily separated from inclusion bodies during centrifugation.

After cell disruption, the addition of DNase (e.g., 10 to 20 µg/ml) will reduce the amount of contaminating DNA and the addition of a detergent (e.g., 1% Triton X-100) reduces the amount of membrane material associated with the inclusion bodies..

Isolation of inclusion bodies using centrifugation

Buffer

Wash buffer: PBS containing 1% Triton X-100 and 1 mM EDTA, pH 7.4.

Procedure

1. Dilute the cell lysate in wash buffer, or add Triton X-100 and EDTA to concentrations of 1% and 1 mM, respectively. Lysis can also be done directly in the wash buffer.
2. Centrifuge at 10 000 to 30 000 × g for 10 to 30 min.
3. Save the pellet, suspend and wash twice with the same buffer and centrifuge at 10 000 to 30 000 × g for 10 to 30 min.
4. Collect the pellet, which contains the inclusion bodies.

In some published protocols, low concentrations of denaturing agents (e.g., 1-2 M urea) and/or a higher concentration of NaCl is included in the wash buffer to increase washing efficiency.

Solubilization

Useful starting points for solubilization of inclusion bodies are given in Table 3.2.

Table 3.2. Experimental starting points for solubilization of inclusion bodies

	Buffer	Denaturant	Inclusion body conc. (mg/ml, wet weight)	Temp (°C)	Time	Reducing agent
Start condition	50 mM Tris-HCl, pH 8.0	8 M urea	10–20	Ambient	60 min	–
Variation range	(Not critical)	6–8 M urea 6–8 M guanidinium hydrochloride		4–95	15 min–12 h	1–10 mM DTT (if the protein contains disulfide bonds)

Many alternative solubilization protocols have been published (e.g., REFOLD database). Options include the use of SDS (10%), N-laurylsarcosine, or other detergents and extremes of pH.

Refolding

Following solubilization, proteins must be properly refolded to regain function. This is done by removing denaturing agents to allow folding of the protein and formation of the correct intramolecular associations. Refolding is the most critical step in the inclusion body workflow. The refolding process is often described as a pathway starting with an unfolded protein and ending with a native protein (Fig. 3.2) where competing pathways can lead to the formation of aggregates.

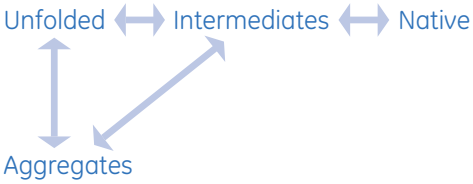


Fig. 3.2. Competing pathways during the refolding process.

The secret to successful refolding thus lies in promoting the main pathway and suppressing the competing pathways that lead to aggregation. There are no known general rules for how to achieve this in practice. Suitable refolding conditions for a given protein thus have to be established empirically. Refolding success is often dependent on the careful optimization of a number of parameters:

- protein concentration
- temperature
- reaction time
- disulfide exchange reagents (see below)
- buffer additives

The buffer additives are believed to act by either suppressing aggregation (urea or guanidinium hydrochloride) or by enhancing folding (arginine, glycerol, salts) Table 3.3 lists some common additives to refolding buffers.

Table 3.3. Common additives in refolding buffers (1)

Additive	Concentration
Urea	2 M
Guanidinium hydrochloride	1 M
Arginine	0.5 M
Glycerol	0.4 M
Sucrose	0.4 M
Lauryl maltoside	0.3 mM
Polyethylene glycol 3550	0.05% (w/v)
Tris	0.5 M
Triton X-100	10 mM
n-hexanol	5 mM
Salts	0.5 mM

Refolding of proteins with disulfide bridges

It is unlikely that immediate formation of the correct intramolecular disulfide bonds occurs randomly if more than two cysteine residues are present. The number of possible combinations increases dramatically with an increasing number of disulfide bonds (15 possible combinations for 3 disulfide bonds, 105 for 4, 945 for 5 etc.).

When disulfide bonds are necessary for correct folding, a reducing agent (e.g., 1 to 10 mM DTT) is added during solubilization. The reducing agent is replaced by disulfide exchange reagents for refolding. The disulfide exchange reagents (e.g., oxidized + reduced glutathione or cysteamine + cystamin) are added to provide a suitable redox potential. The reagents form mixed disulfide intermediates with cysteine residues. This reaction is reversible and the protein disulfide bonds are formed or broken until the most favorable protein disulfide bond formation has occurred. The process is known as oxidative folding.

Yields for the oxidative folding process are strongly dependent on the concentration of redox reagents, and on pH.

Refolding methods

Different methods for refolding are compared in Table 3.4.

Table 3.4 Comparison of methods for protein refolding

Refolding technique	Advantages	Disadvantages
Dialysis	Simple	Slow Uses large volumes of buffer
Dilution	Simple	Slow The protein is diluted to very low concentrations (10–50 µg/ml)
Gel filtration	Straightforward to automate Refolding and aggregate removal in a single operation	Sample volume is limiting
Immobilized metal ion affinity chromatography (IMAC)	Fast and simple Straightforward to automate Refolding and purification in a single operation	Requires histidine-tagged protein
Ion exchange chromatography	Fast and simple Straightforward to automate	Additives with opposite charge to the ion exchanger should be avoided

The focus of this chapter is on chromatographic refolding methods, sometimes also referred to as matrix-assisted refolding or on-column refolding methods.

Refolding using gel filtration

Conceptually, refolding with gel filtration takes advantage of the fact that the gel matrix restricts aggregation through a "cage-like" effect (3). In addition, the constant removal by the gel filtration mechanism of aggregates that may form continuously during the process may be crucial for a high refolding yield. The reason is that the presence of aggregates, even small amounts, is believed to induce an accelerated aggregation. Theoretically then, gel filtration could provide far more efficient refolding (% yields) than simple dilution or dialysis.

Two different experimental strategies are used. One involves equilibrating the column with the refolding buffer. The sample containing the solubilized inclusion bodies in solubilization buffer is then applied. During the passage through the column, solubilization buffer components are retarded and the protein will therefore be transferred to a denaturant-free environment.

The second strategy involves equilibrating the column with solubilization buffer, or introducing into the column a decreasing gradient from solubilization buffer to the refolding buffer before sample application. The refolding buffer is then used to elute the protein. The latter approach involves a more gradual transfer of the protein to a denaturant-free environment, which has proven beneficial in many cases. An example of this latter approach is shown in Fig. 3.3.

