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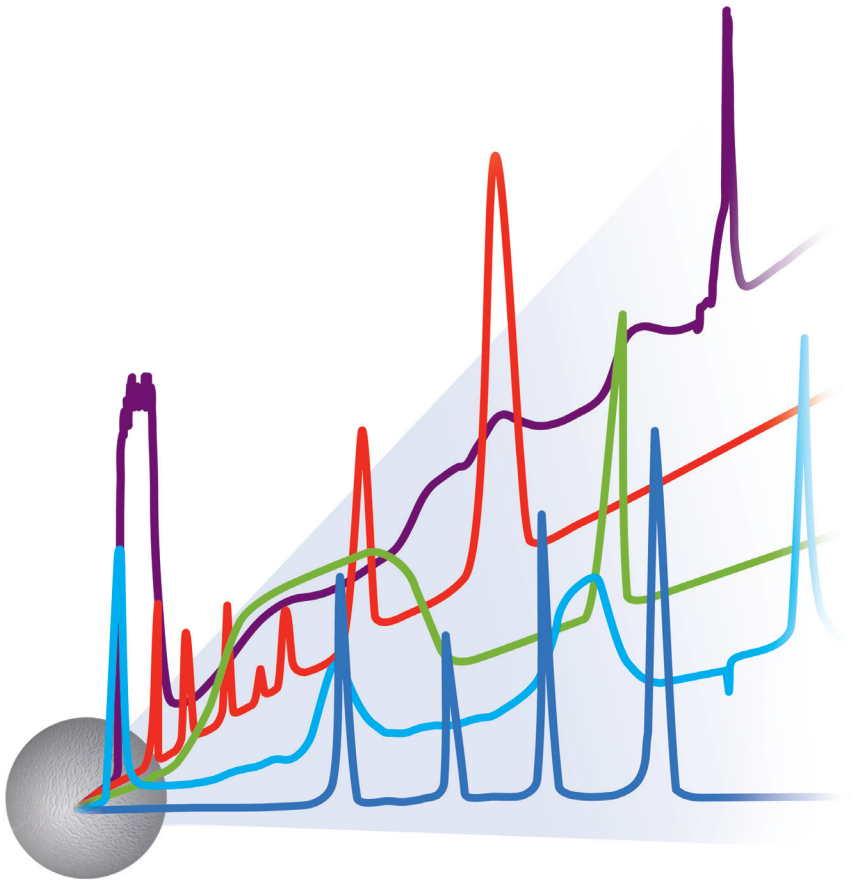

Strategies for Protein Purification

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Strategies for Protein Purification

Handbook

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Introduction

Strategies for Protein Purification is focused on chromatographic purification of proteins at research scale. Beginners can use the handbook to get a quick start when entering the field; experienced protein purifiers may also find valuable information.

The handbook describes how to design a protein purification procedure, how to select and combine chromatography methods and purification formats, and how to monitor and evaluate protein purification. It gives an overview of the available methods and provides advice on how to avoid pitfalls in all operations, from initial sample preparation to final analysis of the purified protein. The handbook is a complement to the series of handbooks from GE Healthcare that describe the different chromatographic purification methods in more detail. The Recombinant Protein Purification Handbook, another handbook from GE Healthcare, gives a large number of detailed protocols for the purification of recombinant proteins. For further reading on the various purification methods, see the list of all handbooks in the "Related literature" section at the end of this book.

Most purifications are today performed with affinity-tagged proteins. This greatly simplifies purification of many proteins. It is, however, relatively common that the target protein is unstable under the conditions used for purification, or it may be difficult to obtain in sufficiently pure form. In such situations, more thorough purification protocol development may be needed to establish suitable conditions for purification of active protein. Not all proteins to be purified are affinity tagged. Sometimes proteins are overexpressed without a tag because the tag would interfere with subsequent use of the protein. In other cases proteins from natural sources are purified. Under these circumstances, several chromatographic purification steps are usually required to obtain sufficient purity. This handbook describes a strategy for development of such protocols.

Common acronyms and abbreviations used in this handbook

A_{280}	UV absorbance at specified wavelength, in this example, 280 nanometers
AAA	amino acid analysis
Ab	antibody
AC	affinity chromatography
AEBSF	aminoethyl benzylsulfonyl fluoride (serine protease inhibitor)
AIEX	anion exchange chromatography
APMSF	4-aminophenyl-methylsulfonyl fluoride (serine protease inhibitor)
BCA	bicinchoninic acid
BSA	bovine serum albumin
CDN	1-chloro-2,4-dinitrobenzene
CF	chromatofocusing
cGMP	current good manufacturing practices
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (detergent)
CIEX	cation exchange chromatography
CIP	cleaning-in-place
CIPP	Capture, Intermediate Purification, and Polishing
CMC	critical micellar concentration
cP	centiPoise
CV	column volume
CYMAL™-7	cyclohexyl-n-heptyl-β-D-maltoside (detergent)
DAOCS	deacetoxycephalosporin C synthase
DDM	n-dodecyl-β-D-maltoside (detergent)
DIGE	differential gel electrophoresis (sometimes referred to as 2-D DIGE)

DM	n-decyl-β-D-maltoside (detergent)
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DS	desalting (here usually group separation by gel filtration; sometimes referred to as buffer exchange)
DSC	differential scanning calorimetry
DTT	dithiothreitol, also 1,4 dithiothreitol (reducing agent, Cleland's reagent)
DTE	dithioerythritol, also 1,4 dithioerythritol (reducing agent)
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid (chelating agent)
EGTA	ethylene glycol tetraacetic acid (chelating agent)
ELISA	enzyme-linked immunosorbent assay
ESI-MS	electrospray ionization-mass spectrometry
FF	Fast Flow
FC12	Fos-choline™-12 (detergent)
FPLC™	fast protein liquid chromatography
GF	gel filtration (sometimes referred to as SEC; size exclusion chromatography)
GFP	green fluorescent protein
GSH	reduced glutathione
GST	glutathione S-transferase
Gua-HCl	guanidine hydrochloride
HAC	hydroxyapatite chromatography
HECAMEG™	6-O-(N-heptylcarbamoyl)methyl-α-D-glucoside (detergent)
HIC	hydrophobic interaction chromatography
HMW	high molecular weight
HP	high performance
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HTPD	high-throughput process development
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 (detergent)
LMW	low molecular weight
LPS	lipopolysaccharides
MAb	monoclonal antibody
MALDI-MS	matrix assisted laser desorption ionization-mass spectrometry
MBP	maltose binding protein
MMC	Multimodal cation exchange(r)
MPa	megaPascal
M_r	relative molecular weight
mRNA	messenger RNA
MS	mass spectrometry
MW	molecular weight
MWCO	molecular weight cutoff
N	number of theoretical plates (column efficiency)
N/m	number of theoretical plates per meter (column efficiency)
NG	n-nonyl-β-D-glucoside (detergent)
NHS	N-hydroxysuccinimide (reagent for covalent coupling of proteins to chromatography media)
OG	n-octyl-β-D-glucoside

PBS	phosphate buffered saline
PEG	polyethylene glycol
PTM	post-translational modification
pI	isoelectric point, the pH at which a protein has zero net surface charge
PMSF	phenylmethylsulfonyl fluoride (serine protease inhibitor)
PVDF	polyvinylidene fluoride
RNase	ribonuclease
RPC	reversed phase chromatography
R_s	resolution, the degree of separation between peaks
SDS	sodium dodecyl sulfate (detergent)
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography (same as gel filtration, GF)
SPR	surface plasmon resonance
STI	soybean trypsin inhibitor
TAP	tandem affinity purification
TB	terrific broth
TCA	trichloroacetic acid
TCEP	tris(2-carboxyethyl)phosphine hydrochloride (reducing agent)
TEV	tobacco etch virus
T_m	transition midpoint in DSC
TMB	3,3',5,5'-tetramethyl benzidine (substrate for HRP)
u	unit (unit for activity of an enzyme)
UV	ultraviolet light
V_e	elution volume
Vis	visible light
V_0	void volume (the elution volume of molecules that are excluded from entering the pores of a chromatography medium)
V_t	total volume

Chromatography terminology

Adapter	Often used for the movable end pieces of columns; contains filter, flow distributor, and possibility to connect tubing.
Adsorption	Binding. The process of interaction between the solute (for example, a protein) and the stationary phase.
Affinity chromatography	A group of methods based on various types of specific affinities between target molecule(s), for example, a protein and a specific ligand coupled to a chromatography medium.
Asymmetry (Asymmetry factor)	Factor describing the shape of a chromatographic peak.
Backpressure	The pressure drop across a column and/or a chromatography system.
Band broadening	The widening of a zone of solute (for example, a protein) when passing through a column or a chromatography system. Gives rise to dilution of the solute and reduces resolution. Also often called peak broadening or zone broadening.
Binding	Adsorption. The process of interaction between a solute (for example, a protein) and the stationary phase.
Binding buffer	Buffer/solution/eluent used for equilibration of the column before sample loading.

Binding capacity	The maximum amount of material that can be bound per ml of chromatography medium. See also Dynamic binding capacity.
Capacity factor	The degree of retention of a solute (for example, a protein) relative to an unretained peak.
Chromatofocusing	Method that separates proteins on the basis of pI.
Chromatogram	A graphical presentation of detector response(s) indicating the concentration of the solutes coming out of the column during the purification (volume or time).
Chromatography	From Greek chroma, color, and graphein, to write.
Chromatography medium/media	The stationary phase, also called resin. The chromatography medium is composed of a porous matrix that is usually functionalized by coupling of ligands to it. The matrix is in the form of particles (beads) or, rarely, a single polymer block (monolith).
CIP (cleaning-in-place)	Common term for cleaning chromatography columns and/or systems with the purpose of removing unwanted/ nonspecifically bound material.
Column	Usually column hardware packed with chromatography medium.
Column equilibration	Passage of buffer/solution through the chromatography column to establish conditions suitable for binding of selected sample components. For example, to establish correct pH and ionic strength, and ensure that proper counter ions or counter ligands are present.
Column hardware	The column tube and adapters. All pieces of the column except the chromatography medium/the packed bed.
Column hardware pressure	The pressure inside the column. Column hardware pressure that is too high can break the column.
Column packing	Controlled filling of the column hardware with chromatography medium to obtain a packed bed.
Column volume	The geometrical volume of the column interior/the chromatography bed.
Counter ion	Ion of opposite charge that interacts with an ion exchange chromatography medium after the column equilibration. The counter ion is displaced by a protein that binds to the ion exchanger. If a high concentration of the counter ion is applied, it will compete with the bound protein and elute it from the chromatography column.
Counter ligand	Substances that interact with ligands of a chromatography medium and can be displaced by a solute (for example, protein) binding to the ligand.
Dead volume	The volume outside the packed chromatography bed. Can be column dead volume or chromatography system dead volume. The dead volume contributes to band broadening.
Degassing	Removal of dissolved air from buffers/solutions.
Desorption	Elution. Release or removal of bound substances from the chromatography medium.

Dynamic binding capacity	The binding capacity determined by applying the target using flow through a column, as opposed to equilibrium binding capacity determined by batch experiment.
Efficiency	Measured as number of theoretical plates. High efficiency means that sharp peaks will be obtained.
Effluent	The mobile phase leaving the column (= eluate).
Eluate	The mobile phase leaving the column (= effluent).
Eluent	The buffer/solution used during chromatography (= mobile phase).
Elution buffer	Buffer/solution used for elution (desorption) of bound solutes (for example, proteins) from a column.
Elution volume	The volume of buffer/solution (eluent) required to elute the solute for example, a protein (= retention volume).
Elution time	The time required for elution of a solute (protein) (= retention time).
Flow rate	Volumetric flow (ml/min) or linear flow rate (cm/h). Measurement of flow through a column and/or chromatography system.
Flowthrough	Material passing the column during sample loading (without being bound).
Frit	Type of deep filter often used at top and bottom of columns.
Gel filtration (GF)	Size-exclusion chromatography. Separates solutes (for example, proteins) according to size.
Gradient elution	Continuous increased or decreased concentration of a substance (in the eluent) that causes elution of bound solutes (for example, proteins).
Hydrophobic interaction chromatography (HIC)	Method based on the hydrophobic interaction between solutes (for example, proteins) and the chromatography medium in the presence of high salt concentration.
Hydroxyapatite chromatography	Mixed-mode ion exchange chromatography method.
Immobilized metal ion affinity chromatography (IMAC)	Method based on the affinity of proteins with His, Cys, or Trp amino residues on their surface and metal ions on the chromatography medium.
Ion exchange chromatography (IEX)	Method based on electrostatic interactions between solutes (for example, proteins) and chromatography medium.
Isocratic elution	Elution of the solutes without changing the composition of the buffer/solution (eluent).
Ligand	The specific molecular group that is coupled to the matrix to give some decided function to the chromatography medium.
Ligand density	Related to ligand concentration. The distribution of ligands on the surfaces (also surfaces inside pores) of the chromatography matrix.
Linear velocity	The flow rate normalized by the column cross section (cm/h).
Mass transfer	Movement of a solute (for example, a protein) in and out of the stationary phase. Important factor for column efficiency.

Matrix	The matrix is the nonfunctional base for the chromatography medium. The matrix has a porous structure that provides a large surface that can be modified with ligands that introduce possibilities for protein binding.
Mobile phase	The fluid (buffer/solution) carrying the solutes during chromatography (= eluent).
Peak broadening	Same as band broadening.
Peak capacity	The number of peaks that can be separated using a chromatography column.
Peak tailing	Broadening at the end of a peak due to additional delay of a fraction of the solute. Results in increased asymmetry factor.
Pore	Cavity in a chromatography matrix.
Pore volume	The total volume of the pores in a chromatography medium.
Pressure over the packed bed	The pressure drop across the packed bed upon passage of solution through the column. Caused by flow resistance in the packed bed.
Recovery	The relative amount of target protein that is retrieved after purification compared with amount loaded on the column.
Resin	The term is sometimes used instead of the more generic term, chromatography medium.
Resolution	Measurement of the ability of a packed column to separate two solutes (peaks).
Retention volume	Same as elution volume.
Retention time	Same as elution time.
Reversed phase chromatography (RPC)	Method based on hydrophobic interactions between solutes (sample components) and ligands coupled to the chromatography medium. Organic modifiers (for example, acetonitrile) in the eluent are used for elution.
Sample	The material loaded on the chromatography column/medium, or to be analyzed.
Sample application	Applying/loading sample on the column.
Sample loading	Loading/applying sample on the column.
Sample volume	Usually the volume of the sample loaded on the chromatography column/medium.
Selectivity	Measure of the relative retention of two solutes in a column. Related to the distance between two peaks.
Solute	The dissolved substance (for example, a protein) in, for example, the mobile phase.
Stationary phase	Often called resin, chromatography beads, chromatography material, chromatography medium or media.
Step gradient elution	Stepwise increase in concentration of the substance that affects elution of bound solutes.
Void volume	The elution volume of solutes that do not enter the pores or interact with the chromatography medium, thus passing between the beads in the packed bed.

Wash	Wash step. Removal of unbound or weakly bound material from a column after the sample loading.
Wash buffer	Buffer/solution used for washing the column after sample loading.
Wash volume	Volume of buffer/solution used for the wash step.
Yield	Amount of target protein (or other solute) obtained after a purification step, or after the entire purification (multiple steps).
Zone broadening	Same as peak broadening.

Symbols used in this handbook

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

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

Chapter 1
Brief history of protein purification

This chapter presents a brief and somewhat narrow description of the history of laboratory-scale protein purification. Opinion may differ regarding details of when and how a contribution or breakthrough was made and by whom.

The term "protein" comes from the Greek, "standing in front." The term was suggested by Jöns Jacob Berzelius in 1838. At the time there were ongoing discussions about whether proteins were macromolecules or colloidal aggregates, a debate that lasted until 1930. In 1901 Hermann Emil Fischer synthesized a dipeptide, demonstrating that amino acids can be linked together, and the following year Franz Hofmeister suggested that proteins are amino acids linked by peptide bonds. Many years later (1926) James B. Sumner crystallized the enzyme urease and showed by chemical analysis that the substance was a protein. This was the first proof that enzymes are proteins. Linus Pauling and co-workers proposed in 1951 that the primary structural motifs of proteins are α helices and β sheets. In 1957 Christian B. Anfinsen and colleagues showed that ribonuclease (RNase) could be refolded into its native structure and regain its activity after complete unfolding and reduction (disulfide bonds broken). Anfinsen later suggested that the native structure of a protein is the thermodynamically most stable structure with the given amino acid sequence. The same year, Max Perutz and John Kendrew determined the structure of myoglobin.

Advances in methodology

Protein purification has been developed in parallel with the discovery and further studies of proteins. See Table 1.1 for some of the milestones in the history of protein purification. The selection of milestones might be controversial and is biased toward the content of this handbook.

Protein purification has been performed for more than 200 years. In 1789 Antoine Fourcroy prepared substances from plants that had similar properties to egg white (mainly albumin). Until the beginning of the 20th century, the only available separation technologies were methods such as filtration, precipitation, and crystallization. In 1840 Felix Hoppe-Seyler prepared the first crystals of hemoglobin. Repeated crystallization was used by Hofmeister in 1889 to purify ovalbumin. During World War II there was an acute need for blood proteins. Cohn fractionation of plasma was developed for the purification of albumin and other plasma proteins. The method is based on multiple precipitation steps where pH, ethanol concentration, temperature, and protein concentration is varied for each step. This precipitation method and others, for example, ammonium sulfate precipitation, are still used today. In 1903 the botanist Mikhail Tswett described his work on separation of plant pigments on a column of calcium carbonate. Later, in 1906, he introduced the term chromatography. In 1924 Theodor Svedberg showed that proteins can be separated by centrifugation. During the next several decades, other important protein separation methods were developed: electrophoresis and affinity chromatography (AC) in the 1930s and ion exchange chromatography (IEX) in the 1940s. Hydroxyapatite, which can be regarded as a mixed-mode ion exchanger, was developed in 1956 by Arne Tiselius and co-workers.

New chromatography media

During the 1950s and 1960s several new hydrophilic chromatography matrices were invented. The matrices are the solid (often porous) material to which functional groups (ligands) are coupled to obtain a chromatography medium. In 1955 starch was used to separate proteins based on differences in size. In 1959, Jerker Porath, Per Flodin, and Bjorn Ingelman developed

cross-linked dextran, which was more suitable for this purpose. During the 1960s other hydrophilic materials for electrophoresis and chromatography where developed: polyacrylamide, methacrylate, porous silica, and agarose. The introduction of cyanogen bromide activation by Rolf Axén and co-workers (1967) allowed ligand coupling to polysaccharides such as in agarose. This discovery is the foundation of modern AC media. The AC method (1968) is attributed to Pedro Cuatrecasas and co-workers, who described the concept in 1968. In the 1960s and 1970s reversed phase chromatography (RPC), hydrophobic interaction chromatography (HIC), and immobilized metal ion affinity chromatography (IMAC) were developed.

During the 1970s and 1980s a large number of chromatography media were developed, and protein purification was developed in parallel. In 1982, Pharmacia (now GE Healthcare) launched a completely integrated chromatography system called FPLC System (Fast Protein Liquid Chromatography). FPLC has since become synonymous with reproducible chromatographic purification of proteins. During this time most purifications were undertaken starting with natural sources, where extremely low concentration of target protein in the source organisms often made purification of even a few micrograms difficult and time consuming. Process-scale protein purification—for large amounts of proteins used in laundry detergents or for enzymatic synthesis of complicated substances, as well as in biopharmaceuticals—is now common. Kilograms or even tonnes of a protein are now manufactured each year in some purification facilities.

Genomics and proteomics

In the 1970s recombinant DNA technology was developed. Molecular cloning allowed modification and heterologous overexpression of a selected protein. During the 1980s and 1990s affinity tagging of proteins became popular, and it allowed efficient AC purification. Because the same affinity tag could be used on many different proteins, generic and time-saving purification protocols could be developed. This was important for the efficiency of structural genomics initiatives that were started during the 1990s and 2000s with the aim of determining the structure of thousands of proteins. The generic protocols allowed the throughput in the protein purification process to be further enhanced by automation of up to four purification steps. Monoclonal antibody technology (1975) involves the establishment of stable cell lines producing a single, selected antibody. This method, in conjunction with highly selective chromatography media based on Protein A and Protein G ligands, has made monoclonal antibodies available for a large number of applications in research and as pharmaceuticals.

The Human Genome Project (1990 to 2003) and other DNA sequencing initiatives have made available complete genome sequences for several organisms. In order to understand the molecular biology of an organism, the genome sequence information must be combined with gene expression information in different cells or tissue, such as messenger RNA (mRNA) levels and protein levels, as well as protein structure and function. An important development was therefore peptide mass fingerprinting, where mass spectrometry (MS) of peptide fragments of proteins combined with searches of databases with known protein sequences allowed identification of proteins present in a sample. Proteomics (1997) aims at the large-scale study of the structure and function of the proteins in a given organism, the proteome. In proteomics, the protein separation is often aimed at simplifying complex samples to allow qualitative and quantitative analysis of multiple proteins in the same experiment, rather than purifying a single target protein.

Where we are today

Protein purification is now performed in scales from micrograms and milligrams in research laboratories to kilograms and tonnes in industrial settings. In some laboratories, proteins are purified in parallel, using automated chromatography systems. The efficiency gained by the generic purification approaches based on affinity tagging of the target protein has revolutionized protein purification, and today many proteins can be purified very easily and

efficiently. It should be noted, however, that these methods do not always provide sufficient purity, and additional physicochemical-based chromatography methods, for example, gel filtration (GF), IEX, and HIC may thus have to be added to the protocol. Proteins that may be fairly easy to obtain in a pure state are not always stable under the first conditions tested. Some proteins may be very challenging to purify in an active and stable form, for example, integral membrane proteins, unstable protein complexes, proteins expressed as insoluble aggregates, and proteins with a specific set of post-translational modifications. The challenges in protein purification that still remain make it worthwhile to gain solid knowledge about protein purification so that the available methods can be selected and applied in an optimal way.