Column:	Superdex 75 10/300 GL
Sample:	Lysozyme in 8 M urea, 0.2 M DTT
Refolding buffer:	0.1 M Tris-HCl, pH 8.7, 1 mM EDTA, 150 mM NaCl, 3 mM reduced glutathione (GSH)/0.3 mM oxidized glutathione (GSSG)
Column equilibrated with:	6 ml linear gradient from 8 M to 2 M urea in refolding buffer

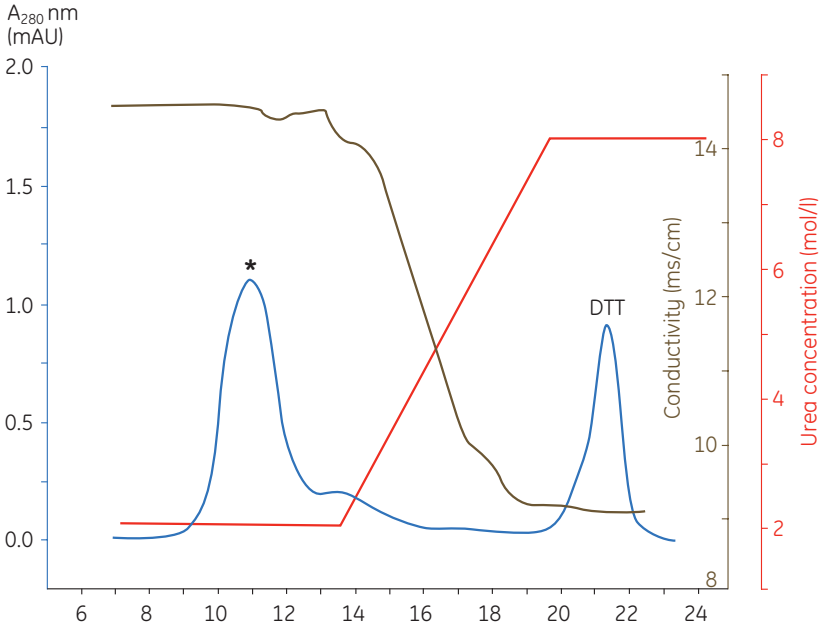


Fig 3.3. Refolding of lysozyme by gel filtration in a urea gradient. The asterisk indicates the elution position for refolded lysozyme. Blue line = absorbance at 280 nm; brown line = conductivity; red line = theoretical urea concentration. From reference 4. Used with permission. Copyright 2001. Elsevier Science BV.

Suitable prepacked gel filtration columns for refolding are listed in Table 3.5.

Table 3.5. Suitable prepacked columns for refolding by gel filtration

Target protein M_r	Column	Recommended sample volume
3 000–70 000	Superdex 75 10/300 GL HiLoad 16/60 Superdex 75 pg HiLoad 26/60 Superdex 75 pg	25-250 µl < 5 ml < 13 ml
10 000–600 000	Superdex 200 10/300 GL HiLoad 16/60 Superdex 200 pg HiLoad 26/60 Superdex 200 pg	25-250 µl < 5 ml < 13 ml
1 000–10 000	HiPrep 16/60 Sephacryl S-100 HR HiPrep 26/60 Sephacryl S-100 HR	< 5 ml < 13 ml
5 000–250 000	HiPrep 16/60 Sephacryl S-200 HR HiPrep 26/60 Sephacryl S-200 HR	< 5 ml < 13 ml
10 000–1 500 000	HiPrep 16/60 Sephacryl S-300 HR HiPrep 26/60 Sephacryl S-300 HR	< 5 ml < 13 ml

Refolding of histidine-tagged proteins using IMAC

Refolding on an adsorptive chromatographic medium (such as an IMAC or an ion exchange medium) involves reversible immobilization of the denatured protein. Immobilization prevents aggregation during the subsequent denaturant removal. Another advantage with the adsorptive techniques is that there is no limitation in sample volume as long as the binding capacity of the medium is not exceeded.

Affinity systems with larger affinity tags and/or protein affinity ligands are often unsuitable for on-column refolding. For such systems, the binding between the tag and the affinity ligand is often dependant on the correct conformation of either the tag, the ligand or both. When this is the case, binding will not occur under denaturing conditions.

Using a histidine-tagged protein enables the use of a simple, but efficient, combined purification and on-column refolding procedure. The workflow shown in Figure 3.4 has been used successfully for several histidine-tagged proteins.

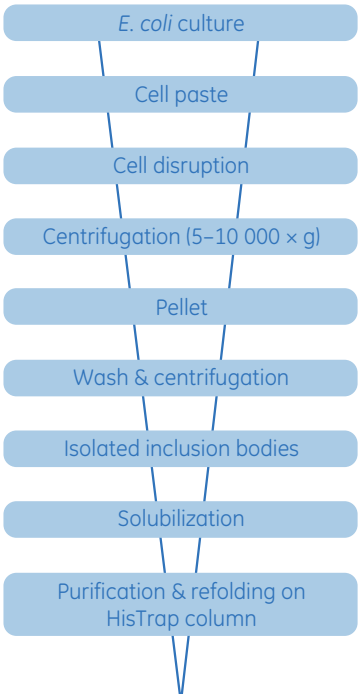


Fig 3.4. General scheme for the extraction, solubilization, and refolding of $(histidine)_6$-tagged proteins produced as inclusion bodies in *E. coli* cells.

High concentrations of chaotropic agents (such as urea or guanidine hydrochloride) are compatible with the binding of the histidine tag to immobilized divalent metal ions. Consequently, histidine-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. Elution is achieved with a gradient of increasing imidazole concentration.

Once refolded, the protein may be purified further by other techniques, such as gel filtration, to remove aggregates if necessary, or if a higher degree of purity is required.

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 can also be used with a HisTrap HP 1 ml or a HisTrap FF crude 1 ml column. The procedure uses ÄKTAprime plus but can also be run on other ÄKTA systems.

Buffers

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

Alternative binding buffers: 5 to 40 mM imidazole can be included in the binding buffer to reduce unwanted 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 s).
3. Centrifuge at 10 000 × g 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 the inclusion body 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 the 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.

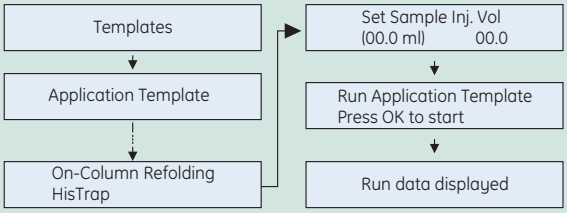
This example uses ÄKTAprime plus. Once the system is prepared, the remaining steps will be performed automatically. A model chromatogram is shown in Figure 3.5.

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.