Table 1. Some milestones in the history of protein purification

Precipitation	1789	Fourcroy
Crystalline ovalbumin	1889	Hofmeister
Chromatography	1903	Tswett*
Ultracentrifugation	1924	Svedberg
Moving boundary electrophoresis	1937	Tiselius
Ion exchange chromatography	1940s	The Manhattan Project
Two-phase partitioning	1955	Albertsson (1)
Size-exclusion chromatography (gel filtration)†	1955, 1959	Lindqvist and Storgårds (2), Porath and Flodin (3), Ingelman
Hydroxyapatite chromatography	1956	Tiselius, *et al.* (4)
Sephadex™ (gel filtration medium)	1959	Pharmacia (now GE Healthcare)
Polyacrylamide gel electrophoresis	1959	Raymond and Weintraub (5)
Isoelectric focusing‡	1959	Kolin (6)
High-performance liquid chromatography (HPLC)§	1941; 1966	Martin and Synge (7)
Sepharose™	1967	Pharmacia (now GE Healthcare)
SDS-PAGE	1967	Shapiro, *et al.* (8)
Affinity-ligand coupling chemistry for affinity chromatography¶	1967	Axén, *et al.* (9)
Affinity chromatography	1968	Cuatrecasas, *et al.* (10)
Reversed-phase chromatography	1970s	Kirkland (11); Molnar and Horvath (12)
Protein A adsorbent for IgG purification	1972	Hjelm, *et al.* (13); Kronvall (14)
Hydrophobic interaction chromatography	1973	Porath (15); Hjertén (16)
Two-dimensional chromatography	1975	O'Farrel l(17)
Immobilized metal ion affinity chromatography	1975	Porath, *et al.* (18)
Chromatofocusing	1977**	Sluyterman and Wijdenes (19)
Capillary electrophoresis	1981††	Jorgenson and Lukacs (20)
Fast Protein Liquid Chromatography	1982	Pharmacia (now GE Healthcare)
Histidine affinity tagging	1988‡‡	Smith *et al.* (21), Hochuli *et al.* (22)
ÄKTA™ design	1996	Pharmacia Biotech (now GE Healthcare)

* *Separation of plant pigments on a chalk (calcium carbonate) column*

† *In 1955, size-exclusion separation was performed on starch. See reference 2. In 1959, cross-linked dextran was introduced by Porath and Flodin. This material became useful in practice for protein purification. See reference 3*

‡ *Performed using a sucrose/buffer gradient*

§ *Martin and Synge suggested use of small particles and high pressure for high-performance separation. See reference 7 Horvath coined the term high-performance liquid chromatography (HPLC) in 1966*

¶ *CNBr-method for coupling affinity ligands to chromatography materials. See reference 9*

** *See, for example, reference 19*

†† *Others, for example, Hjertén, performed electrophoresis in narrow tubing even earlier. See also reference 20*

‡‡ *Smith et al. presented the concept of a peptide affinity tag for IMAC in 1986. In 1988 the histidine tag was developed. See references 21 and 22*

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Chapter 2
Protein purification methods

The most common methods for preparative purification of proteins all involve chromatography. The methods separate according to differences between the properties of the protein to be purified (the target protein) and the properties of other substances in the sample. Examples of protein properties used in different chromatography methods are given in Table 2.1.

Table 2.1. Protein properties used for chromatographic purification

Protein property	Method
Specific ligand recognition (biospecific or nonbiospecific)	Affinity chromatography (AC)
Metal ion binding	Immobilized metal ion affinity chromatography (IMAC)
Charge	Ion exchange chromatography (IEX)
Size	Gel filtration (GF)
Hydrophobicity	Hydrophobic interaction chromatography (HIC) Reversed phase chromatography (RPC)
Isoelectric point	Chromatofocusing

AC is the common first purification step when the target protein is affinity tagged. If further purification is needed, additional methods are used to remove remaining impurities. Purification of untagged proteins often requires the use of several purification steps applying different methods in suitable order. This chapter gives a brief overview of the key purification methods presented in Table 2.1. Further details regarding method descriptions and practical advice, including many examples, can be found in other handbooks from GE Healthcare.

Affinity chromatography (AC)

AC separates proteins on the basis of a reversible interaction between the target protein (or group of proteins) and a specific ligand attached to a chromatography matrix (Fig 2.1). The interaction can be biospecific, for example, antibodies binding Protein A or a receptor binding a hormone, or nonbiospecific, for example, a protein binding a dye substance or histidine-containing proteins binding metal ions (as in IMAC, which, due to its importance will be described separately in the following section). AC offers high selectivity, hence high resolution, and intermediate to high capacity. Elution can often be performed under mild conditions.

In AC, the target protein 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 out of the column, and bound target protein is recovered by changing conditions to those favoring elution. Elution is performed specifically, using a competitive ligand, or nonspecifically, by changing, for example, pH, ionic strength, or polarity. The target protein is eluted in a purified and concentrated form. The key stages in an AC separation are shown in Figure 2.2.

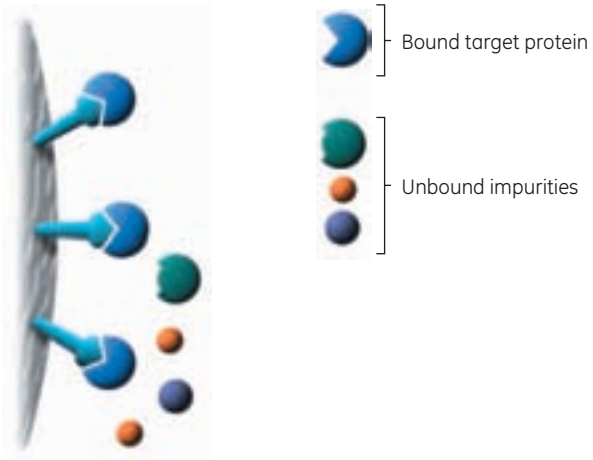

Fig 2.1. Schematic depicting AC.

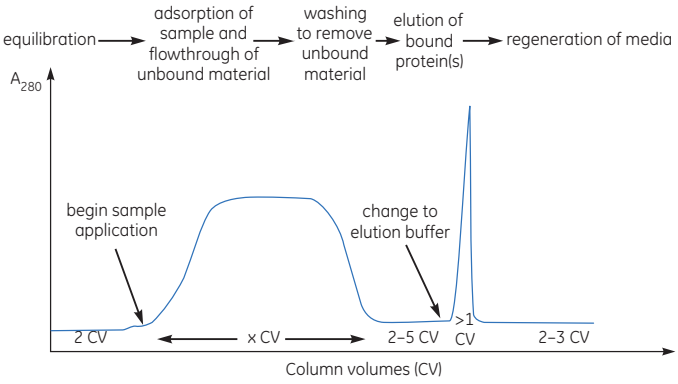

Fig 2.2. Typical affinity purification.

AC can sometimes be used for single-step purification because of high selectivity or in instances when some impurities can be accepted. It is, however, more common for AC to be used as the first purification step (capture step; see Chapter 4), followed by a second purification step (polishing step) to remove remaining impurities or aggregates. In other cases when very high purity is required, one or more additional purification steps may need to be applied.

Today, most laboratory-scale purifications are performed with affinity-tagged proteins. A large number of different affinity tags as well as chromatography media and prepacked columns are available to allow selection of optimal conditions for each target protein and purification task. The most common is purification of histidine-tagged proteins using IMAC or glutathione S-transferase (GST)-tagged proteins using media with immobilized glutathione. AC is also used to remove specific contaminants, for example, Benzamidine Sepharose 4 Fast Flow for removal of serine proteases. Pre-activated chromatography media can be used for covalent coupling of various ligands, for example, antibodies can be raised to the target protein and coupled on NHS-activated Sepharose for immunoaffinity purification of the desired protein. Table 2.2 shows examples of prepacked columns available from GE Healthcare; see ordering information for more details. Other formats for purification are also available; see Chapter 7.

Table 2.2. HiTrap™, HiPrep™, and HiScreen™ affinity columns for laboratory-scale purification

Application	Columns
Purification of immunoglobulins	
IgG, fragments, and subclasses	HiTrap MabSelect™ SuRe™, 1 ml and 5 ml
	HiTrap MabSelect , 1 ml and 5 ml
	HiTrap MabSelect Xtra™, 1 ml and 5 ml
	HiTrap rProtein A FF, 1 ml and 5 ml
	HiTrap Protein A HP, 1 ml and 5 ml
	HiScreen MabSelect SuRe, 4.7 ml
	HiScreen MabSelect, 4.7 ml
	HiScreen MabSelect Xtra, 4.7 ml
IgG, fragments, and subclasses including human IgG_3	HiTrap Protein G HP, 1 ml and 5 ml
Strong affinity for monoclonal mouse IgG_1 and rat IgG	MAbTrap™ Kit
Avian IgY from egg yolk	HiTrap IgY Purification HP, 5 ml
Mouse and human IgM	HiTrap IgM Purification HP, 1 ml
Purification of recombinant proteins	
Histidine-tagged proteins	HisTrap™ FF, 1 ml and 5 ml
	HisTrap HP, 1 ml and 5 ml
	HisTrap FF crude, 1 ml and 5 ml
	HisTrap FF crude Kit
	HisPrep™ FF 16/10, 20 ml
GST-tagged proteins	GSTrap™ 4B, 1 ml and 5 ml
	GSTrap FF, 1 ml and 5 ml
	GSTrap HP, 1 ml and 5 ml
	GSTPrep™ FF 16/10, 20 ml
MBP-tagged proteins	MBPTrap™ HP, 1 ml and 5 ml
Strep-tag™ II proteins	StrepTrap™ HP, 1 ml and 5 ml
Group-specific purification	
Albumin and nucleotide-requiring enzymes	HiTrap Blue HP, 1 ml and 5 ml
Proteins and peptides with exposed His, Cys, or Trp	IMAC columns that are not precharged with metal ion:
	HiTrap Chelating HP, 1 ml and 5 ml
	HiTrap IMAC HP, 1 ml and 5 ml
	HiTrap IMAC FF, 1 ml and 5 ml
Biotinylated substances	HiTrap Streptavidin HP, 1 ml
DNA binding proteins and coagulation factors	HiTrap Heparin HP, 1 ml and 5 ml
	HiPrep Heparin FF 16/10, 20 ml
Trypsin-like serine proteases including Factor Xa, thrombin, and trypsin	HiTrap Benzamidine FF (high sub), 1 ml and 5 ml
Phosphorylated proteins and peptides	IMAC columns charged with Fe^{3+}
	Titanium oxide based separation can be performed using TiO_2 Mag Sepharose
Matrix for preparation of affinity media. Coupling via primary amines.	HiTrap NHS-activated HP, 1 ml and 5 ml

For further information please refer to *Affinity Chromatography Handbook: Principles and Methods*, *Antibody Purification Handbook*, and *Recombinant Protein Purification Handbook: Principles and Methods* from GE Healthcare (see also the "Related literature" section at the end of this handbook).

Immobilized metal ion affinity chromatography (IMAC)

IMAC is based on the interaction of proteins with histidine residues (or Trp and Cys) on their surface with divalent metal ions (e.g., Ni^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}) immobilized via a chelating ligand. Histidine-tagged proteins have an extra high affinity in IMAC because of the multiple (6 to 10) histidine residues and are usually the strongest binder among all the proteins in a crude sample extract (e.g., a bacterial lysate), while other cellular proteins will not bind or will bind weakly.

IMAC purification begins with equilibration of the column with a binding buffer containing a low concentration of imidazole. When using columns packed with Ni Sepharose, 20 to 40 mM imidazole should be used. Columns from other suppliers usually use much lower concentrations. The imidazole binds to the immobilized metal ion and becomes the counter ligand. The sample should be adjusted to the same imidazole concentration as the binding buffer before being loaded on the column. Proteins with histidines bind the column while displacing the imidazole counter ligands. The column is washed using the binding buffer. Elution of bound proteins is performed using a gradient of imidazole up to 100 to 500 mM or by step elution. Gradient elution (Fig 2.3) often gives two peaks, an early peak corresponding to naturally binding proteins in the lysate and a later peak corresponding to the histidine-tagged protein, which has higher affinity for the medium. Step elution (Fig 2.4) gives a single peak, with the histidine-tagged protein often of slightly lower purity, but it is a powerful capture step that can be combined with a second purification step, for example, GF, to obtain higher purity.

We recommend binding at neutral to slightly alkaline pH (pH 7 to 8) in the presence of 500 mM NaCl. Including salt in the buffers and samples eliminates ion-exchange effects.

Avoid chelating agents such as EDTA or citrate in buffers, because they may strip off the metal ions from the chromatography medium.

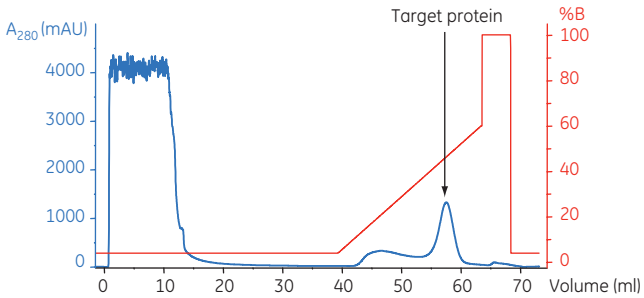

Fig 2.3. Typical IMAC purification with gradient elution.

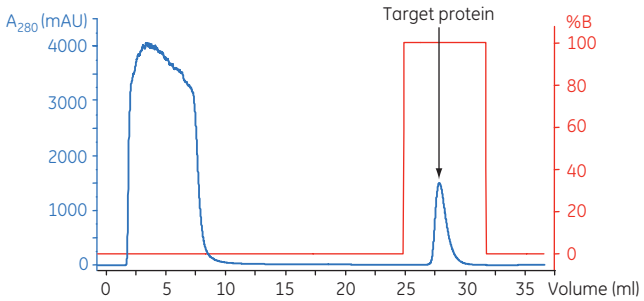

Fig 2.4. Typical IMAC purification with step elution.

Histidine tags are small and generally less disruptive than other tags to the properties of the proteins on which they are attached. Because of this, tag removal after purification of a histidine-tagged protein may not always be of high priority. Histidine-tagged protein expressed in *E. coli* can accumulate in two main forms, as biologically functional soluble proteins or as large insoluble aggregates of more or less unfolded, inactive target protein. Preparation of active protein from such inclusion bodies will be addressed in Chapters 6 and 9.

For further information please refer to *Affinity Chromatography Handbook: Principles and Methods* and *Recombinant Protein Purification Handbook: Principles and Methods* from GE Healthcare (see also the "Related literature" section at the end of this handbook).

Gel filtration (GF)

GF is simple to use and allows separation of substances with differences in molecular size, under mild conditions. GF is also called size-exclusion chromatography, which more closely describes the separation mechanism. GF can be used for protein purification (Fig. 2.6) or for group separation in which the sample is separated in two major groups (Fig. 2.7). Group separation is mainly used for desalting and buffer exchange of samples; see Chapter 3.

GF is a non-binding method (Fig 2.5), which means that no concentration of the sample components takes place. In fact, the sample zone is broadened during the passage through the column, resulting in dilution of the sample. The loaded sample volume must be kept small. In preparative GF, maximum resolution can be obtained with sample volumes of 0.5% to 2% of the total column volume; however, up to 5% may give acceptable separation. Even larger samples volumes can be appropriate if the resolution between target protein and the impurities to be removed is high. To increase capacity, the sample can be concentrated before GF. Avoid concentrations above 70 mg/ml, because viscosity effects may cause severe band broadening (so-called viscous fingering) that reduces the resolution.

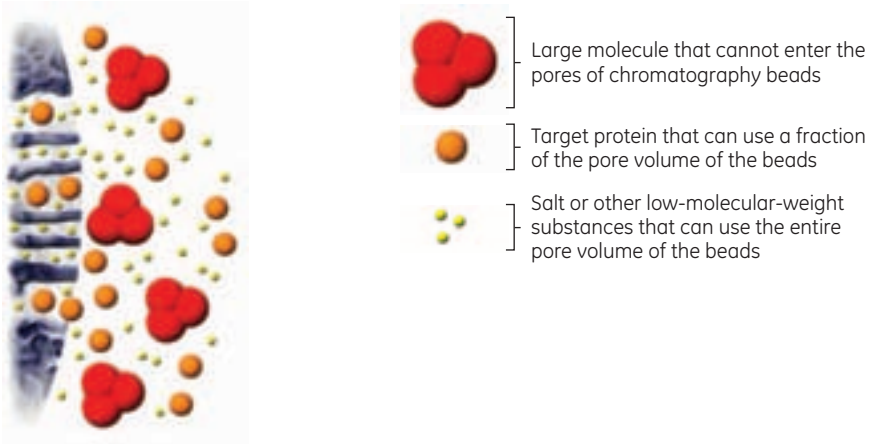

Fig 2.5. Schematic depicting GF.

Sample components are eluted isocratically (single buffer, no gradient). Separation can be performed within a broad pH, ionic strength, and temperature range, and the medium accepts a variety of additives: co-factor, protein stabilizers, detergents, urea, and guanidine hydrochloride. The buffer composition does not usually affect resolution, although including a low concentration of salt, for example, 25 to 150 mM NaCl, is recommended to eliminate any weak electrostatic interactions between proteins and the GF matrix. Buffer conditions are selected to suit the sample type and to maintain target protein activity, because the proteins are transferred to the

buffer used for equilibration of the column. Equilibration buffer can thus be selected according to conditions required for further purification, analysis, storage, or use.

The selection of chromatography medium is the key parameter for optimization of resolution in GF.

- The loading sample volume is the most important factor for high resolution.
- Select conditions that maintain target protein stability and that are suitable for subsequent work.
- Capacity can be increased by concentrating the sample.
- Resolution can be increased by lowering the flow rate.
- Avoid sample volumes larger than 4% of the total column volume when separating proteins by GF. Group separations allow sample volumes of up to 30% of the column volume.
- Avoid protein concentrations above approximately 70 mg/ml, because high viscosity may severely affect resolution.

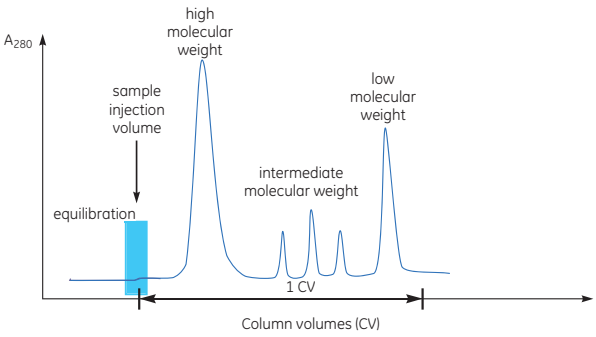

Fig 2.6. Principles of GF purification.

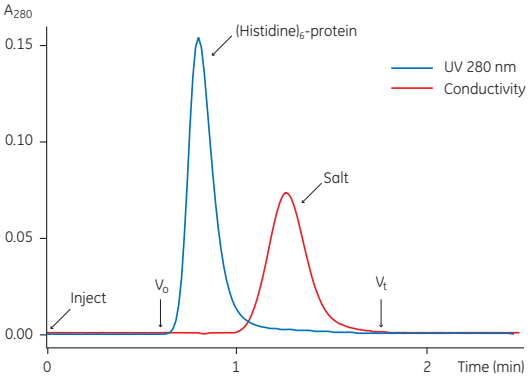

Fig 2.7. Typical example of group separation used for desalting of protein.

GF is a powerful method for purification of proteins that have passed one or several initial purification steps. After those steps, the material has been concentrated and bulk impurities have been removed. GF can now be used to remove remaining impurities; it will also remove oligomers or aggregates of the target protein. The purified target protein obtained after GF will thus also be homogeneous in size. GF is rarely used as a first purification step, but can be useful for small samples with moderate complexity.

For further information please refer to *Gel Filtration Handbook: Principles and Methods* from GE Healthcare (see also the "Related literature" section at the end of this handbook).

Ion exchange chromatography (IEX)

IEX separates proteins with differences in surface charge to give high-resolution separation with high sample loading capacity (Fig 2.8). The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatography medium. Target proteins are concentrated during binding and collected in a purified, concentrated form. IEX media can be used at high flow rates because binding kinetics for IEX are fast, and rigid chromatography particles can be used.

The net surface charge of proteins varies according to the surrounding pH (Fig. 2.9). Typically, when above its isoelectric point (pI) a protein will bind to a positively charged anion exchanger; when below its pI a protein will bind to a negatively charged, cation exchanger. It should be noted, however, that even if the protein has a net negative charge it may bind to a negatively charged cation exchanger, because there might be positively charged clusters on the protein surface that could be sufficient for binding. This may of course also be true for the opposite case, with a net positively charged protein binding to a positively charged anion exchanger. Unexpected binding or absence of binding can also be caused by pH changes in the micro-environment inside the beads compared with the bulk buffer (Donnan effect). This can increase the effective pH in anion exchangers and decrease it in cation exchangers.

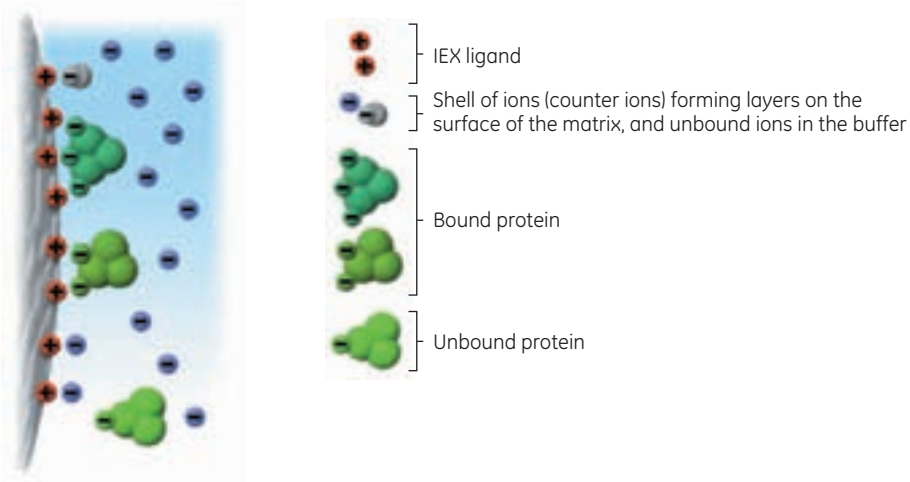

Fig 2.8. Schematic depicting IEX.

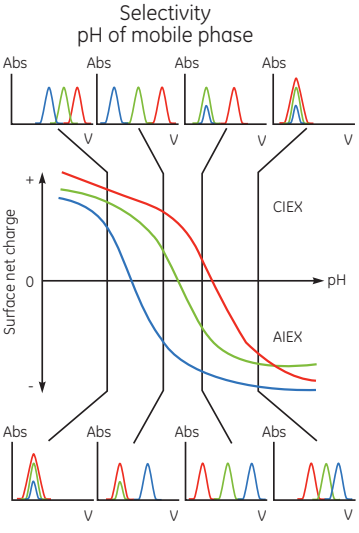

Fig 2.9. Schematic view of the effects of pH on protein elution patterns. The middle diagram shows the surface net charge of three proteins (blue, green, and red). The four chromatograms on top describe the behavior of these proteins in cation exchange chromatography (CIEX) with salt gradient elution run at varying pH values as indicated by the vertical lines. The bottom chromatograms show the behavior in anion exchange chromatography (AIEX).