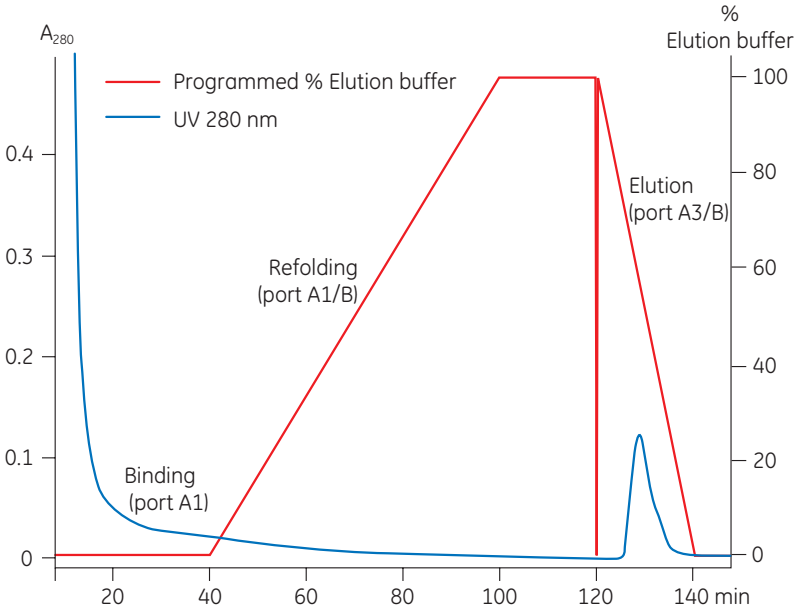


Fig 3.5. Results for on-column refolding of a histidine-tagged protein. For conditions, see the protocol above.

Refolding of histidine-tagged membrane proteins from inclusion bodies using IMAC has also been reported (5).

Suitable prepacked IMAC columns for refolding are listed in Table 3.6.

Table 3.6. Suitable prepacked columns for refolding by IMAC

Column[1]	Column volume	Dynamic binding capacity /ml medium
HisTrap FF	1 ml or 5 ml	Approx 40 mg his-tagged protein
HisTrap HP	1 ml or 5 ml	Approx 40 mg his-tagged protein
HisPrep FF 16/10	20 ml	Approx 40 mg his-tagged protein

[1]The columns are prepacked with Ni Sepharose 6 Fast Flow (FF) or Ni Sepharose High Performance (HP) which are precharged with Ni^{2+} ions.

Screening conditions for refolding using IMAC

To screen for suitable IMAC refolding conditions, a 96-well microplate prepacked with Ni Sepharose can be used (Table 3.7). An example is shown in Fig. 3.6.

Table 3.7. Useful products for screening IMAC refolding conditions

Product	Description
His MultiTrap FF	96-well plate; 800 µl wells filled with 50 µl Ni Sepharose 6 Fast Flow
His MultiTrap HP	96-well plate; 800 µl wells filled with 50 µl Ni Sepharose High Performance

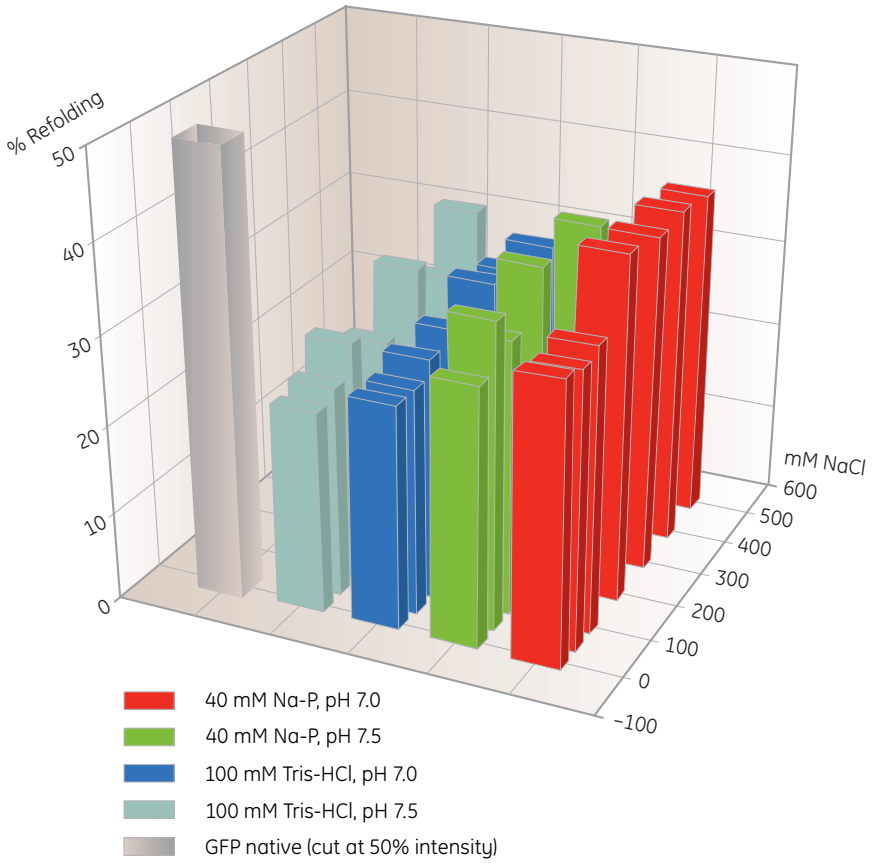


Fig. 3.6. Screening of IMAC refolding conditions for histidine-tagged GFP using His MultiTrap FF. This initial screen covered buffer substances, pH and salt concentrations. Data kindly provided by J. Buchner, M. Haslbeck and T. Dashivets, Munich Technical University, Germany.

Refolding using ion exchange chromatography

As for IMAC, refolding using ion exchange chromatography involves reversible immobilization of the denatured protein. Immobilization prevents aggregation during the subsequent denaturant removal. While adsorption conditions to an IMAC medium for a histidine-tagged protein are essentially generic, the conditions for adsorbing to an ion exchange medium will depend on the charge characteristics of the target protein. Both cation (e.g., SP Sepharose) and anion exchange (e.g., Q Sepharose) media have been used successfully for refolding. Binding to ion exchange media usually requires low ionic strength conditions (i.e., below 0.1). Strong binding to a cation exchange resin is promoted at a low pH while strong binding to an anion exchange resin is promoted at a high pH.

Examples of refolding using anion exchange and cation exchange chromatography are shown in Figures 3.7 and 3.8, respectively. Experiments can be designed in a number of ways. In Figure 3.7, the refolding buffer was applied to the column and the flow stopped for 0 to 40 h in different experiments. In Figure 3.8, refolding was done using a decreasing urea gradient. In both examples, the refolded protein was eluted using a salt gradient.

Once refolded, the protein may be purified further by other techniques, such as gel filtration, if a higher degree of purity is required and to remove aggregates if necessary.

Column: HiTrap Q FF, 1 ml
Sample: 1 ml BSA 0.5-20 mg/ml denatured-reduced (contains 17 disulfide bonds)
Start buffer: 50 mM Tris-HCl, 3 mM EDTA, 8 M urea, pH 8.5
Refolding Buffer: 50 mM Tris-HCl, 1 mM EDTA, 79 mM urea, 1.1 mM oxidized glutathione (GSSG), 2.2 mM reduced glutathione (GSH), pH 8.5
Incubation period: 0 to 40 h
Wash buffer: 50 mM Tris-HCl, 1 mM EDTA, pH 8.5
Elution buffer: 0 to 1M NaCl in 50 mM Tris HCl, pH 8.5

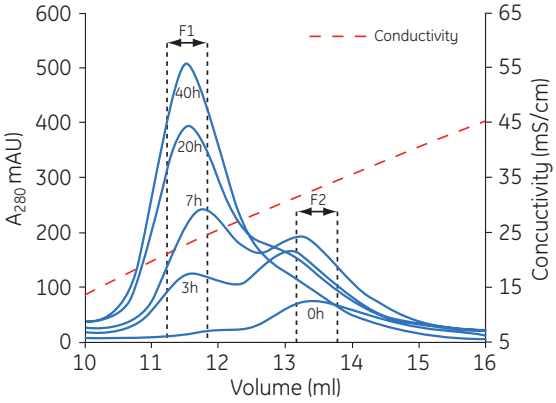


Fig. 3.7. Refolding of albumin using anion exchange chromatography. F1 contains refolded protein. From reference 6. Used with permission. Copyright 2005. Elsevier Science BV.

Column: SP Sepharose HP, 5 ml
Sample: Lysozyme (8 mg)
Start buffer: 50 mM Tris HCl, 6 M urea, 3 mM reduced glutathione (GSH)/0.3 mM oxidized glutathione (GSSG), pH 6.2
Elution buffer: 50 mM Tris HCl, 1 M urea, 300 mM NaCl, 3 mM GSH/0.3 mM GSSG, pH 10
Flow rate: 0.4 ml/min
Instrument: ÄKTApurifier

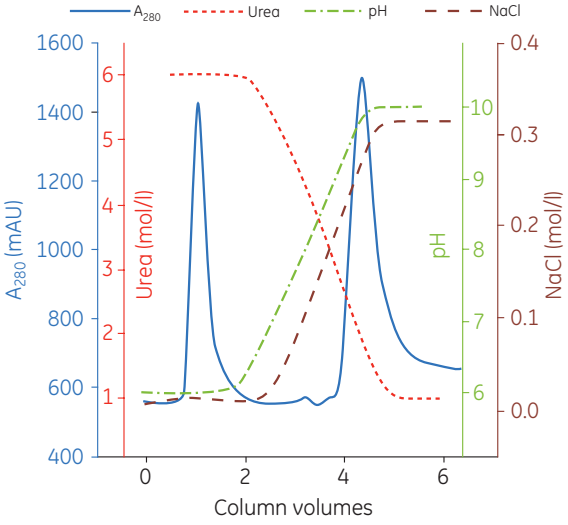


Fig. 3.8. Refolding of lysozyme using cation exchange chromatography. From reference 7. Used with permission. Copyright 2002. Elsevier Science BV.

Suitable prepacked ion exchange chromatography columns for refolding are listed in Table 3.8.

Table 3.8. Suitable prepacked columns for refolding by ion exchange chromatography

Column	Type of ion exchanger	Column volume	Dynamic binding capacity /ml medium
HiTrap Q FF	Anion exchange	1 ml or 5 ml	>100 mg HSA
HiPrep 16/10 Q FF	Anion exchange	20 ml	>100 mg HSA
HiTrap Q HP	Anion exchange	1 ml or 5 ml	70 mg BSA
HiLoad 16/10 and 26/10 Q Sepharose HP	Anion exchange	20 ml or 50 ml	70 mg BSA
HiTrap SP FF	Cation exchange	1 ml or 5 ml	70 mg ribonuclease A
HiPrep 16/10 SP FF	Cation exchange	20 ml	70 mg ribonuclease A
HiTrap SP HP	Cation exchange	1 ml or 5 ml	55 mg ribonuclease A
HiLoad 16/10 and 26/10 SP Sepharose HP	Cation exchange	20 ml or 50 ml	55 mg ribonuclease A

Analysis of refolding

A comprehensive characterization of the outcome of a refolding experiment could include assaying the biological activity and also a physical-chemical characterization of the homogeneity and secondary structure (often using circular dichroism). In many cases, and in particular for proteins with an unknown function, this characterization program is not possible or is impractical to perform.

A homogeneity assay with gel filtration is often sufficient as an indicator of whether refolding was successful. A symmetrical peak corresponding to the expected molecular weight for the protein indicates that refolding has occurred. This assay can be carried out rapidly using the prepacked columns Superdex 75 5/150 GL and Superdex 200 5/150 GL (Table 3.9). These columns can be used for rapid analysis after refolding screening experiments.

Table 3.9. Suitable prepacked column for rapid protein homogeneity assay after refolding experiments

Column	Target protein M_r	Recommended sample volume	Typical run time
Superdex 200 5/150 GL	10 000–600 000	4–50 µl	12 min
Superdex 75 5/150 GL	3 000–70 000	4–50 µl	12 min

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Appendix 1
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 A1.1. Affinity chromatography is also used to remove specific contaminants; for example, Benzamidine Sepharose 4 Fast Flow can remove serine proteases.

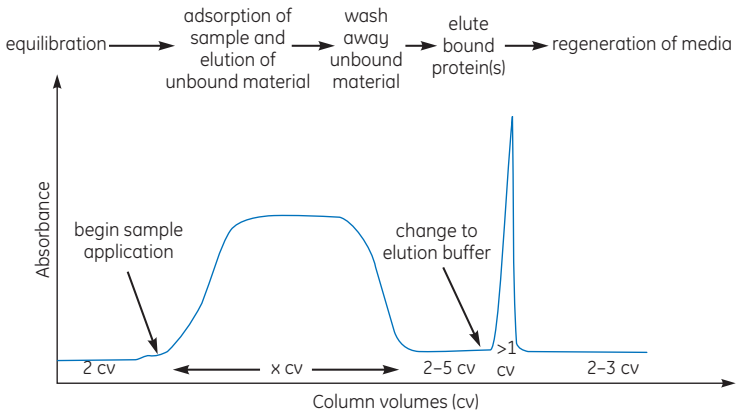


Fig A1.1 Typical affinity purification.