Proteins bind as they are loaded onto a column at low ionic strength (Fig 2.10). The conditions are then altered so that bound substances are desorbed differentially. Elution is usually performed by increasing salt concentration or changing pH in a gradient, or stepwise (Fig. 2.11). The most common salt is NaCl, but other salts can also be used to modulate separation, for example, K^+, Ca^{2+}, Mg^{2+}, CH_3COO^-, SO_4^{2-}, I^-, Br^-. The buffer used may also impact separation. Ions that bind to the protein may change its behavior in IEX.

A strong ion exchange medium has the same charge density on its surface over a broad pH range, whereas the charge density of a weak ion exchanger changes with pH. The selectivity and the capacity of a weak ion exchanger are different at different pH values.

- First try strong ion exchangers, and if other selectivity is desired try a weak ion exchanger.
- Start by testing binding of small amounts of sample at different pH.
- Use a buffer concentration that is sufficient to maintain buffering capacity and constant pH, typically 20 to 50 mM.
- For efficient binding, the column should be thoroughly equilibrated with the binding buffer, and the sample should have the same pH and ionic strength.
- Avoid EDTA in AIEX because it may bind to the medium and give disturbances in the pH.
- Avoid using buffer substances with a charge that is opposite the charge of the ion exchange medium to be used. See Appendix 1 for suitable buffers for AIEX and CIEX.

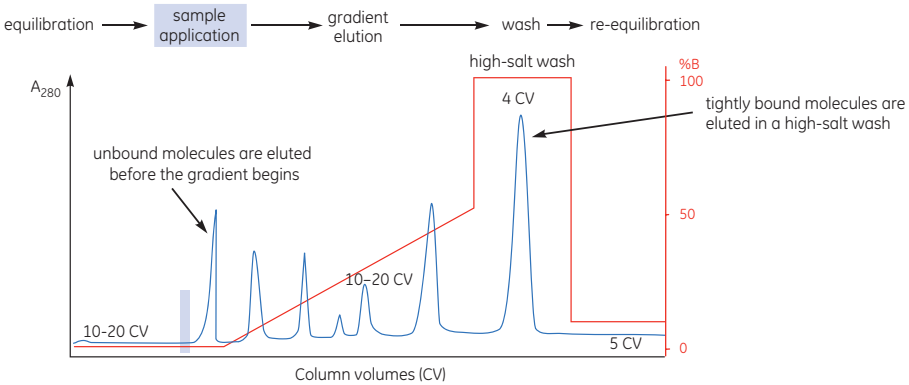

Fig 2.10. Typical IEX purification with gradient elution.

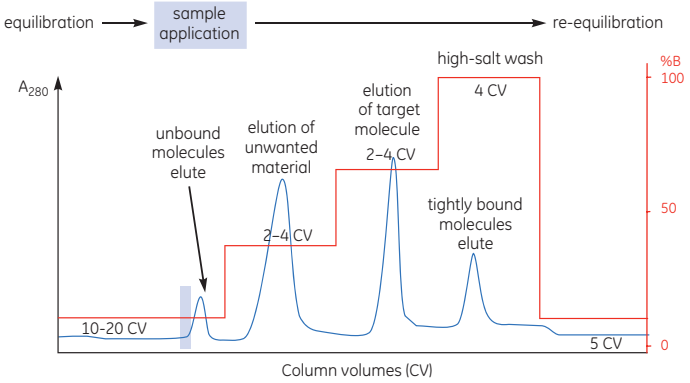

Fig 2.11. Typical IEX purification with step elution.

IEX can be used in any part of a multistep purification procedure: as a first step, in which high binding capacity and high flow rates allow capturing of both target protein and bulk impurities from a large-volume sample, as an intermediate purification step, or as a final step for high-resolution purification to remove the remaining impurities. Typically IEX is used to bind the target molecule, but it can also be used to bind impurities, letting the target protein pass the column. IEX can be repeated at different pH values to separate several proteins that have distinctly different charge properties. Alternatively, a purification step using CIEX can be followed by a second purification step using AIEX at the same pH.

Mixed-mode ion exchangers, Capto™ adhere and Capto MMC, have been developed to offer novel selectivities. The charged ligands are complemented with additional functional groups that introduce additional cooperative interactions (combinations of hydrogen bonds, hydrophobic and van der Waals interactions). Capto adhere can be used for the removal of aggregated monoclonal antibodies to obtain pure monomers.

Hydroxyapatite chromatography (HAC) can also be considered a mixed-mode ion exchange method. Crystals of hydroxyapatite $Ca_3(PO_4)_3OH$ can be used as chromatography medium. Proteins are believed to bind cooperatively to both calcium and phosphate ions on the hydroxyapatite. The hydroxyapatite has a negative charge at neutral pH, and proteins that bind AIEX media tend to also bind to hydroxyapatite. HAC is a less common purification method, partly because of its unpredictable separation mechanism and low binding capacity.

For further information please refer to *Ion Exchange Chromatography and Chromatofocusing Handbook: Principles and Methods* from GE Healthcare (see also the "Related literature" section at the end of this handbook).

Hydrophobic interaction chromatography (HIC)

HIC separates proteins with differences in hydrophobicity. The method 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 chromatography medium (Fig 2.12). This interaction is enhanced by high-ionic-strength buffer, which makes HIC an excellent purification step after precipitation with ammonium sulfate or elution in high salt during IEX. There is no universally accepted theory on the mechanisms involved in HIC. For a brief discussion of the mechanisms, see *Hydrophobic Interaction Chromatography and Reversed Phase Handbook: Principles and Methods* from GE Healthcare (see also the "Related literature" section at the end of this handbook).

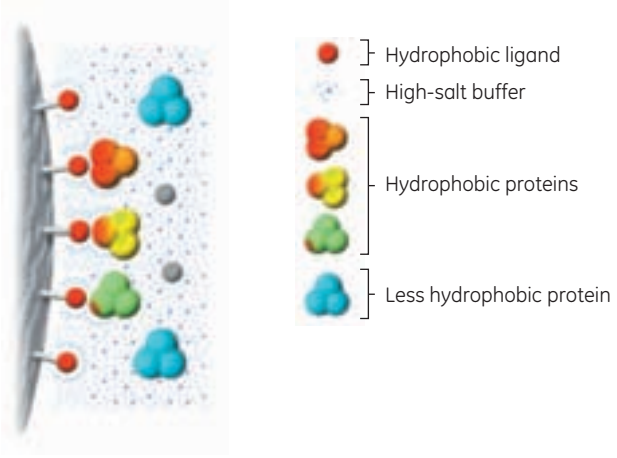

Fig 2.12. Schematic depicting HIC.

Many sample components bind a HIC column in high-ionic-strength solution, typically 1 to 2 M ammonium sulfate or 3 M NaCl. Conditions are then altered so that the bound substances are eluted differentially. Elution is usually performed by decreasing the salt concentration. Changes are made with a continuous decreasing salt gradient (Fig 2.13) or stepwise (Fig 2.14). Most commonly, samples are eluted with a decreasing gradient of ammonium sulfate. Target proteins are concentrated during binding and are collected in a purified and concentrated form. Other elution procedures include reducing eluent polarity (ethylene glycol gradient up to 50%), adding chaotropes (urea, guanidine hydrochloride) or detergents, or changing pH or temperature. Optimization involves screening several HIC media with different ligands and ligand concentrations of the HIC medium, and scouting conditions for best binding selectivity and capacity. High concentrations of salt, especially ammonium sulfate, may precipitate proteins. Therefore, check the solubility of the target protein under the binding conditions to be used.

Screen several HIC media and conditions for best selectivity and capacity.

Scout conditions for protein solubility and for best selectivity and capacity.

Avoid working above pH 8 when using ammonium sulfate to avoid decomposition into ammonia. Use sodium sulfate as an alternative at high pH.

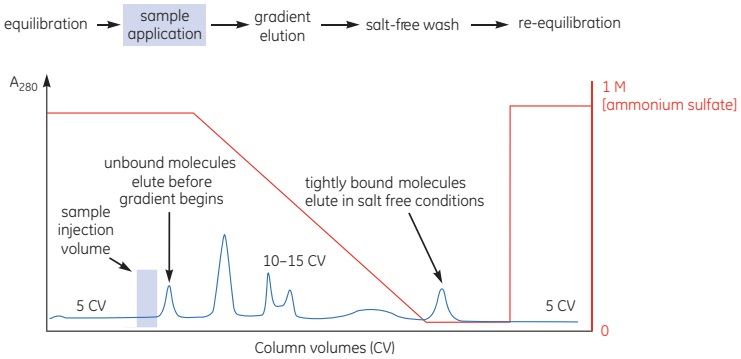

Fig 2.13. Typical HIC purification with gradient elution.

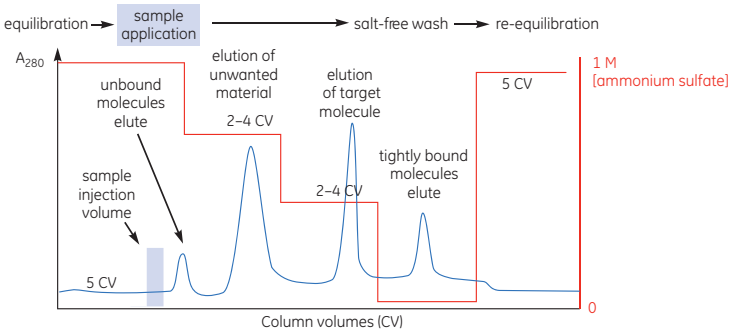

Fig 2.14. Typical HIC purification with step elution.

HIC can be used as a first purification step, as an intermediate step, or as the final step to remove remaining impurities. HIC may be an ideal purification step after ammonium sulfate precipitation.

For further information please refer to *Hydrophobic Interaction Chromatography and Reversed Phase Handbook: Principles and Methods* from GE Healthcare (see also the "Related literature" section at the end of this handbook).

Chromatofocusing (CF)

Chromatofocusing separates proteins according to differences in their isoelectric point (pI). It is a powerful method and can resolve very small differences in pI (down to 0.02 pH units) and thus separate very similar proteins. The capacity of the method is, however, low, and it should ideally be used for partially pure samples. A pH gradient is generated on the column as buffer and chromatography medium interact. The medium is a weak anion exchanger, and the buffer is composed of a large number of buffering substances as in Polybuffer™. Proteins with different pI values migrate at different rates down the column as the pH gradient develops, continually binding and dissociating while being focused into narrow bands and finally eluted. The protein with the highest pI elutes first, and the protein with the lowest pI elutes last. The upper limit of the gradient is defined by the pH of the start buffer, and the lower limit of the gradient is defined by the pH of the elution buffer (Fig 2.15). Polybuffers perform best over pH intervals of 3 pH units or less, and the narrowest pH intervals are likely to give the highest resolution.

A chromatofocusing medium is equilibrated with start buffer (standard buffer with low ionic strength) at a pH slightly above the highest pH required. An elution buffer (Polybuffer adjusted to the lowest pH required) is passed through the column and begins to titrate the amines on the medium and the proteins. As the buffer flows through the column, the pH is lowered, and a moving, descending pH gradient is generated. After a pre-gradient volume of elution buffer has passed, sample (in start buffer) is applied to the column. The proteins in the sample are titrated (pH adjusted) as soon as they are introduced into the column. Proteins in the sample that are at a pH above their pI are negatively charged and bind near the top of the column. Any proteins that are at a pH below their pI begin to migrate down the column with the buffer flow and will not bind until they reach a zone where the pH is above their pI. As the pH continues to decrease inside the column, any protein that drops below its pI becomes positively charged, is repelled by the positively charged amine groups of the medium, and begins to migrate down the column with the elution buffer, traveling faster than the speed at which the pH gradient moves down the column. The process continues until the proteins have been eluted from the column at a pH near their pI. The protein with the highest pI is eluted first and the protein with the lowest pI is eluted last.

Sample:	Partially purified hemoglobins
Column:	Mono P™, 0.5 cm × 20 cm
Start buffer:	0.025 M triethanolamine-methanesulfonic acid, pH 8.1
Flow rate:	1 ml/min (305 cm/h)
Elution buffer:	Polybuffer 96-methanesulfonic acid, diluted in distilled water 1:16, pH 6.65

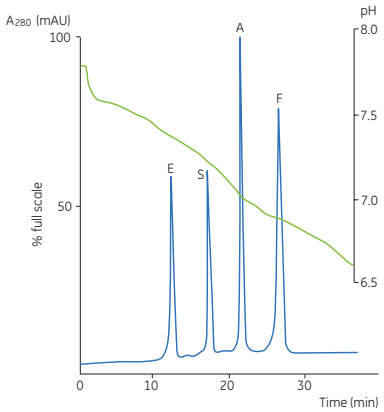

Fig 2.15. Example of chromatofocusing purification. Green line represents pH in eluted fraction. The peaks E, S, A, and F represent subgroups of hemoglobins that are very well separated even though the pI between the A and F subgroup differs by only 0.05 pH unit.

Use chromatofocusing for high-resolution, analytical separations and in preparative purification if IEX or other methods do not give a satisfactory purification.

Avoid using chromatofocusing for proteins that easily precipitate at their isoelectric point. Such proteins are likely to precipitate on the column if they reach a concentration that is too high.

For further information please refer to *Ion Exchange Chromatography and Chromatofocusing Handbook: Principles and Methods* from GE Healthcare (see also the "Related literature" section at the end of this handbook).

Reversed phase chromatography (RPC)

RPC separates proteins and peptides on the basis of hydrophobicity (Fig 2.16). RPC is a high-resolution method, requiring the use of organic solvents.

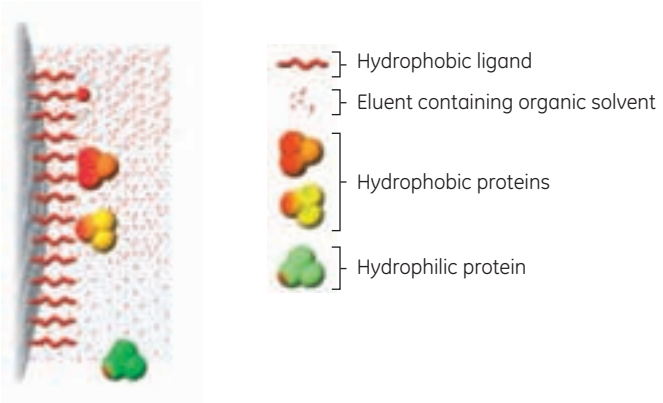

Fig 2.16. Schematic depicting RPC.

The method is widely used for purity check analyses when activity and tertiary structure are not a focus. Because many proteins are denatured by organic solvents, the method is not generally recommended for preparative protein purification—the recovery of activity and native tertiary structure often is compromised. Proteins tend to denature and bind strongly to the RPC medium, and can be very difficult to elute. However, in the polishing phase, when the majority of protein impurities have been removed, RPC is excellent, particularly for small target proteins that are less commonly denatured by organic solvents.

Sample components bind as they are loaded onto the chromatography column. Conditions are then altered so that the bound substances are eluted differentially. Due to the nature of the reversed phase matrices, 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 an increase in organic solvent concentration. Acetonitrile, methanol, ethanol, and propanol are

most commonly used. The target protein is purified and concentrated in the process. The key stages in a separation are shown in Figure 2.17.

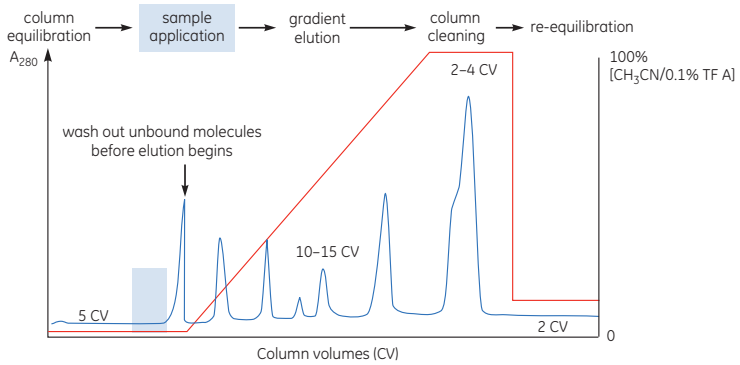

Fig 2.17. Typical RPC gradient elution.

Use RPC for the final purification step of proteins that are stable in the organic solvents used.

For further information please refer to *Hydrophobic Interaction and Reversed Phase Chromatography Handbook: Principles and Methods* from GE Healthcare (see also the "Related literature" section at the end of this handbook).

Chapter 3
Desalting, buffer exchange, and protein concentration

In this chapter, we will focus on two key aspects of protein purification: desalting and similar applications using GF, and protein concentration by ultrafiltration. Other methods will be addressed briefly.

GF for desalting, removal of low-molecular-weight substances, and buffer exchange

During purification work, there is often a need to change the composition of samples or of preparations of purified protein before the end application. Examples include:

- Before IEX to remove salt, for example, for the purpose of decreasing the ionic strength of a sample to allow binding to the column, or after IEX to remove the salt used for elution
- Before any purification step to remove a low-molecular-weight substance that interferes with the purification, or to change pH or exchange one buffer component for another
- After AC to remove a low-molecular-weight component used for elution, which may be important if the substance interferes with stability or activity of the target protein or with subsequent purification
- After low-pH elution in a purification step, to restore pH
- After purification to make a final adjustment of conditions of the purified protein
- After labeling or other chemical modification of a protein, to stop the reaction or to remove excess reagents
- To remove inhibitors from enzymes

Desalting at laboratory scale is a well-proven, simple, and very fast method that, in a single step, will rapidly remove low-molecular-weight contaminants and transfer the sample into the desired buffer.

GE Healthcare offers a range of prepacked chromatography columns and 96-well filter plates that can be used manually, together with a chromatography system, or in high-throughput applications (Table 3.1 and Fig 3.1). The majority of these products contain Sephadex G-25, a GF medium that allows effective removal of low-molecular-weight substances from proteins with M_r > 5000. PD MiniTrap™ G-10 and PD MidiTrap™ G-10 columns contain Sephadex G-10. These prepacked, single-use gravity columns allow desalting/buffer exchange of smaller proteins with M_r > 700.

Use desalting/buffer exchange when needed: before purification, between purification steps, and/or after purification. These are very fast methods compared with dialysis, but remember that each extra step can reduce yield and that desalting often dilutes the sample (centrifugation protocols for desalting do not dilute samples).

Use Sephadex G-25 products to remove salts and other low-molecular-weight compounds from proteins with M_r > 5000 and Sephadex G-10 products for proteins and peptides with M_r > 700.

Purified fractions may have a concentration of target protein that is too low or be in a volume that is too large. In such cases, Vivaspin™ sample concentrators (see next section) can be used for concentrating the sample before buffer exchange.

Desalting provides several advantages over dialysis. Dialysis is generally a slow method that requires large volumes of buffer and carries the risk that material and target protein activity will be lost during handling. When desalting, sample volumes of up to 30% of the total volume of the desalting column can be processed. The high speed and capacity of the separation allows even relatively large sample volumes to be processed rapidly and efficiently in the laboratory. Sample concentration does not influence the separation as long as the concentration of proteins does not exceed approximately 70 mg/ml when using normal aqueous buffers, and provided that the target protein is stable and soluble at the concentration used. Use 100 mM ammonium acetate or 100 mM ammonium hydrogen carbonate if volatile buffers are required.

Consider whether the conditions of the sample can be adjusted simply by additions or dilution of the sample. For AC or IEX, it may be sufficient to adjust the pH of the sample and, if necessary, the ionic strength. Before HIC, ammonium sulfate is normally added and the pH adjusted.

There are a number of different formats available for desalting; see Fig 3.1 and Table 3.1.

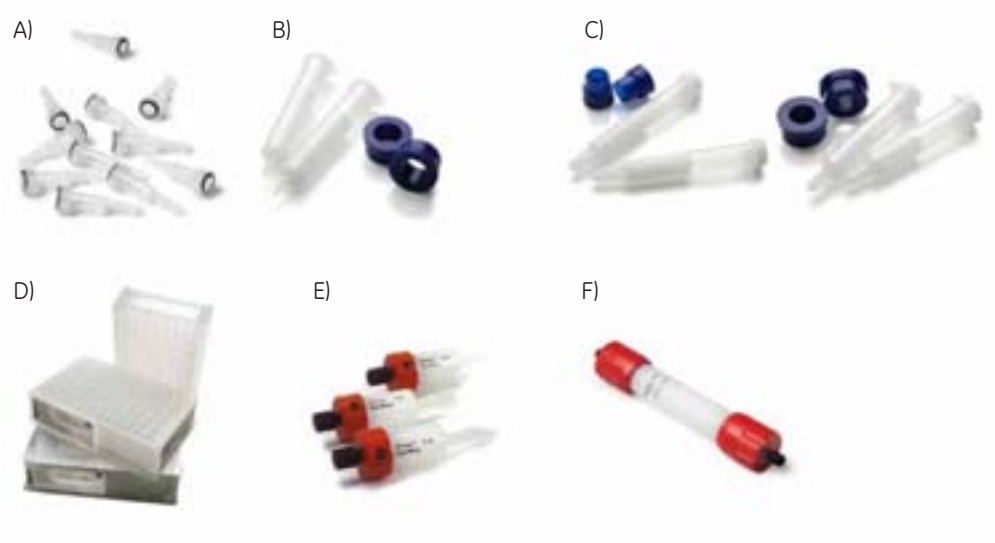

Fig 3.1. Different formats for desalting. (A) PD SpinTrap™ G-25 single or parallel use (70 to 130 µl sample) by centrifugation. (B) PD-10 for single or parallel use (1 to 2.5 ml sample) by gravity or centrifugation. (C) PD MiniTrap (left) and PD MidiTrap (right) for single or parallel use by gravity or centrifugation. (D) PD MultiTrap™ G-25 96-well plates for parallel use (70 to 130 µl sample), either by centrifugation manually or by a robotic system. (E) HiTrap Desalting for single samples (0.1 to 1.5 ml) using a syringe, pump, or chromatography system. (F) HiPrep Desalting for single samples (up to 15 ml) using a pump or chromatography system.

Table 3.1. Selection table for desalting/buffer exchange columns

Columns and 96-well plates	Chromatography medium	Loaded volume (ml)	Eluted volume (ml)	Dilution factor	Operation
PD SpinTrap G-25	Sephadex G-25 Medium	0.07–0.13	0.07–0.13*	No dilution	Centrifuge
PD MultiTrap G-25	Sephadex G-25 Medium	0.07–0.13	0.07–0.13*	No dilution	Centrifuge
PD MiniTrap G-25	Sephadex G-25 Medium	0.2–0.5	0.1–0.5	No dilution	Centrifuge
		0.1-0.5	1.0	2-10	Gravity flow
PD MidiTrap G-25	Sephadex G-25 Medium	0.75-1.0	0.5–1.0	No dilution	Centrifuge
		0.5-1.0	1.5	1.5-3	Gravity flow
PD-10 Desalting columns	Sephadex G-25 Medium	1.75-2.5	1.0–2.5	No dilution	Centrifuge
		1.0-2.5	3.5	1.5-3.5	Gravity flow
PD MiniTrap G-10	Sephadex G-10 Medium	0.1-0.3	0.5	1.7-5	Gravity flow
PD MidiTrap G-10	Sephadex G-10 Medium	0.4-1.0	1.2	1.2-3	Gravity flow
HiTrap Desalting	Sephadex G-25 Superfine	0.25	1.0	4 (approx)	Syringe/pump/system
		0.5	1.5	3 (approx)	Syringe/pump/system
		1.0	2.0	2 (approx)	Syringe/pump/system
		1.5 (max.)	2.0	1.3 (approx)	Syringe/pump/system
2× HiTrap Desalting	Sephadex G-25 Superfine	3.0 (max.)	4.0–5.0	1.3–1.7	Syringe/pump/system
3× HiTrap Desalting	Sephadex G-25 Superfine	4.5 (max.)	6.0–7.0	1.3–1.7	Syringe/pump/system
HiPrep 26/10	Sephadex G-25 Fine	10	10–15	1.0–1.5	Pump/system
		15 (max.)	15–20	1.0–1.3	Pump/system
2× HiPrep 26/10	Sephadex G-25 Fine	30 (max.)	30–40	1.0–1.3	Pump/system
3× HiPrep 26/10	Sephadex G-25 Fine	45 (max.)	45–55	1.0–1.2	Pump/system
4× HiPrep 26/10	Sephadex G-25 Fine	60 (max.)	60–70	1.0–1.2	Pump/system

Contains Sephadex G-25 Medium
Contains Sephadex G-10 Medium
Contains Sephadex G-25 Superfine
Contains Sephadex G-25 Fine

* *Applied volume = eluted volume; For sample volumes less than 100 µl it is recommended to apply a stacker volume of 30 µl equilibration buffer after the sample has fully absorbed*

Method

The desalting column or plate is thoroughly equilibrated with the solution (buffer) into which the sample is to be transferred, and the sample is applied. See Table 3.1 for maximum sample volume for different columns and plates. The same solution is applied to elute the desalted sample. In centrifugation protocols for desalting, the sample is applied and collection is performed directly by centrifugation. No addition of solution (buffer) is required to elute the sample. This method gives essentially no sample dilution. Figures 3.2 and 3.3 show typical examples of laboratory-scale desalting using HiTrap and HiPrep Desalting columns, respectively. Notice the efficient salt removal and the very short processing time. Detailed protocols can be found in the *Recombinant Protein Purification Handbook. Principles and Methods* from GE Healthcare (see also the "Related literature" section at the end of this handbook).

General considerations

Buffer: A salt concentration of at least 25 mM is recommended to prevent possible ionic interactions with the chromatography medium. At salt concentrations above 1.0 M, hydrophobic substances may be delayed or they may bind to the matrix. Volatile buffers can be used, for example, 100 mM ammonium acetate or 100 mM ammonium hydrogen carbonate (see Appendix 1). Protein solubility often depends on pH and/or ionic strength (salt concentration), and the exchange of buffer may sometimes therefore result in precipitation of the protein. Also, protein activity can be lost if the change of pH takes it outside of the range where the protein is active.

Sample: The concentration of the sample does not influence the separation as long as the viscosity does not differ more than a factor of 1.5 from that of the buffer used. This corresponds to a maximum concentration of 70 mg/ml for proteins or 5 mg/ml for high-molecular-weight polymers such as dextran, in normal aqueous buffers at room temperature. The sample should be fully solubilized. Centrifuge or filter (0.45 µm filter) immediately before loading to remove particulate material if necessary.

Column: HiTrap Desalting
Sample: 2 mg/ml BSA in 50 mM sodium phosphate, 500 mM NaCl, pH 7.0
Sample volume: 1.4 ml
Buffer: 50 mM sodium phosphate, 150 mM NaCl, pH 7.0
Flow rate: 10 ml/min

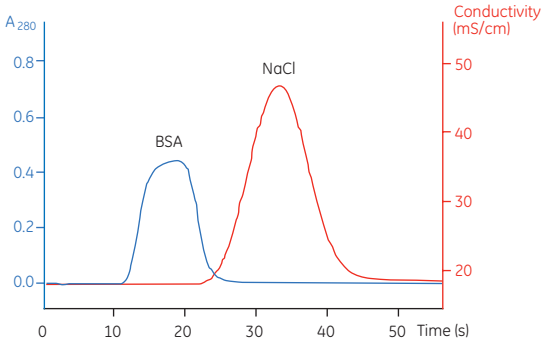

Fig 3.2. Typical desalting using HiTrap Desalting monitored by UV absorption and conductivity.

Column: HiPrep 26/10 Desalting
Sample: 2 mg/ml BSA, 0.07 mg/ml N-Hydroxysuccinimide (NHS) in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.0. Filtered through a 0.45 µm filter
Sample volume: 13 ml
Buffer: 50 mM sodium phosphate, 0.15 M NaCl, pH 7.0
Flow rate: 31 ml/min (350 cm/h)

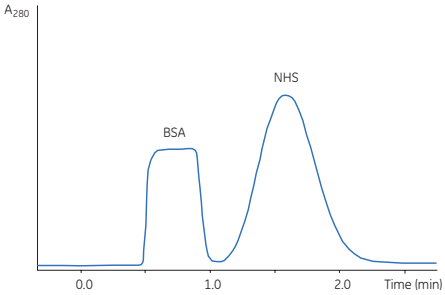

Fig 3.3. Removal of N-Hydroxysuccinimide from BSA.

Scaling up desalting

For desalting of sample volumes larger than 1.5 ml, or to increase the resolution between high- and low-molecular-weight components, up to three 5 ml HiTrap Desalting columns can easily be connected in series. Alternatively, scaling up to HiPrep 26/10 Desalting allows up to a 15 ml sample volume. Further scale-up can be accomplished by connecting up to four HiPrep 26/10 Desalting columns for desalting of up to a 60 ml sample in one run (Fig 3.4). Even larger sample volumes can be handled by repeating runs or by packing Sephadex G-25 medium in a single large column.

Fig 3.4. A 60 ml sample volume can be desalted on four HiPrep 26/10 Desalting columns connected in series.

Automated restoration of pH after low-pH elution

Protein A Sepharose High Performance and Protein G Sepharose High Performance, available in HiTrap columns, are commonly used for purifying antibodies at research scale. Binding is performed at neutral pH and elution at low pH. The elution at low pH, however, may cause conformational changes, and ultimately aggregation of the target antibody. Immediate buffer exchange will minimize such risks. Figure 3.5 shows unattended purification of a monoclonal antibody on HiTrap Protein G HP 1 ml. The eluted material was directly transferred to two HiTrap Desalting 5 ml columns connected together for optimal column volume. The transfer was automatically performed by switching of a valve in the system to change the flow path. This procedure reduced the time that the eluted protein stayed at low pH to a minimum, greatly reducing the risk of denaturation.

Affinity purification

Column:	HiTrap Protein G HP 1 ml
Sample:	3 ml monoclonal mouse IgG_1 in cell culture supernatant. Volume adjusted to 20 ml in binding buffer prior to loading.
Binding buffer:	20 mM sodium phosphate, pH 7.0
Elution buffer:	100 mM glycine HCl, pH 2.7
Flow rate:	1 ml/min

Buffer exchange

Column:	HiTrap Desalting 2 × 5 ml
Sample:	Eluted pool from HiTrap Protein G HP 1 ml
Buffer:	20 mM sodium phosphate, 150 mM NaCl, pH 7.0
Flow rate:	3 ml/min

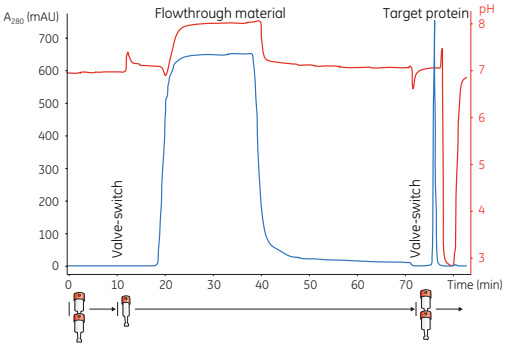

Fig 3.5 Unattended affinity purification and buffer exchange of mouse IgG_1 monoclonal antibody on ÄKTAprime™ plus chromatography system.

Group-separation of larger substances

Removal of contaminants with M_r >5000 cannot be accomplished using Sephadex G-25. Instead, GF media with higher-molecular-weight separation ranges can be used. Chromatofocusing involves the use of Polybuffer. For most applications it is not necessary to remove Polybuffer because the amount that is eluted with any sample is small. Polybuffers do not interfere with enzyme assays or amino acid analysis, but they may interfere with certain protein assays such as Lowry. Polybuffers can be removed from proteins by GF on Superdex™ 75 (Fig 3.6) or Sephacryl™ S-100 HR columns.

Column: Superdex 75 10/300 GL
Sample: Myoglobin (M_r 17 500) + Polybuffer 9–6
Buffer: Phosphate buffered saline
Flow rate: 0.7 ml/min

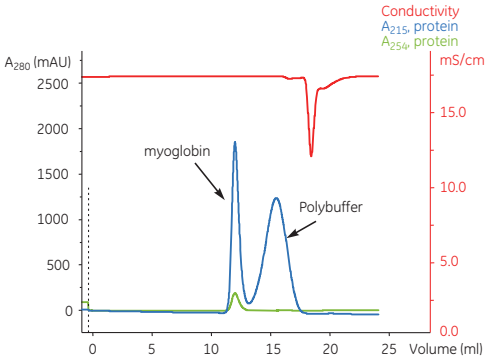

Fig 3.6. Separation of Polybuffer from protein using GF. Notice that Polybuffer can be detected by absorbance measured at 215 nm.

Other methods for desalting and buffer exchange

Chromatography

Unwanted low-molecular-weight impurities are often removed as a side effect of the purification of a protein. It may thus be advantageous to take this possibility into account when selecting purification steps and the order in which to perform them. For example, the sample application can be performed under one set of conditions, and the column can be washed and eluted using another set of conditions. Obviously, different methods may bring different opportunities and limitations to what conditions can be used.

Dialysis

Dialysis is a commonly used method for desalting and buffer exchange of proteins despite the slow speed and large volumes of buffers often required. The sample is placed in a compartment of a semipermeable membrane (M_r cutoff 3000 to 10 000) that is placed in a container with a large volume of buffer having the desired end conditions. Dialysis is typically performed overnight, especially for large samples.

We recommend desalting by Sephadex G-25 columns or plates as a first alternative for desalting, removal of low-molecular-weight substances, or buffer exchange because of the short time required; this reduces the risk of protein aggregation and degradation.

Dialyzing small samples can be difficult and inefficient when the sample must be transferred into and out of dialysis bags and centrifuge tubes. The disposable tubes in Mini Dialysis Kit from GE Healthcare offer a simple solution to the handling problems of small-volume (0.25 to 2 ml) dialysis. The dialysis tubes in the kit each have a cap that incorporates a small disk of dialysis

membrane. Samples are easily pipetted into and removed from the conical bottom of the tube. The capped tube is inserted into a float and then, with the cap and membrane down, placed in a stirred beaker containing the desired dialysis solution. Following dialysis, the tube is centrifuged briefly for easy collection of the sample.

Protein concentration using ultrafiltration

Proteins can be concentrated using ultrafiltration. Centrifugal ultrafiltration is commonly used for sample volumes typical in laboratory-scale protein purification. Vivaspin sample concentrators (Fig 3.7) are designed for fast, nondenaturing, high-recovery concentration of biological samples by membrane ultrafiltration. Up to 30-fold concentration of the sample can be achieved with recovery of the target molecule typically exceeding 95%.

The entire process is performed in a single tube with an upper compartment containing sample and lower compartment separated by a semipermeable membrane with a molecular weight cutoff (MWCO) selected by the user. Centrifugation is applied to force solvent through the membrane, leaving a more concentrated protein solution in the upper chamber. A patented dead-stop technology ensures that samples cannot be concentrated to dryness.

Vivaspin sample concentrators have a tilted vertical polyethersulfone membrane placed in a polycarbonate tube. The materials have been selected to give a minimum of protein adsorption. Vivaspin sample concentrators can be used with a wide range of sample volumes and MWCO values; see Table 3.2.

For maximum recovery select a MWCO at least 50% smaller than the molecular size of the species of interest.

Fig 3.7. Vivaspin sample concentrators provide up to 30-fold concentration of the sample with recovery of the target molecule typically exceeding 95%.

Table 3.2. Vivaspin columns and sample volume ranges. All Vivaspin columns are available with MWCO 3000, 5000, 10 000, 30 000, 50 000, and 100 000.

Product	Volume range
Vivaspin 500	100-500 µl
Vivaspin 2	400 µl to 2 ml
Vivaspin 6	2-6 µl
Vivaspin 20	5-20 ml

Other methods for protein concentration

Lyophilization

Lyophilization (freeze drying) of a protein is a way to obtain a dry powder of the protein. Even though not all proteins are stable enough to survive lyophilization, this is a common method for preserving and shipping proteins. Many proteins can be stored for months or years in dried form.

Lyophilization is performed by freezing the protein solution and then using lyophilization equipment, applying a vacuum to cause sublimation (evaporation) of the solvent. Before freeze drying, the protein is usually transferred, for example, by desalting, to a suitable volatile buffer (see Appendix 1), and stabilizers are often added. The stabilizers are typically sugars, for example, trehalose or sucrose, or polyalcohols. Freezing should be performed quickly, for example, by submersion in liquid nitrogen or a mixture of dry ice and ethanol, to avoid denaturation.

Chromatographic concentration

In protein purification the focus of the first (capture) step, besides purification, is to increase the concentration of target protein (or decrease the sample volume). Any adsorptive chromatography method, for example, AC, IEX, HIC, or RPC, can be used to increase the concentration of a protein. The concentration that can be obtained in an eluted preparation is limited by the binding capacity of the column used and may be reduced by zone broadening during elution. IEX may be especially interesting because of the typically high binding capacities. A drawback with the use of chromatography for concentrating proteins is obviously that the preparation will contain the substance used for elution. If required, the contaminant can be removed by desalting. Adsorption of proteins on columns can give concentrations of more than 100 mg/ml, which risks causing the protein to precipitate.

Precipitation

Precipitation using trichloroacetic acid (TCA) is an efficient procedure for concentrating proteins when there is no need to maintain protein activity. Precipitation using neutral salts, for example, ammonium sulfate, can often be accomplished without loss of activity. This method is relatively common for purification and enrichment of a target protein from extracts (see Chapter 6). Protein crystallization can also be used for protein concentration, but is less common because it is often difficult to find conditions that allow crystal formation.

Chapter 4
Purification strategies

Affinity-tagged proteins and antibodies can often be purified by a single-step protocol using AC to achieve the desired level of purity. However, the purity obtained after a single step is sometimes not sufficient. An additional purification step may be required, in some instances even two or more additional steps.

Untagged proteins usually require a multi-step purification protocol. These proteins may come from natural sources or have been overexpressed without a tag because the presence of a tag would interfere with the use of the protein.

The focus of this chapter is to describe a generic strategy for protein purification that can be used for multi-step purification of untagged proteins as well as single-step purification of affinity-tagged protein. The process of planning the purification; describing the purification requirements, collecting information about the target protein and source material, and setting objectives will be the focus of the following chapter.

Three-stage purification strategy (CIPP)

The need to obtain a protein with sufficient purity and quantity in an efficient and economical way applies to any purification, from preparation of an enriched protein extract for biochemical characterization to large-scale production of a therapeutic recombinant protein. The purification strategy *Capture, Intermediate Purification, and Polishing (CIPP)* (Fig 4.1) has been developed to simplify planning and execution of protein purification. The strategy gives guidelines for how to combine purification methods in the best way to reach the set goals.

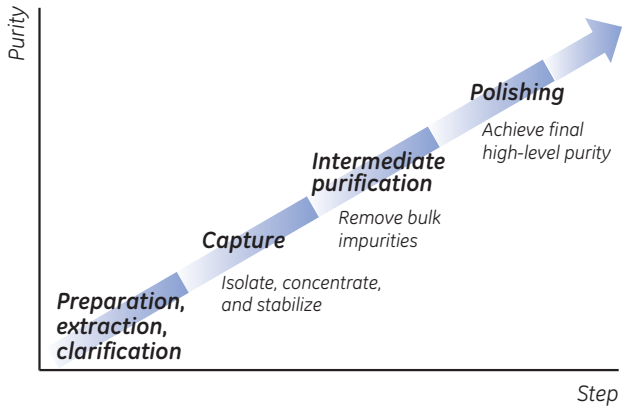

Fig 4.1. The three-stage purification strategy.

Sample preparation is the starting point of the purification strategy (see Chapter 6). The purpose of sample preparation is to obtain a clarified extract of the source material. The extract should be prepared under or adjusted to conditions that are compatible with the first chromatography step.

In the capture stage the objectives are to isolate, concentrate, and stabilize the target product. The product should be concentrated and transferred to an environment that will conserve potency/activity. At best, significant removal of critical contaminants can also be achieved.

During the intermediate purification stage, the key objective is to remove most of the bulk impurities, such as other proteins and nucleic acids, endotoxins, and viruses. If the capture step is efficient, the intermediate purification stage is often omitted in favor of one or more polishing steps.

In the polishing stage, most impurities have already been removed. Now only trace amounts of impurities remain and possibly proteins closely related to the target protein. In the polishing stage, remaining impurities are removed and the target protein may be transferred to conditions suitable for use or storage. The objective is to achieve final purity.

Apply a systematic approach to development of a purification strategy.

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

The three-stage purification strategy does not mean that all protocols must have three purification steps. The number of steps to be included will depend on the purity requirements and intended use of the protein. Increasing the number of purification steps will often decrease the overall protein recovery (Fig 4.2). More steps mean longer purification time, which can be detrimental to activity. For most laboratory-scale work a two- or three-step purification protocol will be sufficient. Difficult purifications may require several additional steps.

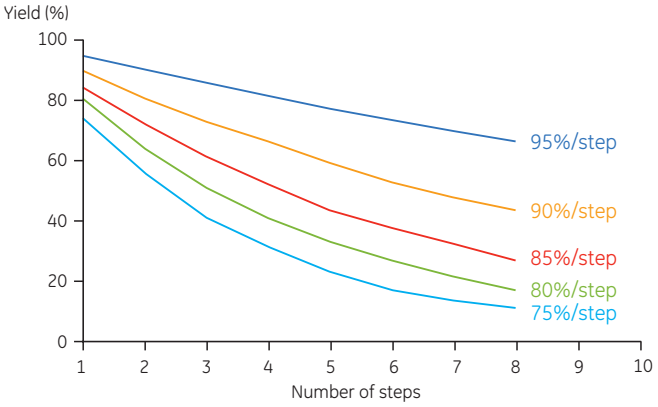

Fig 4.2. Total yield versus number of purification steps.

Limit the number of steps in a purification procedure.

Performance parameters

There are four important performance parameters to consider when planning each purification step: resolution, capacity, speed, and recovery. Optimization of any one of these four parameters can be achieved only at the expense of the others, and each purification step will be a compromise (Fig. 4.3). The importance of each parameter will vary depending on whether a purification step is used for capture, intermediate purification, or polishing. Purification methods should be selected and optimized to meet the objectives for each purification step.

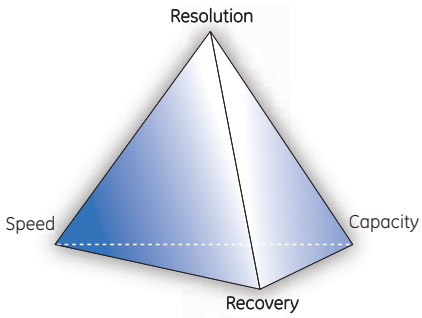

Fig 4.3. Key performance parameters for protein purification. Each purification step should be optimized for one or two of the parameters.

Resolution depends on the selectivity and efficiency of the column, sample, and conditions used. In general, high resolution is more important at the final stage of purification because then the impurities and target protein are likely to have very similar properties.

Capacity refers to how much sample can be loaded onto the column. The amount of sample that can be loaded may be limited by volume (as in GF) or by total amount of target protein and impurities that can be bound to the column without loss or reduction of purity. (Purity may decrease with high sample loads.) The amount of sample and usually also the volume of sample decrease toward the final stage of the purification.

Speed is most important at the beginning of purification where contaminants, such as proteases, must be removed as quickly as possible.

Recovery becomes increasingly important as the purification proceeds because of the increased value of the purified product. Recovery may be decreased by destructive processes in the sample and by unfavorable conditions during the purification.

Capture

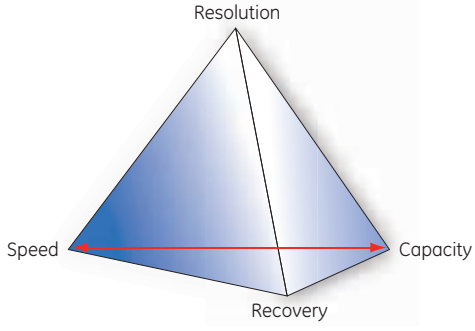

Fig 4.4. The capture stage.

Definition: Initial purification of the target molecule from the source material.
Goal: Rapid isolation, stabilization, and concentration.

In the capture stage (Fig 4.4), the objective is to isolate, concentrate, and stabilize the target product. The purification method is selected and optimized for speed and capacity. A capture step is often a group separation with step elution using, for example, IEX or AC. Ideally, removal of critical contaminants is also achieved. It is sometimes possible to obtain a high level of purification, for example, if a highly selective affinity medium is used.

Conditions are selected to avoid binding of impurities to maximize the binding capacity for the target protein, because impurities that bind use a part of the binding capacity of the column. The target protein should be isolated from critical impurities such as proteases and glycosidases. High speed is desired to reduce sample application time, particularly to remove proteases and other factors to reduce risk of degradation of the target protein. Maximizing capacity and/or speed will be at the expense of some resolution. It is, however, often possible to also achieve considerable resolution, because many sample components will have significant physicochemical differences compared with the target protein. Even though recovery may not be the key parameter to be optimized, it will nonetheless be of concern in any preparative situation, especially for production of a high-value product, and it is then important to assay for recovery during optimization of the capture step. An example of a capture step is shown in Figure 4.5.

- Use a high capacity, concentrating method to reduce sample volume, to enable faster purification and to allow the use of smaller columns.
- Focus on robustness and simplicity in the first purification step. Do not try to solve all problems in one step when handling crude material.
- For the capture step, select the method that binds the target protein while binding as few of the contaminants as possible, that is, the method with the highest selectivity and/or capacity for the target protein.
- Changing a protocol from gradient elution to step elution will increase speed, but at the expense of selectivity during elution.