Further information

Protein Purification Handbook (Code No. 18-1132-29)
Affinity Chromatography Handbook: Principles and Methods (Code No. 18-1022-29)

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 A1.2). Target proteins are concentrated during binding and collected in a purified, concentrated form.

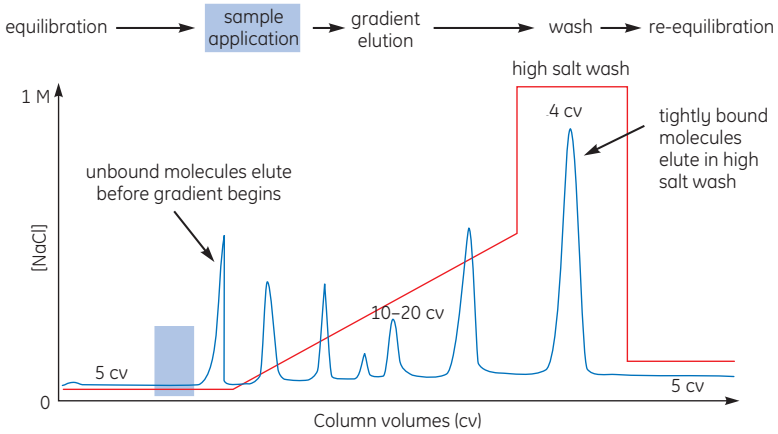


Fig A1.2. Typical IEX gradient elution.

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 A1.3.

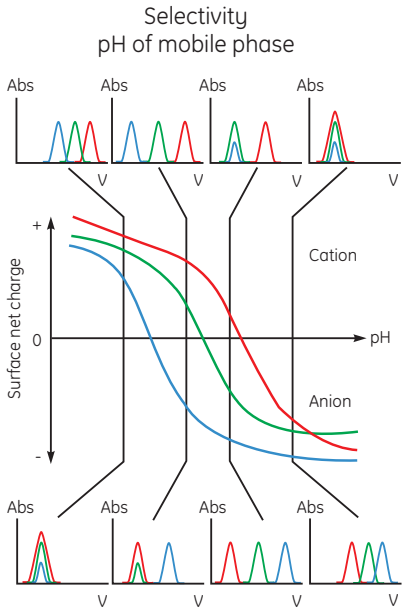


Fig A1.3. Effect of pH on protein elution patterns.

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 A1.4. It is often possible to increase sample loading when using step elution.

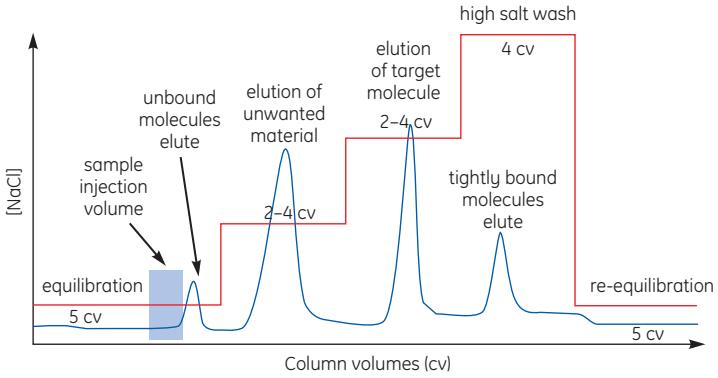


Fig A1.4. Step elution.

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 A1.5). 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.

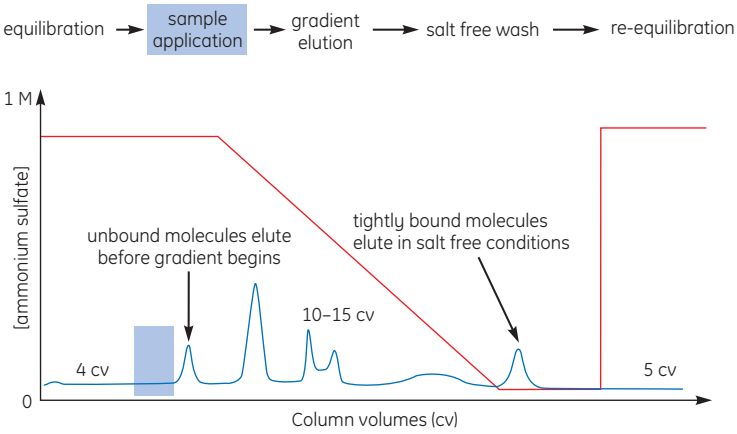


Fig A1.5. Typical HIC gradient elution.

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% elution buffer (0% elution buffer, e.g., 1 M 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 A1.6. It is often possible to increase sample loading when using step elution.

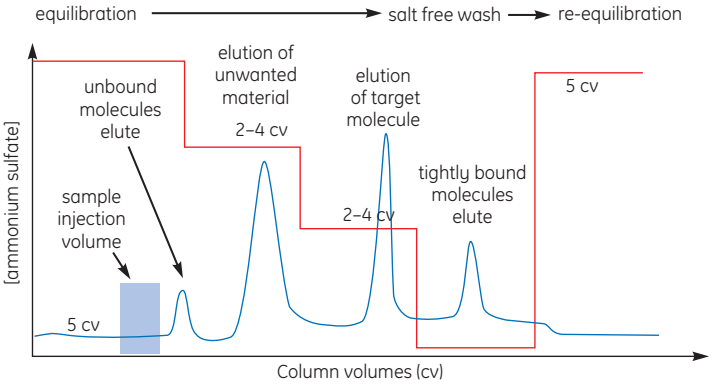


Fig A1.6. Step elution.

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 A1.7). 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.

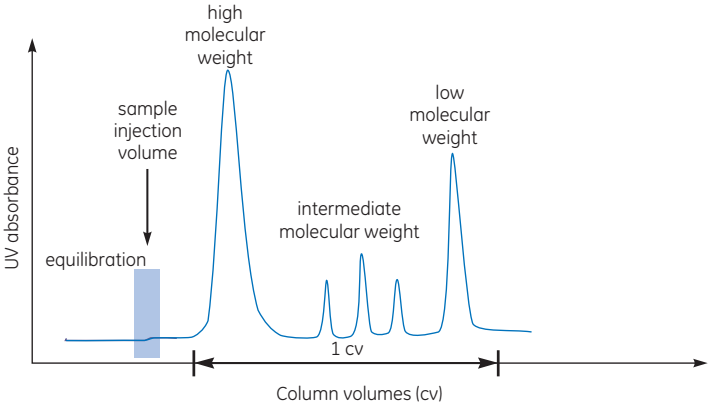


Fig A1.7. Typical GF elution.

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 A1.8.