Column: rProtein A Sepharose Fast Flow, XK16/20, bed height 4.8 cm (9.6 ml)
Sample: 600 ml clarified cell culture containing 87.6 mg of IgG_{2a}
Starting buffer: 20 mM sodium phosphate, pH 7.0
Elution buffer: 20 mM sodium citrate, pH 4.0
Flow rate: 5 ml/min (150 cm/h)

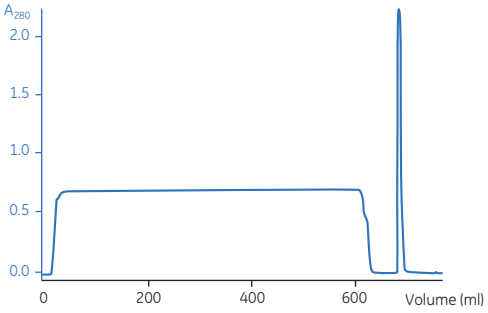

Fig 4.5. Example of capture step: Purification of IgG_{2a} from clarified cell culture.

Intermediate purification

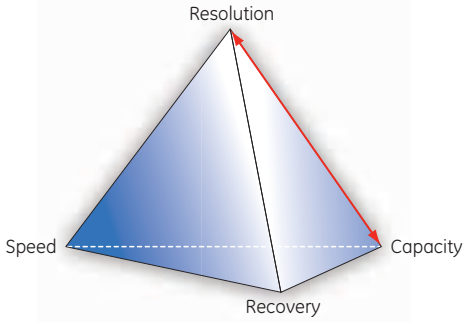

Fig 4.6. The intermediate purification stage.

Definition: Further removal of bulk contaminants.
Goal: Purification and concentration.

The intermediate stage is commonly omitted from the purification protocol, because the capture step is efficient enough by itself (single-step purification) or can be complemented by one or more polishing step(s) to obtain the required purity. In the intermediate purification stage (Fig 4.6), the focus is to separate the target protein from most of the bulk impurities such as other proteins, nucleic acids, endotoxins, and viruses. The ability to chromatographically resolve similar components is of increased importance. The requirements for resolution will depend on the properties of the sample produced from the capture step and the purity requirements for the final product. If the capture step has been efficient, it is often not necessary to include an intermediate purification step. Instead, a high-resolution polishing step may be applied.

The purification method must give a high-resolution separation. Elution by a continuous gradient will often be required.

In the intermediate purification stage, the capacity will still be important to maintain recovery, because there may still be significant amounts of impurities. Speed is often less critical in intermediate purification, because the impurities causing proteolysis or other destructive effects preferably have been removed, and sample volume has been reduced in the capture step. The optimal balance between capacity and resolution must be defined for each case. As in the capture stage, selectivity will be important, not only to achieve high binding capacity for the target protein. However, in contrast to most capture steps, selectivity during elution is important and is usually achieved by applying a continuous gradient or a multi-step elution procedure. An example of intermediate purification steps is shown in Figure 4.7.

Column:	XK 16/20 Butyl Sepharose 4 Fast Flow
Sample:	5 ml of partially purified Annexin V expressed in *E. coli*
Buffer A:	20 mM Sodium phosphate, pH 7.0, 1 M $(NH_4)_2SO_4$
Buffer B:	20 mM Sodium phosphate, pH 7.0
Flow rate:	100 cm/h
Gradient:	0 to 50% B, 20 column volumes

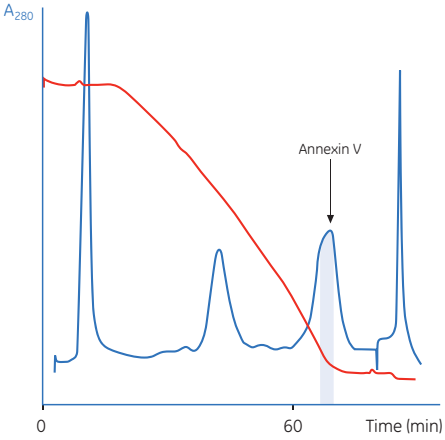

Fig 4.7. Example of an intermediate purification step: Purification of recombinant Annexin V by HIC.

Polishing

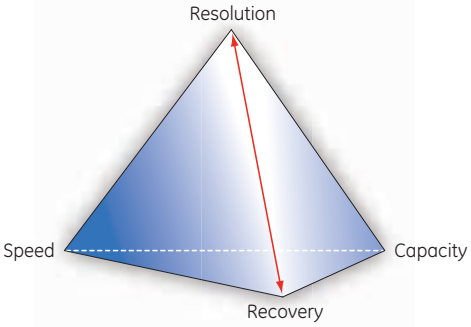

Fig 4.8. The polishing stage.

Definition: Final removal of trace contaminants and adjustment of pH, salts, or additives for use or storage.
Goal: End product of required high-level purity.

In the polishing stage (Fig 4.8), the focus is put on high resolution to achieve final purity. Most contaminants and impurities have already been removed. The impurities remaining can be trace impurities of proteins or other unwanted substances (e. g., endotoxins, nucleic acids, or viruses), and closely related proteins such as microheterogeneous structural variants. A polishing step can also be used to remove fragments or aggregates of the target protein.

The method chosen must discriminate between the target protein and any remaining impurities to be removed. To achieve enough resolution it may be necessary to sacrifice sample load (overload may decrease purity) and recovery by narrow peak fractionation. High resolution usually requires selection of a high-efficiency chromatography medium with small, uniform bead sizes. Recovery of the final product is also a high priority, and a method must be selected that ensures the highest possible recovery. Product losses at this stage are more costly than in earlier stages. Ideally, the product should be recovered in buffer conditions ready for the next procedure.

High-resolution GF is often used for polishing. In GF, the size of the column determines the volume of sample that can be applied. It is thus most efficient to apply this method at the end of the purification protocol, when the sample volume has been decreased by the earlier purification steps. GF has the additional advantage that fragments and aggregates of the target protein can also often be removed (Fig 4.9), and the protein is transferred into any desired buffer. To remove impurities of similar size as the target protein, an alternative high-resolution method is recommended, for example IEX (Fig 4.10).

Column:	XK 16/60 packed with Superdex 75 prep grade
Sample:	1.0 ml of partially purified ZZ-brain IGF
Buffer:	300 mM ammonium acetate, pH 6.0
Flow rate:	0.5 ml/min (15 cm/h)

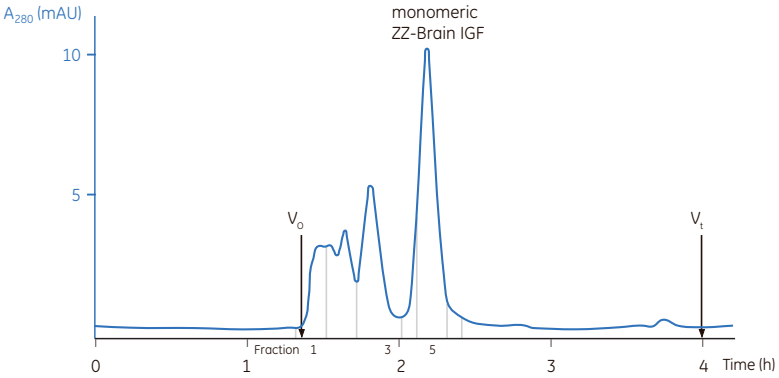

Fig 4.9. Example of polishing step: removal of dimers and multimers by GF.

Column:	Mono S™ 5/50 GL
Sample:	14.5 ml of partially purified and desalted transposase TniA
Binding buffer:	20 mM MES pH 6.5, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT
Elution buffer:	20 mM MES pH 6.5, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 1 M NaCl
Flow rate:	1 ml/min
Gradient:	0%–100% elution buffer, 20 CV

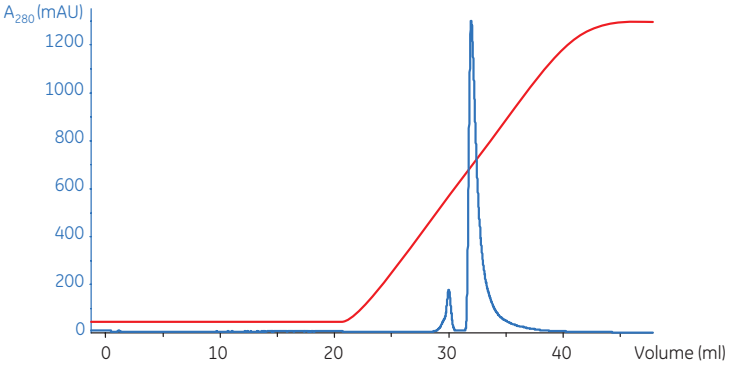

Fig 4.10. Example of polishing: removal of trace contaminants by high-resolution CIEX. Purification of the transposase TniA.

Selection and combination of purification methods

The optimum selection and combination of purification methods is crucial for an efficient purification process. The key chromatography methods available for protein purification were presented in Chapter 2 (see also the relevant method handbooks listed in "Related literature" at the end of this handbook). Table 4.1. shows the basic protein properties utilized in each method.

Table 4.1. Protein properties used during purification

Protein property	Method
Specific ligand recognition (biospecific or nonbiospecific)	AC
Metal binding	IMAC
Charge	IEX
Size	GF
Hydrophobicity	HIC RPC
Isoelectric point	Chromatofocusing

Each method has inherent characteristics that determine how it can be used to optimize for the key performance parameters described earlier: resolution, speed, capacity, and recovery. Table 4.2 is a guide to the suitability of each purification method for the stages in CIPP. In addition to this, the choice of chromatography medium within each method greatly affects performance, because chromatography media may differ in physical characteristics such as bead size (flow resistance and efficiency) and ligand type and concentration (selectivity and capacity). The efficiency and selectivity together affect resolution. Selection of specific medium for a method will be addressed later in this chapter.

Table 4.2. Suitability of purification methods for CIPP

	Typical characteristics		Purification phase				
Method	Resolution	Capacity	Capture	Intermediate	Polishing	Sample start conditions	Sample end conditions
AC	+++ or ++	+++ or ++	+++	++	+	Various binding conditions	Specific elution conditions
IMAC	+++	++	+++	++	+	For purifying histidine-tagged proteins using Ni Sepharose columns:: 20-40 mM imidazole; pH > 7; 500 mM NaCl; no chelators Other proteins: low concentration of imidazole	High concentration of imidazole, pH > 7, 500 mM NaCl
GF	++	+	+		+++	Most conditions acceptable, limited sample volume	Buffer exchange possible, diluted sample
IEX	+++	+++	+++	+++	+++	Low ionic strength. pH depends on protein and IEX type	High ionic strength or pH changed
HIC	+++	++	++	+++	+++	High ionic strength, addition of salt required	Low ionic strength
Chromato-focusing	+++	+			++	Low ionic strength	Polybuffer Low ionic strength
RPC	+++	++		+	++	Ion-pair reagents and organic modifiers may be required	Organic solvents (risk for loss of biological activity)

AC is a desirable and the most common capture step when the target protein is affinity tagged. AC can often combine speed, capacity, resolution, and recovery in a single purification step, although it is more common that it is followed by at least one polishing step.

IMAC is an excellent capture step for histidine-tagged proteins and is used with or without a subsequent polishing step.

GF is seldom used as a capture step because of limitation in sample volume, but is possibly the most common polishing step.

IEX is a common method for any purification stage. IEX columns are suitable for the capture stage because they have high binding capacity, allow high flow rates, and are resistant to harsh cleaning conditions that may be needed after purification of crude samples. IEX is frequently used as a polishing step.

HIC can be an excellent capture step, especially after ammonium sulfate precipitation. The salt concentration and the total sample volume will be significantly reduced after elution from the HIC column. HIC can be used at any stage.

Chromatofocusing is a high-resolution method with moderate binding capacity and is therefore rarely used as a capture step. The method can be used for the polishing stage.

RPC is very rarely used as a capture step because the method will usually bind too many of the sample components in an extract. If the target protein is sufficiently stable, RPC can be efficient for polishing.

- Choose logical combinations of purification methods based on the main benefits of the method and the conditions of the sample at the beginning or end of each step.
- Combine methods that apply different separation mechanisms.
- Minimize sample handling between purification steps by combining methods to avoid the need for sample conditioning before the next step. The product should be eluted from the first column in a buffer suitable for the starting conditions required for the next method.

Figure 4.11 shows common combinations of purification methods. These are separated vertically based on the capture step (AC, IEX, or HIC). From left to right in the figure:

AC: Single-step purification of affinity-tagged proteins using AC may give sufficient purity for some applications.

AC-GF: Complementing an AC step with polishing by GF is very common, and can often be used for generic setups when regularly purifying multiple proteins.

AC-IEX-GF: For high-purity requirements in affinity-tagged protein purification, AC-IEX-GF is a powerful and convenient combination.

Untagged proteins can usually be sufficiently purified by combining purification methods that separate on the basis of different physicochemical characteristics of the proteins (orthogonal methods).

IEX-HIC-GF: The combination IEX-HIC-GF is a very often used three-step purification because the high-salt conditions after the first step can simply be adjusted with additional salt for HIC purification and followed by GF for polishing and salt removal.

HIC-IEX-GF: If ammonium sulfate precipitation has been performed, the combination HIC-IEX-GF is suitable because HIC requires high-salt conditions for binding and gives elution in a relatively low salt concentration in a significantly smaller volume. Dilution or desalting can then be used to remove remaining salt, so that the sample can be bound to an IEX column.

The combinations given here will be efficient for the vast majority of protein purifications if selected and optimized properly. Other combinations not given here or combinations that include more than three steps may be necessary in some instances. IEX is a method that offers different selectivity by using either cation (CIEX) or anion (AIEX) exchangers. A purification protocol can thus be designed to include a combination of CIEX and AIEX. The order of the IEX columns may be important, and one should consider using the first column for binding of impurities only (the target protein does not bind) and binding the target protein on the second IEX column. This eliminates the need for desalting or other adjustments between these purification steps.

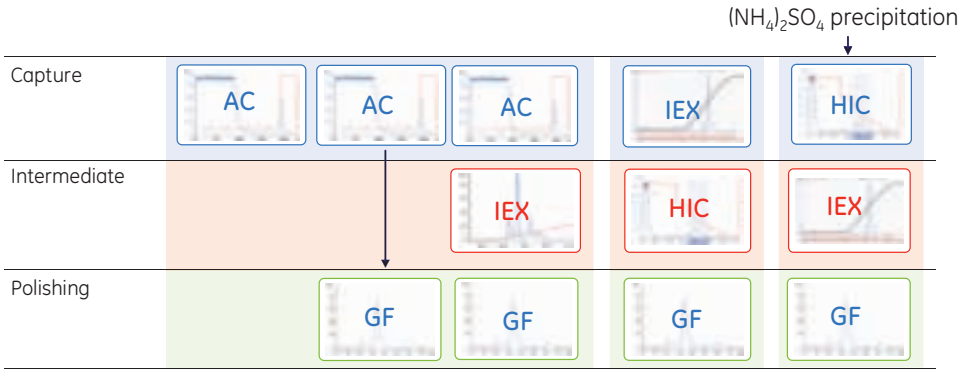

Fig 4.11. Examples of logical combinations of chromatography steps.

If the protein does not have an affinity tag and if little is known about it, use IEX-HIC-GF. This combination of methods can be regarded as a standard protocol.

GF can be used after any of the concentrating methods, for example, IEX, HIC, AC, because it can be used under most buffer conditions. GF is a nonbinding method with limited volume capacity.

HIC is a good capture step after ammonium sulfate precipitation. The salt concentration and the total sample volume will be significantly reduced after elution from a HIC column. Dilution or rapid desalting will prepare it for the next IEX or AC step.

Consider the use of both AIEX and CIEX chromatography to give different selectivities within the same purification strategy. Also consider the order of the methods, because this will often make a difference in purification.

Consider RPC for a polishing step provided that the target protein can withstand the purification conditions and is not irreversibly bound or denatured.

Keep in mind the interplay between "required purity" and "required yield." In general, every added purification step (except for desalting) will increase purity but decrease total recovery and yield.

Chromatography media

In addition to the different selectivities available through the various purification methods, the purification efficiency depends strongly on the selection of different chromatography media available for each method. The efficiency, flow resistance, selectivity, and capacity differ between media. The particle size of the medium strongly affects efficiency and flow resistance. A medium with large beads give columns with low efficiency (the peaks are broad) and low backpressure, whereas small beads give high efficiency and high backpressure.

Early in the purification process (e.g., the capture stage) high speed is often required because the sample volume is large and the sample quickly needs to be stabilized. There is less focus on the resolution. Chromatography media with large particles should be selected that give low backpressure at high flow rates. In the polishing stage focus is put on high purity, which can be obtained with chromatography media with high efficiency, that is, small beads. These media give higher backpressure that may require lower flow rates and columns that resist high pressure. These limitations are acceptable because the sample volume and amounts in this stage are smaller.

Figure 4.12 shows the general principle of choosing chromatography media with larger bead sizes for early purification, and smaller sizes for later steps when demands on purity increase. Inserted chromatograms show the separation results of applying a small, complex sample to columns with IEX media with different bead sizes. The importance of bead size is greatest in large-scale purification and slightly less in laboratory-scale purification. In laboratory scale, it is common that intermediate sized beads (e.g., Sepharose High Performance, 34 µm) are used in the capture stage as well.

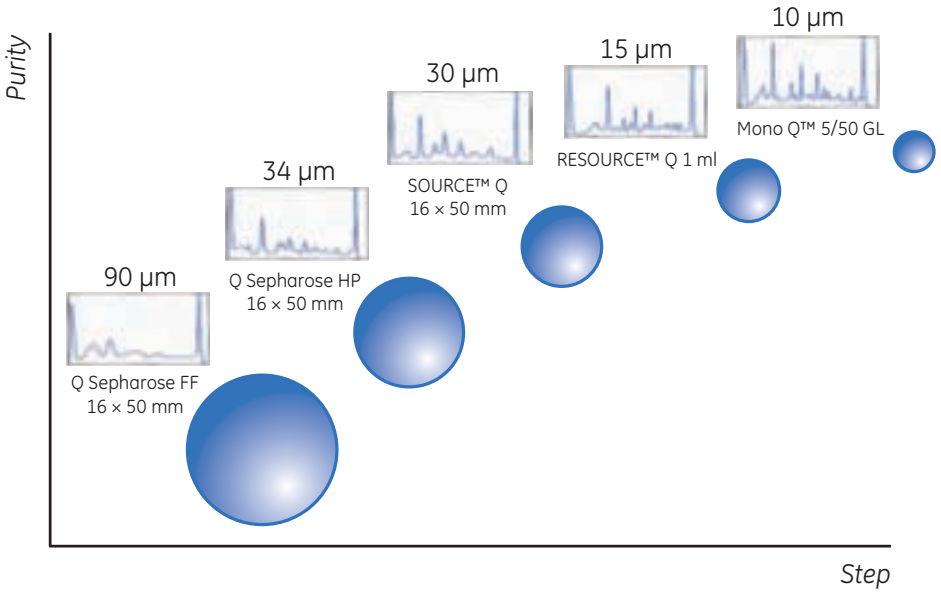

Fig 4.12. Principle of selection of chromatography bead size.

Table 4.3. Summary of chromatography media for CIPP

Capture	Intermediate purification	Polishing	Base matrix	Method	Bead size (µm)	Flow/ velocity (cm/h)	Availability
■			Sepharose XL	IEX	90	100-500	Prepacked or bulk media
■	■		Sepharose Fast Flow	IEX, HIC, AC, IMAC	90	100-300	Prepacked or bulk media
■			MabSelect	AC	85	100-500	Prepacked or bulk media
■			MabSelect SuRe	AC	85	100-500	Prepacked or bulk media
■			MabSelect Xtra	AC	75	100-300	Prepacked or bulk media
■	■	■	Capto	IEX, HIC, AC	75	< 600	Prepacked or bulk media
■	■	■	SOURCE 30	IEX, HIC, RPC	30	< 2000	Bulk media
■	■	■	Sepharose High Performance	IEX, HIC, AC, IMAC	34	< 150	Prepacked or bulk media
	■	■	SOURCE 15	IEX, HIC, RPC	15	150-1800	Prepacked or bulk media
	■	■	Superdex	GF	13	< 75	Prepacked
	■	■	Superdex pg	GF	34	< 75	Prepacked or bulk media
	■	■	MonoBeads™	IEX	10	150-600	Prepacked
Micropurification			MiniBeads™	IEX	3	< 700	Prepacked

■ *common use*
■ *rarely used*
□ *not recommended*

For a purification scheme that is not intended for scale-up (i.e., only milligram quantities of product are needed), use high-performance media such as Sepharose High Performance (IEX, HIC) or MonoBeads (IEX), or SOURCE (IEX, HIC). All these media are available in prepacked columns.

For microscale purification, use MonoBeads (IEX) or MiniBeads (IEX) and Superdex (GF), and other media as appropriate.

If the starting material is relatively pure, a single-step purification at high resolution, for example, using MonoBeads (IEX) or Superdex (GF), may be sufficient to achieve the required purity at laboratory scale.

Use the convenient selection kits HiTrap IEX Selection Kit, HiTrap Capto IEX Selection Kit, HiTrap HIC Selection Kit, and RESOURCE HIC Test Kits for media screening and simple method optimization.

If a biospecific ligand is available, consider using AC in the capture stage. If the medium is to be used routinely, ensure that any bound impurity from the crude sample can be removed by column regeneration procedures that do not damage the affinity ligand.

Chapter 5
Planning

The scope of a protein purification plan will vary depending on the target protein. Purification of affinity-tagged recombinant proteins does not require extensive planning. High purity is often obtained in a single AC purification step by following the instructions, and planning of the purification work can be kept to a minimum. Purification of difficult affinity-tagged proteins or proteins without a tag may be more challenging. A more thorough plan is then required for successful purification. This chapter lists a number of considerations that may or may not be relevant, depending on the situation. It is a good rule not to spend too much time on planning before starting the experiments, because new information will inevitably appear that cannot be foreseen or planned for. Consider the following guidelines:

- **Define objectives**
  for purity, activity, and quantity required of the final product to avoid over- or under-developing a method.
- **Describe properties of the target protein and critical impurities**
  to simplify method selection and optimization.
- **Develop analytical assays**
  for fast detection of protein activity/recovery and critical contaminants.
- **Minimize sample handling at every stage**
  to avoid lengthy procedures that risk losing activity/reducing recovery.
- **Minimize use of additives**
  because additives may need to be removed in an extra purification step or may interfere with activity assays.
- **Remove damaging impurities early**
  for example, proteases.
- **Use different methods at each step**
  to take advantage of sample characteristics that can be used for separation (size, charge, hydrophobicity, ligand specificity).
- **Minimize the number of steps**
  because extra steps reduce yield and increase time; combine steps logically.

KEEP IT SIMPLE!

Define objectives

Goal: To set minimum objectives for the purification.

Start the planning process by considering how the purified protein will be used. Understanding its use will help in setting the objectives. Estimating how much protein is needed at a given time will determine the amount of protein to be prepared, and when. Other important questions to be answered could be: What purity is required? Are there any impurities that may interfere with the usage? Is protein activity required? Are tags acceptable? How can the purified protein be stored? Is shipping required?

- Define purity requirements according to the final use of the product.
- Identify key impurities.
- Identify the nature of prevalent remaining impurities as soon as possible.

The key objectives in most purifications are purity, activity, quantity, and recovery. Examples of other requirements are listed in Table 5.1.

Table 5.1. Examples of parameters for purification objectives

Objectives	Examples
Correct protein identity	Identified by, for example, MS
Amount	
Concentration	
Purity and activity	Polypeptide purity
	Specific activity
	Level of specific impurities
	Level of contaminants
Protein homogeneity	Polypeptide sequence homogeneity
	Size homogeneity (monomeric, dimeric, aggregates, etc.)
	Protein complex composition/intactness (which subunits must be present)
	Cofactors
	Coenzymes
	Post-translational modifications
Lifetime of preparation	Stable activity
Storage stability	Stable size homogeneity (no aggregation)
Protein in suitable form	What conditions (pH, ionic strength, additives, etc.)
	Protein freeze dried

Purity

The statement that a protein is > 95% pure (i.e., target protein constitutes 95% of total protein) is far from a guarantee that the purity is sufficient for an intended application. The same is true for the common statement that "the protein was homogeneous by Coomassie™-stained SDS-PAGE." Purity of 95% may in some cases be acceptable if the remaining 5% consists of harmless impurities.

However, even minor impurities that may be biologically active could cause significant problems in both research and therapeutic applications. It is therefore important to differentiate between impurities that must be removed completely and those that can be reduced to acceptable levels.

Because different types of starting material will contain different impurities, they will present different purification challenges. The purity requirement may differ dramatically between usages of the purified protein (Fig 5.1). Although the number of purification steps should be minimized, the quality of the end product should not be compromised. Subsequent results might be questioned if sample purity is low and contaminants are unknown.

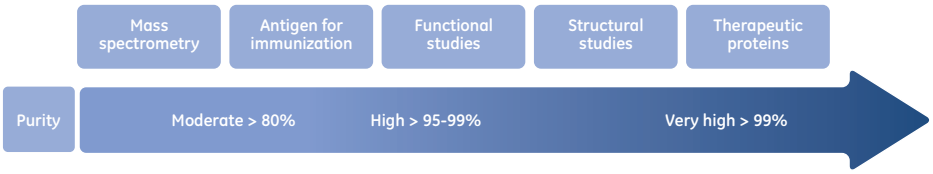

Fig 5.1. Applications require different protein purities.

Activity

It is usually an absolute requirement that the protein is active after purification. This is obvious for functional studies, and many other analyses also require that the protein retain its native structure. However, for many chemical analyses, activity may not be important. In such cases purification can even be performed using harsh purification methods (e.g., acetone or heat precipitation) and conditions (e.g., strong detergents/denaturants, extreme pH, salt concentration).

Some applications of the purified protein may require long-term stability, for example, crystallographic studies. When long-term stability is required, the focus is placed on finding purification end conditions that stabilize the protein and may affect the choice of purification steps and their order in the protocol.

Quantity

The amount of protein required for different use varies greatly, from picograms to kilograms or even tonnes (Fig 5.2). The scale of the purification will depend on the amount of target protein required but also on the sample volume and amount of contaminants that have to be handled (bound) by the column. In addition, the required amount of purified protein can possibly be obtained by multiple purification cycles at a smaller scale.

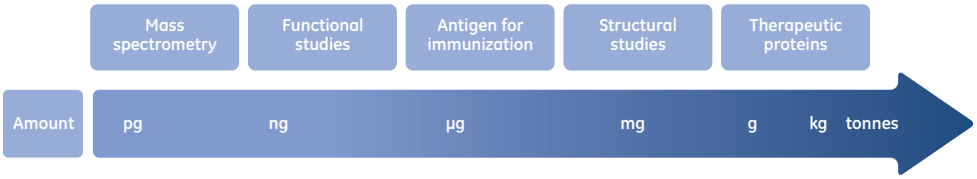

Fig 5.2. Applications require different protein quantities.

Protein homogeneity

It is not always sufficient to obtain a protein at high purity; often the protein must have a high degree of homogeneity as well. A protein preparation can be heterogeneous in various ways. Fragments of the target protein can be derived from preterminated translation, protease action during expression, or proteolysis during purification. The protein may form oligomers or aggregates. Post-translational modifications may be heterogeneously distributed among proteins and be heterogeneous themselves, for example, glycosylations. Protein complexes may tend to lose one or more subunits, causing heterogeneities.

Describe properties of the target protein and critical impurities

The properties of the target protein will affect the purification methods to be applied and set limits for what purification objectives can be fulfilled. The critical impurities to be removed depend on the type of starting material used (for example, expression system).

Describe the target protein

Goal: To determine the properties of the target protein, to allow selection of purification methods and conditions, as well as analytical methods.

Information concerning the target protein will help to guide the choice of separation methods and experimental conditions that can be used for purification. Key properties include:

Molecular weight

The molecular weight of the protein polypeptide can easily be calculated from the amino acid composition in the case of a cloned protein. This information is useful if SDS-PAGE is used for the initial expression level estimate (in case the expression is high enough to give an extra protein band upon induction). Note that the apparent molecular weight obtained from SDS-PAGE (which is an indirect, calibration-dependent method) can sometimes deviate considerably from the calculated one. The molecular weight is also important when selecting a GF medium. Normally GF is performed under native conditions that keep oligomeric proteins and protein complexes intact. It is therefore useful to know whether the target protein is composed of one or several identical or nonidentical polypeptides. Similar considerations can be made for large oligosaccharide parts of glycoproteins. If the amino acid sequence of the target protein is not known, a combination of GF and specific detection such as activity assay or immunoassays can be used to estimate the molecular weight.

Isoelectric point, pI

The isoelectric point of the protein can be theoretically estimated from the amino acid composition or be experimentally determined by isoelectric focusing, provided that the protein can be obtained pure enough or be specifically detected in the gel. Knowledge of the pI value can be used for several purposes: Proteins tend to have lowest solubility at pH values close to the pI. The pI value gives a hint about suitable IEX media for purification and also about what binding conditions (especially pH but sometimes also ionic strength) should work (see Chapter 2). Theoretical charge versus pH curves can be calculated using the amino acid composition to further guide the selection of conditions.

Solubility

Protein solubility is a key parameter in protein purification. Low solubility may depend on association of the protein in active form, or inactivation and unfolding causing aggregation due to exposure of hydrophobic parts of the polypeptide that are hidden in the interior of the active protein. For many (active) proteins, the solubility is lowest at pH values close to the pI of the protein. The presence of salt, for example, NaCl, often affects solubility. Changes of pH and ionic strength during different purification steps should be considered to avoid aggregation and precipitation of the protein.

Stability

The stability of a protein in terms of protein activity, aggregation, and subunit composition and of chemical and physical characteristics is usually important during purification, storage, and use. It is thus important that the stability window for the protein is determined. The stability of a protein varies depending on conditions (pH, ionic strength, and presence of additives). During purification, conditions can change due to differences in the start and end conditions of the purification step. A short time for purification, storage, or use reduces problems with instability. See Chapter 6 for tips and hints regarding protein stabilization.

Describe the starting material

Goal: To determine the properties of the starting material, clarify sample preparation requirements, and identify harmful impurities.

The type of expression system that is used will greatly affect the purification steps, such as the cell disruption procedure (if required), clarification of the sample, and requirements on conditions and stabilizing additives. The starting material will contain a large number of substances that will have to be removed during purification. It is useful to identify the components in the sample that may be most damaging to the target protein, for example, proteases. These should be removed or inhibited as soon as possible.

The protein may be located internally in the cytoplasm, in organelles, in cell walls, or in the periplasm of bacteria; it may also be secreted out of the cell. High overexpression of proteins in bacteria frequently yields inclusion bodies, which are large aggregates composed mainly of the target protein, often in inactive form. The location of the target protein in the host affects the extraction procedure.

The source material may contain substances that interfere with purification, for example, nucleic acids, lipids, and particulate matter. These substances should if possible be eliminated before applying the sample on a chromatography column. The presence of proteases in the starting material may require addition of inhibitors or fast removal of the proteases. The pI and M_r distribution can be investigated by 2-D electrophoresis. Table 5.2 lists a number of factors to be considered. More information about protein sources will be provided in Chapter 6.

Table 5.2. Properties of the starting material that may affect purification

Key factors	Impact on purification method
Critical impurities	May require a specific purification method.
Fouling components	Clarification method.
pI distribution/titration curve	Effect on selection of medium and conditions for IEX.
M_r distribution	Selection of GF medium (in rare cases when it is used as first purification step).
Ionic strength	If the ionic strength is too high, the sample may have to be desalted or diluted before IEX.
pH	Selection of IEX chromatography medium.
	pH adjustment may be needed.
Volume	Selection of chromatography method.
	GF limits sample volume that can be applied in each run.
Protein concentration	Scale/column size or division of samples for repetition of the purification step.
Proteases	Early removal, high speed, low temperature, addition of inhibitors.
Substances that may affect separation	Any chromatography method.

Develop analytical assays

Goal: To monitor the progress during development of the purification protocol and the quality of the final product after purification.

The importance of a reliable assay for the target protein cannot be overemphasized. Identify what analysis methods are required based on the objectives of the purification. The key parameters to be assessed are:

- Target protein concentration
- Purity
- Total protein concentration
- Concentration of critical impurities

Analysis and characterization in the protein purification context will be further addressed in Chapter 8.

Create a purification table

Goal: To evaluate the outcome of each of the purification steps of the purification protocol.

A purification table allows a summary of the results of the purification. For example, if there is an extensive loss of target protein in a purification step, the step may have to be further optimized or even exchanged for another one. If the purification factor decreases in a step, this is probably due to extensive loss of activity or amount of target protein. This outcome may be a reason for removing this purification step. On the other hand, the purification step may be required to remove a specific impurity, and the loss of target protein may have to be accepted.

Table 5.3 is an example of a purification table. Data in columns one (Volume), two (Protein concentration), and four (Total activity) are measured experimentally and are used for calculating the other data in the table:

1. Total *Protein concentration* can be determined using standard methods (e.g., absorbance at 280 nm, Bradford or Lowry assays).
2. *Amount of total protein* can be calculated from columns one and two.
3. *Total activity* is determined experimentally (= U/ml obtained for the small sample taken to assay × the *Volume*). Alternatively, the Total amount of target protein is determined.
4. The purity measured as *Specific activity* is calculated by dividing *Total activity* by *Amount of total protein*. Alternatively, purity is calculated as *Total amount of target protein* divided by *Amount of total protein*, for example, estimated by SDS-PAGE.
5. *Recovery* is calculated by first setting the *Total activity* of the homogenate (or any starting material) to 100% and then calculating the percentage of activity remaining compared with the starting material for each step.
6. *Purification factor* is calculated using the *Specific activity* column to calculate how many times the specific activity increased after each purification step as compared with the specific activity in the starting material.

Table 5.3. Example of protein purification table

Purification step	Volume (ml)	Protein conc. (mg/ml)	Amount total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification factor
Homogenate	220	1.7	373	107	0.29	100	
AC	30	0.14	4.2	93	22	87	76
Concentration	2.2	1.8	4.0	84	21	79	72
GF	8	0.38	3.0	76	25	71	86

Chapter 6
Sample preparation

Chromatographic purification requires, with few exceptions, that the sample contain the target protein in solution and that there are no particles that can clog the column. The sample preparation needed depends on the source. Some source materials are essentially clear liquids that can be directly applied, possibly after clarification, to the first chromatography column, for example, samples such as serum, plasma, or cell supernatants. In other cases the source material is a cell paste or a tissue, and several sample preparation steps are required to obtain a sample suitable for chromatography, with the target protein properly extracted.

The steps required for sample preparation depend on the source material and the characteristics of the target protein and its location (Fig 6.1).

The purpose of sample preparation is to:

- Release the target protein from the source material and get it into solution
- Remove any particles from the sample
- Stabilize the target protein
- Adjust conditions of the sample to fit the first chromatographic purification step
- Eliminate impurities that can interfere with subsequent purification

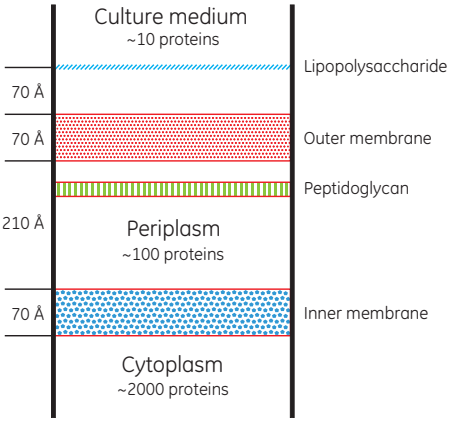

Fig 6.1. Schematic cross-section of the cell wall and typical number of protein species in *E. coli*. (Data from reference 1.)

The most common steps in sample preparation from cultured cells are cell harvest, cell disruption, and clarification, but additional steps may be required depending on the location of the target protein (Fig 6.2). Preparation of secreted proteins obviously does not need cell disruption. Proteins expressed as insoluble aggregates (inclusion bodies) will usually be prepared by isolation and solubilization of the inclusion bodies followed by refolding of the target protein. Membrane protein samples are usually obtained by preparation of the biological membrane and solubilization of the membrane proteins using detergents. Preparation of water-soluble proteins from animals or plants begins with homogenization of the tissue material. The homogenate can then be handled essentially as cultured cells, that is, using cell disruption, clarification, etc. The method for cell disruption depends on the strength of the cell wall, and varies between cell types. Fractional precipitation can be used as a sample preparation step to remove bulk impurities.

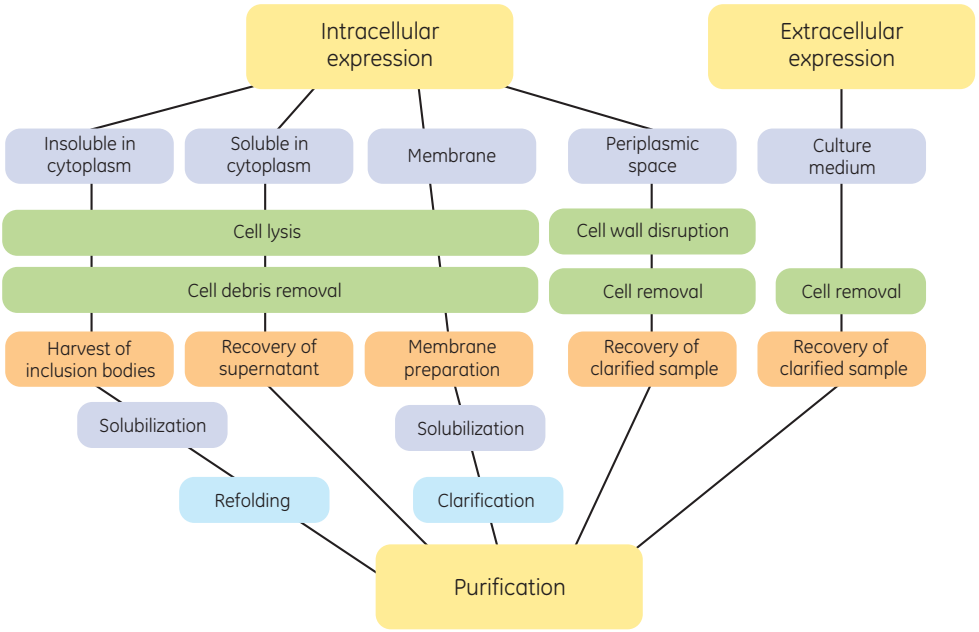

Fig 6.2. Overview of sample preparation from bacteria.

Sample preparation requires tradeoffs between efficient extraction and risk of protein denaturation. Performing the sample preparation quickly, in the cold, under suitable conditions, and by using stabilizing additives will reduce the risk of target protein denaturation.

Source materials

Purification of proteins from natural sources is still relatively common, but the vast majority are overexpressed, recombinant proteins. There are many host systems for protein over-expression, including bacteria, yeast, plants, filamentous fungi, insect or mammalian cells grown in culture, and transgenic animals or plants. In addition to these, cell-free systems are also available. Each host system has its own advantages and disadvantages. The choice of host affects post-translational modification and expression levels, but also the way in which the protein can be purified. Table 6.1 summarizes features of several expression systems.

Table 6.1 Features of expression systems

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

+ = Yes
– = No

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

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

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

The expression of recombinant proteins is highly variable and is affected by the nature of the target protein, the host cell, and culture conditions. Recombinant protein yields in *E. coli* can range from 0 to 10 g/l of culture. Table 6.2 can be used to approximate culture volumes based on an average yield of 2.5 g/l.

Table 6.2. Typical recombinant protein yields in *E. coli*

Protein	12.5 mg	50 mg	1 g	10 g	50 g
Culture volume	5 ml	20 ml	400 ml	4 l	20 l
Volume of lysate	0.5 ml	1 ml	20 ml	200 ml	1000 ml

Cell harvesting and extraction

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

Selection of extraction method depends on the equipment available and scale of operation as well as on the type of sample. Examples of common extraction processes for recombinant proteins are shown in Table 6.3. In many situations a combination of these methods gives optimal results. The results obtained from cell lysis depend on several factors, including sample volume, cell concentration, time, temperature, energy input (speed of agitation, pressure, etc.), and physical properties of the cell lysis apparatus.

- Use procedures that are as gentle as possible because too vigorous cell or tissue disruption may denature the target protein or lead to release of proteolytic enzymes and general acidification.
- Extraction should be performed quickly, and in the cold, for example, at 4°C or on ice, in the presence of a suitable buffer to maintain pH and ionic strength and to stabilize the sample.
- Add protease inhibitors before cell disruption. Fractional precipitation may reduce the presence of proteases.
- The release of nucleic acids may cause viscosity problems. Add nucleases to break down the nucleic acids; see Table 6.5.

Table 6.3. Homogenization and cell disruption methods

Extraction process	Typical conditions	Protein source	Comment
Gentle			
Cell lysis (osmotic shock)	Two volumes water to one volume packed, prewashed cells.	Erythrocytes, *E. coli* (periplasmic proteins).	Reduced protease release, but lower product yield.
Enzymatic digestion	Lysozyme 0.2 mg/ml.	Good for Gram-positive bacteria. For Gram-negative bacteria (e.g., *E. coli*), combine with chemical and osmotic-shock lysis.	Laboratory scale only, often combined with mechanical disruption.
Chemical lysis	Detergents.	Eukaryotes, *E. coli.*	
Hand homogenization	Follow equipment instructions.	Liver tissue, etc.	
Mincing (grinding)	Follow equipment instructions.	Muscle, etc.	
Moderate			
Blade homogenizer	Follow equipment instructions.	Muscle tissue, most animal tissue, plant tissue.	
Grinding with abrasive, e.g., glass beads	Add glass beads to prewashed cells, vortex, centrifuge, repeat up to five times, pooling supernatants.	Bacteria, plant tissue.	Physical method. Chemical conditions are less important for cell lysis but may be important for subsequent removal of cell debris and purification steps.
Freeze/thaw	Freeze cells, thaw (repeat several times), resuspend pellet by pipetting or gentle vortexing in room-temperature lysis buffer. Incubate, centrifuge, retain supernatant.		Several cycles.
Vigorous			
Ultrasonication or bead milling	Follow equipment instructions.	Cell suspensions: intracellular proteins in cytoplasm, periplasm, inclusion bodies.	Small scale; release of nucleic acids may cause viscosity problems; if so, add DNase. Inclusion bodies must be resolubilized.
Manton-Gaulin homogenizer	Follow equipment instructions.	Cell suspensions.	Large scale.
French press	Follow equipment instructions.	Bacteria, plant cells.	Laboratory scale.

Clarification

Samples for chromatographic purification should in general be clear and free from particulate matter. There is one exception from this, the specially designed HisTrap FF crude column, which accepts unclarified extracts (see Chapter 7).

Simple steps taken to clarify a sample before beginning purification will avoid clogging the column, may reduce the need for stringent column washing procedures, and can extend the life of the column. Centrifugation and filtration are standard laboratory methods for sample clarification. A clarified sample that is not used immediately may within minutes start to precipitate. Although the target protein may co-precipitate, this is often not the case, and a reclarification of the sample before application on the chromatography column can often save it.

It is highly recommended to clarify the sample using centrifugation and/or filtration immediately prior to chromatographic purification.

Centrifugation

Centrifugation is the most common clarification method for extracts in laboratory scale. Centrifugation removes most particulate matter and cell debris, but depending on source and extraction procedure, there may be various amounts of particles that cannot be sedimented or that float on top. Centrifugation should preferably be performed in the cold.

For cell lysates, centrifuge at 40 000 to 50 000 × g for 30 min (may be reduced to 10 to 15 min if a short handling time is required).

For small sample volumes, centrifuge at the highest available g-force, such as 15 000 × g for 15 min in a benchtop centrifuge.

Use the cooling function of the centrifuge and precool the rotor by storing it in the cold room or by starting to cool the centrifuge well in advance with the rotor in place. If the sample is still not clear after centrifugation, use a 5 µm filter as a first step and one of the filters listed in Table 6.4 as a second step.

Filtration

Filtration removes particulate matter. Small samples can sometimes be clarified by passage through a filter fitted to a syringe, thus avoiding a more time-consuming centrifugation run. Larger syringe filters can often be used conveniently for relatively large volumes, up to 50 to 100 ml. It is good practice to also filter samples already clarified by centrifugation prior to application on the chromatography column. Membrane filters that give the least amount of nonspecific binding of proteins are composed of cellulose acetate, polyether sulfone (PES), or polyvinylidene fluoride (PVDF). A first filtration through a filter with large pores, for example, a pore size of 5 µm, may remove the bulk of the particles, thus avoiding clogging the final filter. This final filter should be selected in relation to the bead size of the chromatography medium as shown in Table 6.4. Filters become saturated, that is, they have a certain capacity. It may be necessary to check the sample volume capacity when setting up a protocol.

Some proteins may bind nonspecifically to filter surfaces. If extensive loss of protein upon filtration is suspected, check the recovery of the target protein in a test filtration.

For protocol development, consider checking sample capacity of the filter.