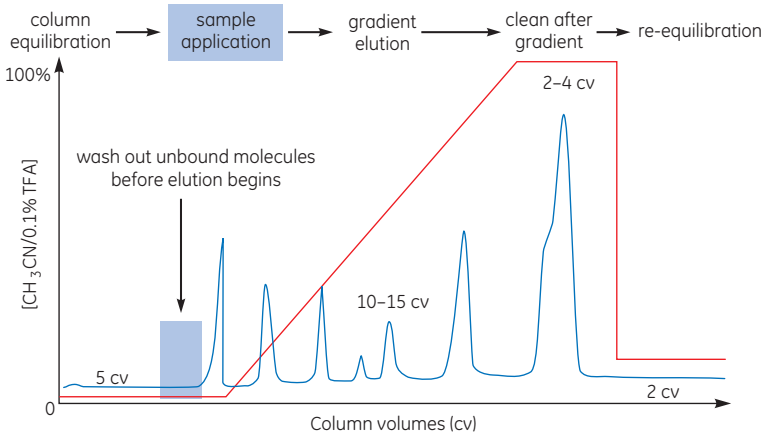


Fig A1.8. Typical RPC gradient elution.

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)

Appendix 2
Manual and automated purification

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, 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 A2.1) is an economical and easy-to-learn system for the purification of 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 A2.1. ÄKTAprime plus.

Fig A2.2. ÄKTAexplorer.

Purification of proteins can also be performed on more advanced chromatography systems. ÄKTAexplorer (Fig A2.2) is a system where multiple samples (up to eight) 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 A2.3) 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 4 samples per module. Proteins can be purified using protocols containing up to four chromatographic steps. The purification protocols may begin with AC or IEX 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 100 mg of tagged protein of > 95% purity. These purified samples are suitable, for example, for use in functional or structural studies.

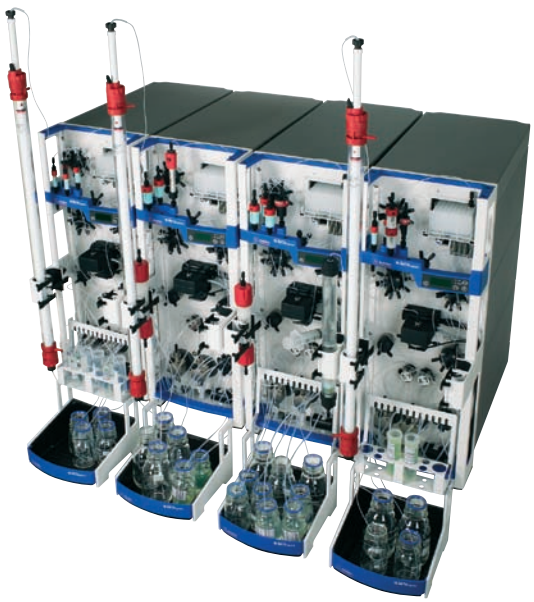


Fig A2.3. Four modules of ÄKTAxpress system.

There are other ÄKTAdesign systems available that can also be used for the purification of tagged proteins. Standard ÄKTAdesign configurations are given in Table A2.1 and Figure A2.4.

More details about methods for purification are given in Appendix 1.

Table A2.1. Ways of working with standard ÄKTAdesign configurations.

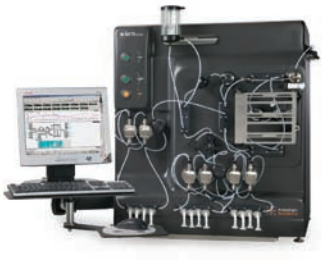
Way of working	Standard ÄKTAdesign configurations						
	ÄKTA prime plus	ÄKTA purifier™	ÄKTA explorer	ÄKTA xpress	ÄKTA pilot™	ÄKTA crossflow™	ÄKTA process™
Manufacturing and production					•		•
UNICORN™ software		•	•	•	•	•	•
One-step simple purification	•			•			
Reproducible performance for routine purification	•	•	•	•	•	•	•
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 A2.4. The standard ÄKTAdesign configurations.

Appendix 3
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 an affinity 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.

Chromatographic media for purifying proteins can be packed in, for example, 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").

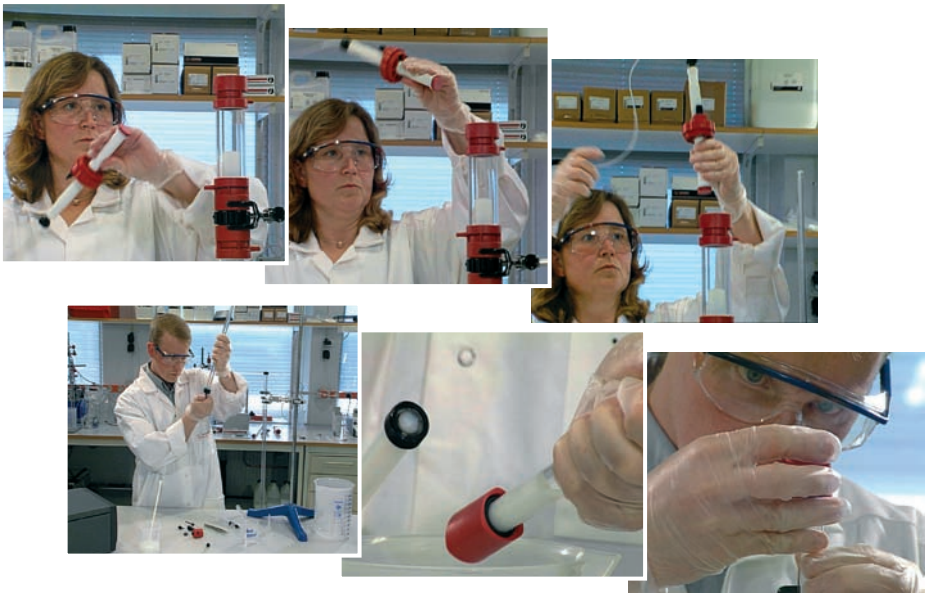


Fig A3.1. *Column Packing — The Movie* provides a step-by-step demonstration of column packing.

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 to 2 cm of buffer in the column.
3. Gently resuspend the medium.