Table 6.4. Selecting filter pore sizes

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

Protein stability—selection of conditions

The biological activity and structural integrity of the purified target protein are often of interest and need to be retained throughout the entire purification procedure. Sample preparation is a critical step to achieve this goal. Cell disruption releases proteases and other modifying enzymes, and may expose the target protein to harsh physical and chemical conditions. It is thus important to transfer the target protein to a friendlier environment as quickly as possible.

Proteins generally have unique tertiary structures, kept together by van der Waals' forces, ionic and hydrophobic interactions, and hydrogen bonding. Any conditions capable of destabilizing these forces may cause denaturation and/or precipitation. By contrast, peptides tolerate a much wider range of conditions than proteins. This basic difference in native structures is also reflected in that proteins are not easily renatured, while peptides often renature spontaneously. It is important to check the stability limits of the sample and to work within these limits during sample preparation and purification.

Perform stability tests as early as possible.

The list below may be used as a basis for stability testing:

- Test pH stability in steps of one pH unit between pH 2 and pH 9.
- Test salt stability with 0 to 2 M NaCl and 0 to 2 M $(NH_4)_2SO_4$ in steps of 0.5 M.
- Test the stability toward acetonitrile and methanol in 10% steps between 0% and 50%.
- Test the temperature stability in 10°C steps from 4°C to 40°C.
- Test the stability and occurrence of proteolytic activity by leaving an aliquot of the sample at room temperature overnight.

Centrifuge each sample and measure activity and UV absorbance at 280 nm in the supernatant.

There are many possible symptoms of protein instability, for example:

- Loss of enzyme activity or ligand binding
- Loss of target protein (precipitation or binding to surfaces)
- Conformational changes; loss of secondary structure (cf. circular dichroism spectroscopy)
- Presence of aggregates or oligomers
- Loss of subunits from protein complexes

The target protein can also be affected by chemical modifications, for example:

- Deamidation
- Racemization
- Hydrolysis
- Beta-elimination
- Disulfide exchange
- Oxidation

Conditions for sample preparation and different purification steps should be selected so that the target protein is stabilized and the conditions are compatible with the subsequent purification method.

Use only additives that are proven to be essential for stabilization of the product or to improve extraction. If possible, select additives that are easily removed.

The conditions for sample preparation and subsequent purification may have to be changed in later purification steps or for storage of the purified protein. An additive required during sample preparation can often be omitted during the following purification steps because it is not needed anymore or it interferes with the purification method.

Buffer and salt

The selection of buffer (see Appendix 1) and salts depends on several factors. The pH should be selected so that it matches the pH optimum of the target protein. The solubility of a protein is often lowest at its pI. To obtain good solubility of the target protein, select a pH that is not near the pI. The buffer substance should have a pK_a value as close as possible to the selected pH, to give maximum buffer capacity. A typical concentration of buffer substance is 20 to 100 mM. The pK_a of some buffer substances are highly dependent on temperature. For example, Tris buffer is notable with a dpK_a/dt of 0.028, which means that a solution prepared at 25°C to pH 7.4 will change to pH 8.0 if cooled to 4°C. Good buffers (2) were developed to give a minimum of side effects seen for traditional buffers. For example, phosphate may inhibit enzymes, and borate may react with mono- or oligosaccharides. Buffers may interact with metals ions, for example, phosphate precipitates with some divalent metal ions. However, phosphate buffer can be used for IMAC because the metal ions present are immobilized on the chromatography medium, thus are not available for precipitation. Good buffers are usually compatible with metal ions. Some buffers may interfere with protein determination assays. If a broad pH range using the same buffering system is desired, multiple buffer substances with different pK_a values can be mixed. Some selections can allow essentially constant ionic strength over the pH range (3). The activity and solubility of proteins vary with ionic strength. The optimum ionic strength should be tested early together with the pH optimum mentioned earlier.

Temperature

Sample preparation is often performed at low temperature for best stability, and samples are often kept on ice before application on the chromatography column, independent of whether chromatography is performed at room temperature or in the cold room.

Application of large chilled samples on a room-temperature column could lead to problems with air bubbles, which may give disturbances in the in-line detection after the column.

Stabilizing additives

There are a number of substances that can be added to the solutions used for protein purification in order to stabilize the target protein (Table 6.5).

Always consider whether the added substance may affect the chromatographic separation. In such cases it may be possible to avoid the additive or to remove it prior to the sensitive purification step.

Often the additive is required during sample preparation but can be omitted in the remainder of the purification protocol.

Chelating agents

Traces of metal ions may catalyze oxidation of free cysteines in proteins or interact with specific groups on the protein surface. Addition of ethylenediaminetetraacetic acid (EDTA) protects against oxidation by forming chelate complexes with divalent metal ions. The related ethylene glycol tetraacetic acid (EGTA) forms complexes specifically with Ca^{2+}. Chelate formation does not occur at low pH.

The presence of chelating agents may interfere with separation in IMAC and AIEX. Chelating agents should not be used for proteins that require divalent metal ions for their activity.

Table 6.5. Stabilizing additives

Substance	Concentration	Purpose
EDTA	10 mM	Reduce oxidation damage, chelate metal ions.
Sucrose or glucose	25 mM	Stabilize lysosomal membranes, reduce protease release.
Detergents	Typically 0.01%–1%	Reduce loss due to association with cell components or self-aggregation. Extraction and purification of integral membrane proteins. Solubilization of poorly soluble proteins.
Glycerol	5%–10%	For stabilization, up to 50% can be used if required.
Sucrose	10%	For stabilization.
Nucleases (DNase and RNase)	1–20 µg/ml each (or Benzonase™ according to the manufacturer's recommendations)	Degradation of nucleic acids, reduce viscosity of sample solution.

Glycerol or other polyols

Glycerol (5% to 50%) is very frequently used to stabilize proteins in solution. It is believed to enhance the structure and compactness of the protein and reduce interactions of hydrophobic patches of the protein, thus reducing the risk of aggregation. Other polyols (mannitol, sucrose, lactose, and propylene glycol) can be used at typically 10% (4).

Proteases inhibitors

Proteases from the source organism are the key threats to most proteins to be purified. Working quickly at low temperature reduces their action. Proteases should be removed as quickly as possible by an efficient capture step. In addition to these precautions, protease inhibitors (Table 6.6) are usually added during the extraction because this is the most dangerous step regarding proteolysis. Sometimes protease inhibitors should also be added during protein purification, for example, in chromatography eluents, or to samples collected after chromatography. In some cases a specific protease can be inactivated by selecting a pH where it is inactive (but where the target protein is active). Protease inhibitor cocktails are available from different manufacturers. Optionally, EDTA may be added to inhibit metalloproteases. Keep in mind that nucleases used for removal of nucleic acids during sample preparation requires Mg^{2+} ions, and will thus be inhibited by EDTA.

Use protease inhibitors and remove proteases as quickly as possible by an efficient capture step.

Table 6.6. Protease inhibitors[1]

Inhibitor	Concentration	Target	Comments
Phenylmethylsulfonyl fluoride (PMSF)[2]	0.5–1 mM	Inhibits serine proteases and some cysteine proteases	PMSF is an irreversible inhibitor that inactivates serine and some cysteine proteases. PMSF is rapidly inactivated in aqueous solutions. Prepare just prior to use. Less effective in the presence of thiol reagents. PMSF is very toxic.
AEBSF (aminoethyl benzylsulfonyl fluoride)	up to 4 mM	Inhibits serine proteases	More soluble and less toxic than PMSF. Induces modifications that can potentially alter the pI of a protein, cf., 2D-PAGE and mass spectrometry analysis.
4-Aminophenyl-methylsulfonyl fluoride (APMSF)	0.4–4 mM	Inhibits serine proteases	
Benzamidine-HCl	0.2 mM	Inhibits serine proteases	
Pepstatin	1 µM	Inhibits aspartic proteases	
Leupeptin	10–100 µM	Inhibits cysteine and serine proteases	
Chymostatin	10–100 µM	Inhibits chymotrypsin, papain, cysteine proteases	
Antipain-HCl	1–100 µM	Inhibits papain, cysteine and serine proteases	
EDTA	2–10 mM	Inhibits metal-dependent proteases, zinc, and iron	Inhibit nucleases by binding Mg^{2+} added to break down nucleic acids in viscous samples.
EGTA	2–10 mM	Inhibits metal-dependent proteases, e.g., calcium	Does not bind Mg^{2+}, thus does not inhibit nucleases.

[1] *Protease inhibitors are available in premade mixes from several suppliers.*

[2] *PMSF is a hazardous chemical. Half-life time in aqueous solution is 35 min. PMSF is usually stored as a 10 mM or 100 mM stock solution (1.74 or 17.4 mg/ml) in isopropanol at –20°C.*

Details taken from references 1 and 5, and other sources.

Detergents and chaotropic agents

Detergents are used in membrane protein purification, but can also be applied to decrease aggregation or adsorption for water-soluble proteins. During extraction, the target protein may be bound to filters or container surfaces, cell debris, to other proteins, or to itself via hydrophobic regions on its surface. This may cause significant reduction in recovery or cause aggregation of the target protein. The hydrophobic interactions can be reduced by the addition of mild detergents that interact with hydrophobic regions on the target protein and other sample components. Moderately hydrophobic substances such as ethylene glycol can also decrease hydrophobic interactions.

Nucleases

Release of nucleic acids upon cell disruption may lead to very high sample viscosity. The nucleic acids may also cause aggregation of cell debris. Nucleic acid impurities may hinder effective chromatographic purification by binding to the target protein or the chromatography medium.

Nucleic acids can be broken down by addition of nucleases. Viscosity problems due to nucleic acids are less problematic if multiple mechanical cell disruption cycles are run, although addition of nucleases may still be useful. Benzonase is an engineered nuclease that breaks down both RNA and DNA. The nuclease becomes much more efficient and can be used at a lower concentration if added before cell disruption.

Reducing agents

The redox potential is generally lower inside cells than in the surrounding medium or in solutions used for protein purification, because atmospheric oxygen is dissolved in solutions. Intracellular proteins may have free cysteine residues (free thiols) that are sensitive to these oxidizing environments. The cysteine residues in proteins may be problematic because they are susceptible to oxidation; disulfides, sulfinic acid (-SOOH), or sulfonic acid (-SO_2OH) may be formed. The oxidation may be caused by the presence of oxygen and traces of divalent metal ions. Reducing agents can be added to keep free cysteine residues on proteins reduced or to reduce disulfide bonds. Oxidation can be hindered by addition of EDTA and reducing agents.

The protein should not be stored in the presence of β-mercaptoethanol for too long a time, because the oxidized form of β-mercaptoethanol can react with a reduced cysteine to form a disulfide (5). β-mercaptoethanol can thus be used for purification, but for storage any of the other reducing agents are preferable because their oxidized forms are stable and will not react with free cysteines.

Reducing agents generally do not react with other functional groups on proteins. The DTT and DTE isomers behave similarly in protein solutions.

Table 6.7 Reducing agents

Reducing agent	Concentration	Stock solution	Comments
1,4 dithiothreitol (DTT) (Cleland's reagent) or alternatively 1,4 dithioerythritol (DTE)	1–10 mM	1 M in water, store at -20°C (1 year).	Only effective above pH 7. Susceptible to air oxidation. Oxidized form gives increase in absorbance at 280 nm.
β-mercaptoethanol (BME)	5–20 mM or 0.05%	1 M in water, store in dark at 4°C (1 year).	Susceptible to air oxidation, volatile, unstable in aqueous solution above pH 5, higher pH further decrease stability. Sensitive to presence of metal ions. Stabilize by adding EDTA.
Tris (2-carboxyethyl) phosphine (TCEP) (purchase as TCEP-HCl)	5–50 mM	0.5 M in water adjusted to suitable pH. Store at -20°C. TCEP dissolved in water is acidic. TCEP is less stable in phosphate buffers.	Stable to air oxidation. More efficient than DTT below pH 8. Does not affect metal ions during IMAC. Readily soluble, max. 1.1 M, stable in aqueous solution, stable in acidic and basic solution. Odorless, thiol-free, pH range 1.5–9. TCEP can reduce DTT.

Removal of specific impurities

In laboratory-scale purification, a practical approach is to purify the protein to a certain level and perform SDS-PAGE after storage to check for protease degradation. Information on the degree of purity and quantity of aggregates can also be obtained by analytical GF using Superdex 200 columns. For many applications, a high purity as judged by SDS-PAGE is sufficient, but occasionally, specific impurities negatively affect a purification step or the use of the purified protein, and the impurity will have to be removed.

Lipoproteins

Lipoproteins and lipids can rapidly clog chromatography columns and should be removed prior to purification. Precipitation agents such as dextran sulfate and polyvinylpyrrolidone, described under "Fractional precipitation," are recommended to remove high levels of lipoproteins from samples such as ascites fluid.

Low-molecular-weight contaminants

Some low-molecular-weight substances may interfere with purification. Phenol red is a low-molecular-weight pH indicator frequently used in cell cultures at laboratory scale. Although it does not directly interfere with purification, phenol red may bind to certain purification media; for example, it may bind to AIEX media at pH > 7. Use a desalting column to simultaneously remove phenol red and transfer the sample to the correct buffer conditions for further purification, as described in Chapter 3.

Remove interfering low-molecular-weight substances by desalting.

Fractional precipitation

Fractional precipitation is occasionally used at laboratory scale and in small-scale commercial production to remove gross impurities. The success of this potentially powerful procedure depends completely on the properties of the target protein in comparison with the contaminating proteins.

Increased salt concentration can enhance hydrophobic interaction between proteins. Differences in hydrophobicity result in a selective precipitation. Fractional precipitation can be applied in three different ways, as shown in Figure 6.3.

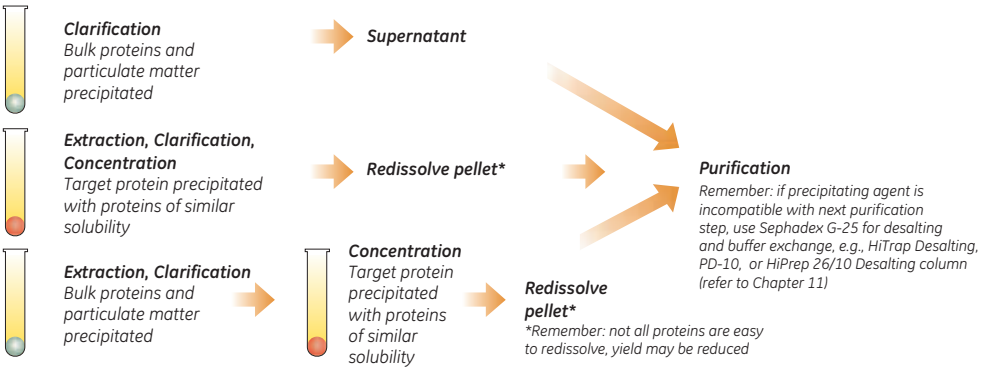

Fig 6.3. Three ways to use precipitation.

Precipitation methods are affected by temperature, pH, and sample concentration. These parameters must be controlled to ensure reproducible results. Examples of precipitation agents are reviewed in Table 6.8. The most common precipitation method using ammonium sulfate is described in more detail.

Table 6.8. Examples of precipitation methods

Precipitation agent	Typical conditions	Sample type	Comment
Ammonium sulfate	As described later in this chapter.	> 1 mg/ml proteins, especially immunoglobulins.	Stabilizes proteins, no denaturation. Supernatant can go directly to HIC. Helps to reduce lipid content.
Dextran sulfate	Add 0.04 ml of 10% dextran sulfate and 1 ml of 1 M $CaCl_2$ per ml of sample, mix 15 min, centrifuge at 10 000 × g, discard pellet.	Samples with high levels of lipoproteins, e.g., ascites.	Precipitates lipoproteins.
Polyvinylpyrrolidone	Add 3% (w/v), stir 4 h, centrifuge at 17 000 × g, discard pellet.	Samples with high levels of lipoprotein, e.g., ascites.	Alternative to dextran sulfate.
Polyethylene glycol (PEG, M_r > 4000)	Up to 20% (w/v).	Plasma proteins.	No denaturation, supernatant goes directly to IEX or AC. Complete removal of PEG may be difficult. Stabilizes proteins.
Acetone (cold)	Up to 80% (v/v) at 0°C. Collect pellet after centrifugation at full speed in chilled microcentrifuge.	Useful for peptide precipitation or concentration of sample for electrophoresis.	May denature protein irreversibly.
Polyethyleneimine	0.1% (w/v).		Precipitates aggregated nucleoproteins.
Protamine sulfate	1% (w/v).		Precipitates aggregated nucleoproteins.
Streptomycin sulfate	1% (w/v).		Precipitates nucleic acids.
Caprylic acid	1:15 (w/w).	Antibody concentration should be > 1 mg/ml.	Precipitates bulk of proteins from sera or ascites, leaving immunoglobulins in solution.

Details taken from references 1 and 5, and other sources.

Ammonium sulfate precipitation

Ammonium sulfate precipitation can be used for initial sample concentration and cleanup. When the concentration of the salt is increased, proteins will begin to "salt out," that is, precipitate. Different proteins are precipitated at different salt concentrations. Proper selection of ammonium sulfate concentration allows removal of selected impurities from the crude extract. Precipitation is often performed in two steps. In the first step, as many impurities as possible are precipitated, without precipitating the target protein. In the second step, the target protein is precipitated by further increase of the salt concentration but with the goal to co-precipitate as few other proteins as possible. If the target protein cannot be safely precipitated and redissolved, only the first step should be employed. HIC is often a suitable next purification step, because the sample already contains a high salt concentration and can be applied directly to the HIC column with little or no additional preparation. The elevated salt level enhances hydrophobic interactions between sample components and the chromatography medium; see Chapter 2. A protocol for ammonium sulfate precipitation at low temperature is given in Appendix 2.

Some proteins may be damaged by ammonium sulfate. Be careful when adding crystalline ammonium sulfate (use slow addition during rapid stirring): high local concentrations may cause contamination of the precipitate with unwanted proteins.

- It may be practical to use HIC as a second step after an initial ammonium sulfate precipitation.
- Many proteins are stabilized in high ammonium sulfate concentrations. Keeping the pellet overnight may be a suitable pause in the purification work.
- For routine, more reproducible purification, precipitation with ammonium sulfate should be avoided in favor of chromatography.
- Precipitation is rarely effective for protein concentrations below 1 mg/ml.

Thermal or pH precipitation

In rare cases, thermal precipitation of contaminants can be performed. Most proteins become inactive and precipitate upon heating, but there are exceptions. A small number of proteins from an organism may be extremely thermostable, as well as the bulk of proteins from thermophilic organisms. Purification of these proteins can be efficiently performed by incubation at 95°C. Most proteins in the extract will be precipitated and can be removed by centrifugation, while the target protein stays active in solution. The method is powerful when proteins from thermophiles have been expressed in *E. coli*. Similarly, fractional precipitation by pH adjustment can sometimes be used, often at pH extremes.

Extraction of membrane proteins

A membrane protein can be purified in the presence of detergent by applying essentially any of the existing protein purification methods 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.

Solubilization is crucial 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. An efficient solubilization dissociates most lipid-protein and protein-protein interactions, thereby allowing the separation of proteins. It is important to have detergents present throughout the entire purification procedure.

Detergents act by disintegrating the lipid bilayer with concomitant formation of detergent complexes with lipids and proteins. The hydrophobic surface areas of the membrane proteins become buried in the hydrophobic interior of the detergent micellar structures, while hydrophilic protein parts are in contact with the aqueous environment.

Some membrane proteins require 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.

- All buffers and solutions used for membrane protein preparations (for solubilization, purification, storage, etc.) should have a detergent concentration above the critical micelle concentration (CMC).

Some examples of detergents recommended for solubilization of membrane proteins are listed in Table 6.9.

For a more detailed description of how to purify membrane proteins, refer to *Purifying Challenging Proteins. Principles and Methods* from GE Healthcare (see also the "Related literature" section at the end of this handbook).

Table 6.9. Some recommended detergents suitable for solubilization of membrane proteins

Detergent	Class*	Fw	CMC† (mM)
Brij™35	N	1200	0.07
$C_{12}E_8$ (Octaethylene glycol monododecyl ether)‡	N	539	0.11
CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate)	Z	615	8
CYMAL-5 (Cyclohexyl-1-pentyl-β-D-maltoside)	N	495	3.5‡
CYMAL-7 (Cyclohexyl-n-heptyl-β-D-maltoside)	N	522	0.19
DM (n-Decyl-β-D-maltoside)	N	483	1.8
Digitonin	N	1229	< 0.5
DDM (n-Dodecyl-β-D-maltoside)	N	511	0.17
FOS12	Z	352	0.12
HECAMEG (6-O-(N-Heptylcarbamoyl)methyl-α-D-glucoside)	N	335	19.5
LDAO (Lauryldimethylamine oxide)	Z	229	1
Nonidet™ P40	N	615	0.25
NG (n-Nonyl-β-D-glucoside)	N	306	6.5
OG (n-Octyl-β-D-glucoside)	N	292	18
Tween™ 20	N	1228	0.06
Triton™ X-100	N	647	0.23

* *Classes: N, nonionic detergent, Z, zwitterionic detergent (both positive and negative charges)*

† *CMC, critical micellar concentration; values depend on conditions (here at 20°C–25°C and 50 mM Na^+)*

‡ *In water*

Extraction of inclusion body proteins

Expression conditions that yield insoluble protein are avoided in many laboratories because the protein, in addition to being insoluble, is usually in a non-native to and thus requires solubilization and refolding into its native structure. Protein refolding usually requires extensive optimization. If refolding is possible, there are several advantages to working with inclusion bodies: High expression levels are possible because production of target polypeptides can be faster and reach higher concentrations. Inclusion bodies often contain well above 50% target protein. The preparation of inclusion bodies can therefore give target protein of high purity. The polypeptides inside the inclusion bodies are well protected against proteolytic action. Inclusion bodies can be easily recovered by centrifugation. Inclusion bodies can be observed by light microscopy (Fig 6.4). Expression as inclusion bodies allows the preparation of large amounts of proteins that would otherwise be toxic to the host cells.

Fig 6.4. Light-microscopic image of *E. coli* with inclusion bodies.

Isolation of inclusion bodies

Inclusion body isolation starts with cell harvest by centrifugation. The cells are usually disrupted by mechanical lysis, for example, sonication. The inclusion bodies can then be washed in 1% Triton (or other detergent) and/or moderate concentrations of urea, multiple times using centrifugation (10 000 × g, 15 min at 4°C). Washing conditions may have to be optimized for each target protein. Target protein purities of more than 90% can often be obtained. The inclusion body preparation can be stored frozen.

Solubilization and denaturation

Protein refolding begins with complete solubilization of the washed inclusion bodies using denaturants such as guanidine hydrochloride and urea. It is recommended to include a reducing agent to reduce any S-S-bonds, because they may have formed erroneously between the denatured protein molecules. The protein may or may not be purified under denaturing conditions before refolding. Refolding is initiated by reduction or removal of the denaturant. Screening of multiple refolding conditions will usually be necessary. The screening work requires a reliable assay for concentration of native protein to allow monitoring of refolding success. Matrix-assisted refolding will briefly be addressed in Chapter 9.