Note that chromatographic 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 adapter into the column at an angle, ensuring that no air is trapped under the net.
12. Slide the adapter slowly down the column (the outlet of the 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 adapter 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 chromatographic media, and a broad range of column dimensions are available (see Table A3.1). 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, or ***www.gelifesciences.com/protein-purification***

Table A3.1. Column bed volumes and heights[1]

	Column size			
	i.d. (mm)	**Length**	**Bed volume (mm)**	**Bed height (cm)**
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 4
Conversion data: proteins, column pressures

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

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 5
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/minπ
d = column inner diameter = 0.5 cm

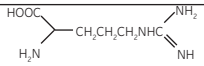
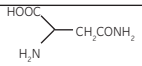
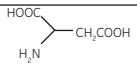
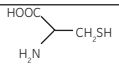
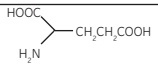
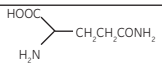
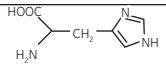
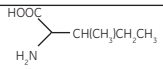
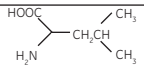
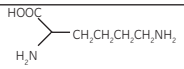
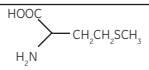
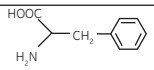
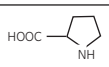
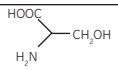
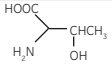
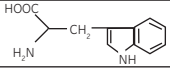
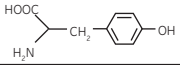
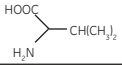
$$\text{Linear flow} = 1 \times 60 \times \frac{4}{\pi \times 0.5 \times 0.5} \text{ cm/h}$$

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

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 6
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	■		

Related literature

	Code No.
Handbooks	
2-D Electrophoresis Using Immobilized pH Gradients: Principles and Methods	80-6429-60
Affinity Chromatography: Principles and Methods	18-1022-29
Antibody Purification	18-1037-46
Gel Filtration: Principles and Methods	18-1022-18
GST Gene Fusion System	18-1157-58
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
Recombinant Protein Purification Handbook: Principles and Methods	18-1142-75
Selection guides/brochures	
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
Glutathione Sepharose—Total solutions for preparation of GST-tagged proteins, selection guide	28-9168-33
Ion Exchange Columns and Media, selection guide	18-1127-31
Ni Sepharose and IMAC Sepharose—Total solutions for preparation of histidine-tagged proteins, selection guide	28-4070-92
Prepacked chromatography columns for ÄKTAdesign systems, selection guide	18-1173-49
Protein purification—applications that meet your needs, application brochure	18-0027-81
The purification platform preferred by 100 000 scientists worldwide, ÄKTAdesign brochure	28-4026-97
Years of experience in every column, columns and media brochure	28-9090-94
Desalting and buffer exchange of proteins in less than 5 minutes, selection guide	18-1128-62
Protein and Peptide Purification, selection guide	18-1128-63
CD	
Column Packing CD—The Movie	18-1165-33
The Protein Purifier—Software-based learning aid for purification strategies	18-1155-49
Application notes	
Addition of imidazole during binding improves purity of histidine-tagged proteins	28-4067-41
Purification and chromatographic characterization of an integral membrane protein	18-1128-92
Purification and renaturation of recombinant proteins produced in *Escherichia coli* as inclusion bodies	18-1112-33
Selective labeling of cell-surface proteins using CyDye DIGE Fluor minimal dyes	11-0033-92

For additional literature, please visit ***www.gelifesciences.com/protein-purification***

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	28-4013-51
IMAC Sepharose High Performance	25 ml	17-0920-06
	100 ml*	17-0920-07
HiTrap IMAC HP	5 x 1 ml	17-0920-03
	5 x 5 ml	17-0920-05
IMAC Sepharose 6 Fast Flow	25 ml	17-0921-07
	100 ml*	17-0921-08
HiTrap IMAC FF	5 x 1 ml	17-0921-02
	5 x 5 ml	17-0921-04
HiPrep IMAC FF 16/10	1 x 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 x 5 ml	17-0409-03
	100 x 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

* Larger quantities are available; please contact GE Healthcare.

† Special pack size delivered on specific customer order

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	25 µg	27-4599-01
All vectors include *E. coli* B21		
Purification		
Glutathione Sepharose High Performance	25 ml	17-5279-01
	100 ml*	17-5279-02
GSTrap HP	5 × 1 ml	17-5281-01
	100 × 1 ml†	17-5281-05
	1 × 5 ml	17-5282-01
	5 × 5 ml	17-5282-02
	100 × 5 ml†	17-5282-05
Glutathione Sepharose 4 Fast Flow	25 ml	17-5132-01
	100 ml	17-5132-02
	500 ml*	17-5132-03
GSTrap FF	2 × 1 ml	17-5130-02
	5 × 1 ml	17-5130-01
	100 × 1 ml†	17-5130-05
	1 × 5 ml	17-5131-01
	5 × 5 ml	17-5131-02
	100 x 5 ml†	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	17-0756-01
	100 ml (function tested)	27-4574-01
	300 ml*	17-0756-04
GSTrap 4B	5 × 1 ml	28-4017-45
	100 × 1 ml†	28-4017-46
	1 × 5 ml	28-4017-47
	5 × 5 ml	28-4017-48
	100 × 5 ml†	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

* Larger quantities are available; please contact GE Healthcare.

† Special pack size delivered on specific customer order

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
Co-immunoprecipitation		
Immunoprecipitation Starter Pack	2 × 2 ml	17-6002-35
Ab SpinTrap	50 × 100 µl	28-4083-47
HiTrap Protein A HP	2 × 1 ml 5 × 1 ml 1 × 5 ml 5 × 5 ml	17-0402-03 17-0402-01 17-0403-01 17-0403-03
HiTrap Protein G HP	2 × 1 ml 5 × 1 ml 1 × 5 ml 5 × 5 ml	17-0404-03 17-0404-01 17-0405-01 17-0405-03
Protein A HP MultiTrap	4 × 96-well filter plates	28-9031-33
Tandem Affinity Purification		
Calmodulin Sepharose 4B	10 ml	17-0529-01
IgG Sepharose 6 Fast Flow	10 ml	17-0969-01
Desalting and buffer exchange		
PD-10 Desalting Columns	30	17-0851-01
HiTrap Desalting	5 × 5 ml 100 x 5 ml†	17-1408-01 11-0003-29
HiPrep 26/10 Desalting	1 × 53 ml 4 x 53 ml	17-5087-01 17-5087-02
illustra MicroSpin G-25 Columns	50	27-5325-01

* Larger quantities are available; please contact GE Healthcare.

† Special pack size delivered on specific customer order

Product	Quantity	Code No.
Gel filtration		
Superdex 75 5/150 GL	1 × 3 ml	28-9205-04
Superdex 75 10/300 GL	1 × 24 ml	17-5174-01
Superdex 200 5/150 GL	1 × 3 ml	28-9065-61
Superdex 200 10/300 GL	1 × 24 ml	17-5175-01
Superose 6 PC 3.2/30	1 × 2.4 ml	17-0673-01
Superose 6 10/300 GL	1 × 24 ml	17-5172-01
HiLoad 16/60 Superdex 75 pg	1 × 120 ml	17-1068-01
HiLoad 26/60 Superdex 75 pg	1 × 320 ml	17-1070-01
HiLoad 16/60 Superdex 200 pg	1 × 120 ml	17-1069-01
HiLoad 26/60 Superdex 200 pg	1 × 320 ml	17-1071-01
HiPrep 16/60 Sephacryl S-100 HR	1 × 120 ml	17-1165-01
HiPrep 26/60 Sephacryl S-100 HR	1 × 320 ml	17-1194-01
HiPrep 16/60 Sephacryl S-200 HR	1 × 120 ml	17-1166-01
HiPrep 26/60 Sephacryl S-200 HR	1 × 320 ml	17-1195-01
HiPrep 16/60 Sephacryl S-300 HR	1 × 120 ml	17-1167-01
HiPrep 26/60 Sephacryl S-300 HR	1 × 320 ml	17-1196-01
Sephacryl S-500 HR	150 ml	17-0613-10
	750 ml	17-0613-01
Sephacryl S-1000 SF	750 ml	17-0476-01
Sephadex LH-20	25 g	17-0090-10
	100 g	17-0090-01
	500 g	17-0090-02
Anion exchange chromatography		
Mono Q 5/50 GL	1 × 1 ml	17-5166-01
Mini Q PC 3.2/3	1 × 0.24 ml	17-0686-01
Mini Q 4.6/50 PE	1 × 0.8 ml	17-5177-01
RESOURCE Q	1 × 1 ml	17-1177-01
	1 × 6 ml	17-1179-01
HiTrap Q HP	5 × 1 ml	17-1153-01
	5 × 5 ml	17-1154-01
HiLoad 16/10 Q Sepharose HP	1 × 20 ml	17-1064-01
HiLoad 26/10 Q Sepharose HP	1 × 53 ml	17-1066-01
HiTrap Q FF	5 × 1 ml	17-5053-01
	5 × 5 ml	17-5156-01
HiPrep 16/10 Q FF	1 × 20 ml	17-5190-01
HiTrap DEAE FF	5 × 1 ml	17-5055-01
	5 × 5 ml	17-5154-01
HiPrep 16/10 DEAE FF	1 × 20 ml	17-5090-01

Product	Quantity	Code No.
Cation exchange chromatography		
Mono S 5/50 GL	1 × 1 ml	17-5168-01
Mini S PC 3.2/3	1 × 0.24 ml	17-0687-01
Mini S 4.6/50 PE	1 × 0.8 ml	17-5178-01
RESOURCE S	1 × 1 ml	17-1178-01
	1 × 6 ml	17-1180-01
HiTrap SP HP	5 × 1 ml	17-1151-01
	5 × 5 ml	17-1152-01
HiLoad 16/10 SP Sepharose HP	1 × 20 ml	17-1137-01
HiLoad 26/10 SP Sepharose HP	1 × 53 ml	17-1138-01
HiTrap SP FF	5 × 1 ml	17-5054-01
	5 × 5 ml	17-5157-01
HiPrep 16/10 SP FF	1 × 20 ml	17-5192-01
Hydrophobic interaction for detergent exchange		
HiTrap Phenyl FF (high sub)	5 × 1 ml	17-1355-01
	5 × 5 ml	17-5193-01
HiTrap Phenyl FF (low sub)	5 × 1 ml	17-1353-01
	5 × 5 ml	17-5194-01
HiTrap Phenyl HP	5 × 1 ml	17-1351-01
	5 × 5 ml	17-5195-01
RESOURCE PHE	1 × 1 ml	17-1186-01
Reversed phase chromatography for detergent removal		
RESOURCE RPC	1 × 1 ml	17-1181-01
RESOURCE RPC	1 × 3 ml	17-1182-01
Purification systems		
ÄKTAprime plus	1	11-0013-13
ÄKTApurifier 10	1	28-4062-64
ÄKTApurifier 100	1	28-4062-66
ÄKTAexplorer 10	1	18-1300-00
ÄKTAexplorer 100	1	18-1112-41
ÄKTAxpress Single	1	28-9089-36
ÄKTAxpress TWIN	1	11-0012-84
ÄKTApilot	1	18-1170-63
Ettan LC	1	18-5050-50
Ettan microLC	1	18-5050-60

Product	Quantity	Code No.
Electrophoresis		
Multiphor II Electrophoresis unit	1	18-1018-06
miniVE Vertical Electrophoresis Systems	1	80-6418-77
4-Gel Caster Complete	1	80-6146-12
EPS 301 Power Supply	1	18-1130-01
EPS 3501 XL Power Supply	1	18-1130-05
MultiTemp III Thermostatic Circulator 115 VAC MultiTemp III Thermostatic Circulator 230 VAC	1 1	18-1102-77 18-1102-78
ExcelGel SDS Gradient 8-18 ExcelGel SDS Buffer Strips (anode and cathode)	6 6 pairs	80-1255-53 17-1342-01
SDS-PAGE Clean-Up Kit	50 samples	80-6484-70
PhastSystem Separation-Control and Development Units 120 VAC	1	18-1018-23
PhastSystem Separation-Control and Development Units 220 VAC	1	18-1018-24
PhastGel Gradient - 8-25	10	17-0542-01
PhastGel Buffer Strips - SDS	10 pairs	17-0516-01
PlusOne Coomassie Tablets, PhastGel Blue R-350	40	17-0518-01
PlusOne Silver Staining Kit, Protein	1	17-1150-01
Deep Purple Total Protein Stain	5 ml 25 ml	RPN6305 RPN6306
CyDye DIGE fluors for cell surface labeling		
CyDye DIGE Fluor, Cy2 minimal dye	5 nmol 10 nmol 25 nmol	25-8010-82 25-8008-60 RPK0272
CyDye DIGE Fluor, Cy3 minimal dye	5 nmol 10 nmol 25 nmol	25-8010-83 25-8008-61 RPK0273
CyDye DIGE Fluor, Cy5 minimal dye	5 nmol 10 nmol 25 nmol	25-8010-85 25-8008-62 RPK0275
Imagers		
Typhoon™ Trio with ImageQuant TL and PC	1	63-0055-88
Ettan DIGE Imager, including installation kit	1	63-0056-42

Product	Quantity	Code No.
Empty columns		
Complete information on the full range of columns, accessories, and spare parts can be found at ***www.gelifesciences.com/protein-purification***		
Tricorn 5/100 column	1	28-4064-10
Tricorn 5/150 column	1	28-4064-11
Tricorn 5/200 column	1	28-4064-12
Tricorn 10/100 column	1	28-4064-15
Tricorn 10/150 column	1	28-4064-16
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	28-4064-17
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
Empty disposable PD-10 Desalting columns	50	17-0435-01

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