Include a reducing agent during solubilization to break any S-S-bonds.

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Chapter 7
Purification formats

There are numerous ways to set up a protein purification experiment. The combination of hardware, chemicals, and the mode of operation is referred to as the purification format. The principal purification formats will be described in this chapter. They are based on chromatography in closed columns (operated by a chromatography system, a stand-alone pump, or sometimes a syringe); in open columns (e.g., gravity flow columns, spin columns, multiwell filter plates); or using batch adsorption (e.g., magnetic beads). Many other formats and hybrids have been described in the literature. The selection of purification format is governed by what the application demands (e.g., sample volume, purity, amount of purified protein required, presence of toxic components, number of samples to be processed) and what equipment and experience are available in the laboratory.

An affinity-tagged protein can often be sufficiently purified on a single column using a syringe, gravity flow, centrifugation, or vacuum, but very high purity usually requires multiple purification steps with high-resolution columns for the polishing purification steps. The higher pressure and narrow peaks generated by these columns require that the runs are handled by a chromatography system. An automated chromatography system provides a constant flow at intermediate or high pressure and allows automatic collection of narrow peaks. Several manual purification formats allow parallel purification of multiple proteins. Automated purification of multiple samples can be performed sequentially, and there are solutions also for automated multistep purifications.

Manual purification formats

Several column formats and methods are available for manual purification (Tables 7.1 and 7.2). A syringe or a stand-alone pump (for larger sample volumes) can be used for HiTrap columns (Fig 7.1). Gravity flow can be used on GraviTrap™ columns ("drop columns"). Centrifugation can be used for SpinTrap, GraviTrap, and MultiTrap formats. The MultiTrap 96-well filter plates can also be used with vacuum (except for PD MultiTrap G-25). Mag Sepharose magnetic beads are primarily used with magnetic apparatuses. Several of the above-mentioned formats can be used for desalting as described in Chapter 3.

Fig 7.1. Using HiTrap columns with a syringe. (A) Prepare buffers and sample. Remove the column's top cap and twist off the end. (B) Equilibrate the column, load the sample, and begin collecting fractions. (C) Wash and elute, continuing to collect fractions. This procedure is particularly suitable for purification of tagged proteins, because a single step is expected to give a high degree of purification.

Table 7.1. Manual purification applications

		Applications					Flow					
Column/plate/bead	**Maximum sample volume[1]**	**Preparative purification**	**Parallel purification**	**Minipreps**	**Screening**	**Multi-parallel screening**	**Syringe**	**Gravity**	**Centrifugation**	**Vacuum**	**Magnet**	**ÄKTA design chromatography**
HiTrap	Unlimited[2]	×			×		×					×
GraviTrap	35 ml	×	×		×			×	×			
SpinTrap	600 µl	×	×	×	×				×			
MicroSpin	150 µl	×	×	×	×				×			
MultiTrap	600 µl	×	×	×	×	×			×	×		
Mag Sepharose beads	Unlimited[3]	×	×	×	×	×					×	

[1] *Several applications of the same sample can be performed as long as the capacity of the column, plate, or beads is not exceeded. Larger sample volumes can thus be handled using the same format*

[2] *The sample volume is limited by binding capacity of the medium used and amount of binding protein in the sample*

[3] *The sample volume limit depends on the amount of beads used in batch adsorption, the binding capacity of the chromatography medium present, and the amount of binding protein in the sample*

Table 7.2. Options for manual, affinity-based purifications (for HiTrap columns see Table 2.2 in Chapter 2)

Applications	**Products**
Purification of immunoglobulins	
IgG, fragments, and subclasses	Protein A HP SpinTrap Protein A HP MultiTrap Protein A Mag Sepharose Protein A Mag Sepharose Xtra
IgG, fragments, and subclasses including human IgG_3, the strong affinity for monoclonal mouse IgG_1 and rat IgG. Immunoprecipitation and antibody purification.	Ab SpinTrap Protein G HP SpinTrap Protein G HP MultiTrap Protein G Mag Sepharose Protein G Mag Sepharose Xtra
Purification of recombinant proteins	
Histidine-tagged proteins	His GraviTrap His SpinTrap His MultiTrap HP His MultiTrap FF His Mag Sepharose Ni
GST-tagged proteins	GST GraviTrap GST SpinTrap GST MultiTrap FF GST MultiTrap 4B
Group-specific purification	
Biotinylated substances	Streptavidin HP SpinTrap Streptavidin HP MultiTrap
Phosphorylated proteins and peptides	Phos SpinTrap Fe (IMAC medium charged with Fe^{3+}) TiO_2 Mag Sepharose
Other	
Immunoprecipitation	Protein A Mag Sepharose, Protein G Mag Sepharose Immunoprecipitation Starter Pack (containing nProtein A Sepharose Fast Flow, and Protein G Sepharose Fast Flow)
Albumin and IgG depletion	Albumin and IgG Depletion SpinTrap
Protein immobilization, for various applications (e.g., pull-down experiments, immunoaffinity chromatography, immunodepletion)	NHS HP SpinTrap NHS Mag Sepharose

Batch adsorption is an alternative to column chromatography; the method is applied (or partly applied) in several of the manual formats, for example, MultiTrap plates and Mag Sepharose magnetic beads. Batch adsorption can also be performed using essentially any binding chromatography technique. The sample and chromatography beads are mixed with careful agitation during binding. The beads, except for Mag beads, are then transferred to an empty column for wash and elution. Mag Sepharose magnetic beads are separated from liquids using a magnetic apparatus. In the MultiTrap plate format, the sample is applied to the wells, which are prefilled with chromatography medium, and the plate is carefully shaken using a shaker during incubations. However, for several MultiTrap protocols, shaking is not required. Batch adsorption can often be used with unclarified samples. Generally, batch adsorption should be performed using longer contact times than when using packed columns. The time required for binding equilibrium depends on the method used (AC, IMAC, IEX, HIC, etc.) and can be from minutes to hours. Overnight incubation is sometimes used for convenience.

Prepacked columns and multiwell filter plates

Fig 7.2. HiTrap columns.

HiTrap 1 ml and 5 ml columns are available prepacked with a large number of different AC, IEX, desalting, and HIC media for clarified samples (Fig 7.2). For manual purification, HiTrap columns can be run using a syringe or a stand-alone pump but preferably using a chromatography system. HiTrap columns allow purification from micrograms to more than 200 mg of target protein with a single column. Columns can also be connected in series for scale-up. HisTrap FF crude is a specially designed column for purification of histidine-tagged proteins from unclarified lysates.

Fig 7.3. SpinTrap columns.

SpinTrap columns are prefilled spin columns (Fig 7.3) designed for protein minipreps using a microcentrifuge. Figure 7.4 shows the principles of purification using SpinTrap columns. Purification can be achieved starting from clarified or unclarified samples and in parallel. SpinTrap columns accept 600 µl samples, but multiple sample applications can be made to handle a larger sample. Up to 0.75 mg of histidine-tagged protein can be purified in 10 min per His SpinTrap column.

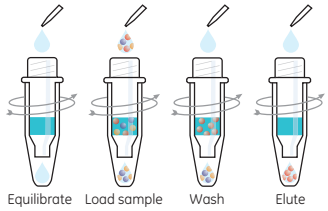


Fig 7.4. Principle of SpinTrap purification.

Fig 7.5. MicroSpin columns.

MicroSpin columns are available prepacked with a selection of media for buffer exchange/desalting and removal of low-molecular-weight components from proteins and nucleic acids. The columns are driven by centrifugation. (Fig 7.5). MicroSpin columns can accept 15 to 150 µl of sample depending on application.

Fig 7.6. GraviTrap columns

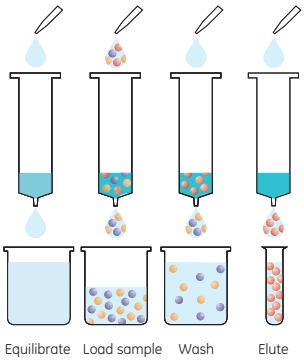


Fig 7.7. Principle of GraviTrap purification.

GraviTrap columns (Fig 7.6) are prepacked columns driven by gravity flow or centrifugation. These columns are designed to allow purification of clarified or unclarified samples, with a binding capacity of up to 20 mg for GST-tagged proteins and 35 mg for histidine-tagged proteins. Large sample volumes can be handled by repeated application of sample, before wash and elution. The ability to process unclarified samples saves time and manual work. The short purification protocol gives reduced loss of sample and minimized degradation and oxidation. Figure 7.7 shows the principles of purification using GraviTrap columns. The product packaging converts into a convenient column stand, enabling 10 samples or more to be processed in parallel by a single person.

Fig 7.8. MultiTrap filter plates.

MultiTrap 96-well filter plates are prefilled with chromatography medium (Fig 7.8). Purification can be performed starting with clarified or unclarified samples, in parallel using a robotic system or manually, with centrifugation or vacuum. MultiTrap plates are typically used for screening expression constructs, in solubility studies, for optimizing purification conditions, or for small-scale preparative purification of multiple target proteins. Each well accepts up to 600 µl of sample and can have a binding capacity of up to 1 mg for some applications. Scale-up can be performed using any of the columns packed with the same medium.

Fig 7.9. PreDictor plates.

PreDictor™ 96-well filter plates for high-throughput process development (Fig 7.9). The plates are prefilled with BioProcess™ chromatography media for IEX and AC.

PreDictor plates support high-throughput process development (HTPD) by allowing parallel screening of chromatography conditions for binding, wash, and elution. They can also be used for determining adsorption isotherms. PreDictor plates can be operated in either a manual or an automated workflow. Assist software supports the PreDictor workflow from set-up of experimental design to data evaluation.

Data generated using PreDictor plates show good correlation with data from chromatography columns, making the plates useful for initial screening of process conditions. PreDictor plates allow increasing process understanding through exploring an enlarged experimental space because of the efficient parallel format. Obtained information can then be used for further optimization using standard prepacked columns such as HiTrap or HiScreen.

Fig 7.10. Mag Beads.

Mag Sepharose beads are magnetic beads designed for simplified handling in the enrichment of target proteins or peptides, and small-scale purification/screening of antibodies or histidine-tagged proteins (Fig 7.10). Antibodies or other proteins can be immobilized on the Mag Sepharose beads via the protein A, protein G, or NHS ligands, for capture of target proteins using immunoprecipitation methods. The TiO_2 Mag Sepharose bead has direct affinity for phosphorylated peptides from digested protein samples. A magnetic apparatus for handling microcentrifuge tubes is available (Fig 7.11); alternatively, a PickPen™ can be used to transfer beads between the tubes. Much larger volumes can be handled with other magnetic apparatuses.

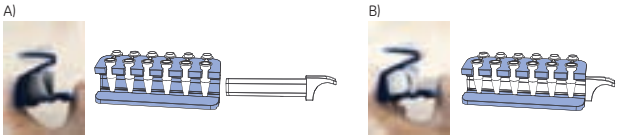


Fig 7.11. General magnetic separation steps. (A) Remove the magnet before adding liquid. The beads are dispersed into the liquid when the magnet is removed. (B) Insert the magnet before removing liquid. Mag Sepharose beads are rapidly drawn to the tube wall when exposed to a magnetic field.

Automated purification using chromatography systems

A chromatography system should be used when reproducible results are important and when manual purification becomes too time-consuming and inefficient. Systems provide more control than manual purification because of the ability to automatically control the flow rate and monitor the progress of the purification as well as to make controlled gradients and fraction collection. Systems can perform simple step-gradient elution as well as high-resolution separations using accurately controlled linear-gradient elution. Following is a description of the range of ÄKTA design chromatography systems suited to purification of proteins.

ÄKTA design chromatography systems

ÄKTA design chromatography systems are designed for protein purification ranges from micrograms to kilograms of target protein. ÄKTAcrossflow™ is a system for ultrafiltration using membranes for separation. All systems are controlled by UNICORN™ software, with the exception of ÄKTAprime plus, which is monitored by PrimeView™ software. UNICORN has the benefits of one common control platform and user interface for all scales of operation in chromatography and filtration. Research-scale ÄKTA design chromatography systems are briefly described on the following pages.

Table 7.3. Standard ÄKTA design configurations

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

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

ÄKTAprime plus ÄKTApurifier ÄKTAxpress (one module) ÄKTA avant ÄKTAmicro

Fig 7.12. The standard ÄKTA design configurations.

Fig 7.13. ÄKTAprime plus system.

ÄKTAprime plus is an economical and easy-to-learn system for the purification of proteins (Fig 7.13). With push button control, it offers simple one-step purification of proteins (Fig 7.14). This system includes preprogrammed methods for the purification of affinity-tagged proteins (histidine, GST, *Strep*-tag II, and MBP tags) and antibodies. There are preprogrammed methods for the use of any HiTrap column. The chromatography runs are monitored with PrimeView software. 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 7.14. Typical procedures using ÄKTAprime plus. (A) Prepare the buffers. (B) Connect the column. (C) Prepare the fraction collector. (D) Load the sample and press start.

Fig 7.15 ÄKTApurifier system.

ÄKTApurifier is designed for versatile FPLC purification of proteins and peptides (Fig 7.15). There are four core ÄKTApurifier systems that can be combined with ÄKTA design automation kits for a tailor-made solution. For purification of proteins at microgram and milligram scale, choose ÄKTApurifier 10 or UPC 10 core systems. Purification of larger, gram-scale quantities of protein is achieved with ÄKTApurifier 100 or UPC 100 systems.

Fig 7.16. ÄKTAxpress systems.

ÄKTAxpress is designed for unattended multistep purification of tagged proteins and antibodies (Fig 7.16). Up to 12 ÄKTAxpress systems can be controlled from one computer. The system allows parallel purification of up to 48 different samples. Due to its small footprint, two systems can fit in a cold cabinet.

The purification protocols consist of up to four purification steps. A typical four-step protocol begins with AC followed by desalting, IEX, and GF. In addition, automatic on-column or off-column tag-removal steps can be integrated in the purification protocols. Extended and automated washing procedures enable processing of a larger number of samples with minimal risk of cross-contamination.

Fig 7.17. ÄKTA avant 25 and ÄKTA avant 150 systems.

ÄKTA avant represents the new generation of ÄKTA systems (Fig 7.17). It incorporates cutting-edge functionality for achieving fast and secure protein purification. ÄKTA avant is available in two versions, with 25 and 150 ml/min pumps. ÄKTA avant 25 is designed for screening of media and method optimization in laboratory-scale purification. ÄKTA avant 150 is designed for scale-up and robustness testing.

ÄKTA avant together with UNICORN 6 contains several features to facilitate and automate the protein purification.

A Design of Experiments (DoE) software tool integrated in UNICORN 6 allows the maximum amount of information from a minimum number of experiments during method optimization.

BufferPro is an advanced online buffer preparation function that enables buffer mixing without manual interaction.

The built-in fraction collector provides security by cooling the purified samples and preventing dust from being introduced.

ÄKTA avant has a versatile valve configuration to facilitate the purification and increase reproducibility: up to eight samples can be automatically purified; the delta pressure over the column is monitored; five columns can be connected in parallel; and built-in air sensors prevent air from being introduced.

Fig 7.18. ÄKTAmicro system.

ÄKTAmicro is designed for micropreparative liquid chromatography applications and for rapid purity analysis in method development and protein characterization (Fig 7.18).

Microscale purifications can be performed starting with samples containing extremely small amounts of target protein using microbore to analytical-scale columns.

The highest possible sample recovery and stability are obtained when the complete flow path is manufactured from inert and biocompatible materials and assembled to give minimal band broadening. The pump design gives a flexible flow range with low pulsation and a broad pressure range, enabling high- as well as low-pressure separations.

Columns for ÄKTA design systems

High-quality column packing is essential for a good separation. A poorly packed column gives rise to poor and uneven flow, peak broadening, and loss of resolution. A wide variety of available columns are described in the next section, covering different principles, matrices, and sizes.

Sometimes there may still be a need for packing your own column. A brief guideline for column packing is given in Appendix 3. A range of empty columns available for packing the chromatography media of choice are described later in this chapter. See Table 7.4 for guidelines on how to combine media and columns.

Prepacked columns

Prepacked columns from GE Healthcare will ensure reproducible results and excellent performance.

Fig 7.19. RESOURCE columns.

RESOURCE columns are prepacked with SOURCE 15 media for IEX, HIC, and RPC. RESOURCE columns are made of PEEK (polyetheretherketone), which has high pressure tolerance and high chemical resistance (Fig 7.19). The RPC media are packed into steel columns. SOURCE media are based on a hydrophilic matrix made from monodispersed, rigid, polystyrene/divinyl benzene. There are SOURCE 15 media for IEX, HIC, and RPC. The media demonstrate very high chemical and physical stability. The small particle size allows fast binding and dissociation to facilitate high resolution, and the uniformity and stability of the particles ensure high flow rates at low backpressure.

Fig 7.20. Precision columns. Examples: Mini Q PC 3.2/3 (at left); and Superdex Peptide 3.2/30 (at right).

Precision columns are designed for micropurification and analysis of proteins and peptides (Fig 7.20). The columns are used extensively in peptide sequencing and protein structure/function studies. They are excellent for the polishing step of small-scale protein purification procedures and for purity check analysis. The small volume of the columns decreases the total area of the prepacked medium, which minimizes nonspecific adsorption and dilution effects. The column volumes have been scaled down 10-fold compared with Tricorn™ columns. Precision columns are available for GF (3.2 × 300 mm with Superdex Peptide, Superdex 75 and 200, and Superose™ 6 and 12 media) and IEX (1.6 × 50 mm with Mono Q and Mono S media and 3.2 × 30 mm with Mini Q™ and Mini S™ media). Precision columns require a special Precision column holder for use on ÄKTA design systems.

Fig 7.21. HiTrap prepacked columns.

HiTrap columns are convenient and reliable columns (1 or 5 ml) for fast and easy preparative purifications, either alone or connected in series (Fig 7.21). They are designed for use with a syringe, peristaltic pump, or chromatography system. There are HiTrap columns for a broad range of chromatography media: for AC, IMAC, IEX, desalting, and HIC purification methods, a range of Sephadex, Sepharose High Performance, Sepharose XL, Sepharose 4B, and Sepharose Fast Flow columns, as well as Capto, MabSelect, MabSelect Xtra, and MabSelect SuRe media. The HiTrap column inlet is molded with 1/16" female threads, and the outlet has 1/16" male threads for direct coupling to ÄKTA design systems without the need for extra connectors. HiTrap columns cannot be opened and repacked. See Table 2.2 for a list of available AC columns.

Fig 7.22. HiScreen columns.

HiScreen columns are part of the process development platform available from GE Healthcare (Fig 7.22). The columns are prepacked with a range of BioProcess chromatography media (for AC, IMAC, IEX, and HIC) and designed for parameter screening and method optimization. HiScreen columns have small bed volumes (4.7 ml) thus requiring low sample and buffer volumes. Process fluid velocities can be applied, because the 10 cm bed height gives enough residence time, and the results can then serve as the basis for linear process scale-up. If necessary, two columns can easily be connected in series to give a bed height of 20 cm. The small volume makes HiScreen columns suitable also for laboratory-scale purification.

Fig 7.23. HiPrep prepacked columns.

HiPrep prepacked columns are designed for convenient scale-up purification (Fig 7.23). HiPrep columns are available for GF, desalting, AC, IEX, and HIC in four different sizes, 20 ml, 53 ml, 120 ml, and 320 ml. HiPrep columns for GF are prepacked with Sephacryl High Resolution media, in 120 ml and 320 ml sizes. The column inlet and outlet is molded with 1/16" female threads for direct connection to ÄKTA design systems. HiPrep columns cannot be opened and repacked. See Table 2.2 for a list of available AC columns.

Fig 7.24. HiLoad columns.

HiLoad™ columns are prepacked with high-performance Superdex media for convenient and reliable GF (Fig 7.24). HiLoad columns are available in 120 ml and 320 ml formats prepacked with Superdex 30 prep grade, Superdex 75 prep grade, and Superdex 200 prep grade to cover a wide range of separation of proteins of different molecular weights.

Fig 7.25. Tricorn columns.

Tricorn high-performance columns are designed for high-resolution protein purification at laboratory scale, making them an excellent choice for the polishing step in multi-step purification protocols. Tricorn columns are available with a range of chromatography media for GF (Superose, Superdex), IEX (Mono Q, Mono S, SOURCE 15Q, and SOURCE 15S), chromatofocusing (Mono P), and HIC (SOURCE 15PHE).

The columns are simple to use, with specially designed fittings for easy connection to ÄKTA design and other high-performance LC systems. The columns are coated with a protective plastic film that protects the column and provides personal safety in the event of breakage. Tricorn columns are also available empty for packing with a chromatography medium of choice (see below).

Empty columns

To obtain a column with high-quality packing and that can resist the pressure caused by the pressure drop across the selected chromatography bed, select the appropriate empty column based on the guidelines given in Table 7.4. During packing, follow the instructions supplied with the medium and empty column.

Fig 7.26. Tricorn columns.

Tricorn columns are designed for high-performance chromatography media such as Sepharose High Performance, Superdex prep grade, and SOURCE (Fig 7.26). When working with capture media such as Capto, MabSelect, or Sepharose Fast Flow, a Tricorn Coarse Filter Kit is recommended to use for reducing the risk of clogging. Tricorn columns are available with an inner diameter of 5 mm with lengths of 20, 50, 100, 150, and 200 mm, with an inner diameter of 10 mm and in lengths of 20, 50, 100, 150, 200, 300, and 600 mm. The maximum pressure is 100 bar for the 5 mm (i. d.) column and 50 bar for the 10 mm (i. d.) column.

Fig 7.27. XK columns.

XK columns are specified to run most chromatography media including Superdex prep grade and Sepharose High Performance (Fig 7.27). They are jacketed and available as 16, 26, and 50 mm (i.d.) columns (XK16, XK26, and XK50) with lengths from 20 to 100 cm. The maximum pressure is 5 bar for XK 16 and XK 26 columns and 3 bar for XK 50 columns. XK columns prepacked with medium go under the name HiLoad.

Fig 7.28. HiScale columns.

HiScale™ columns are designed for preparative laboratory-scale purification and for process development using standard chromatography media (Fig 7.28). HiScale columns are available with inner diameters of 16, 26, and 50 mm and lengths of up to 20 or 40 cm. The maximum pressure is 20 bar. The QuickLock mechanism of the adapter shaft facilitates rapid and easy movement of the adapter, simplifying adjustments as well as disassembly and cleaning. Turning the column end caps enables controlled axial compression of the gel bed, which is suitable during packing of rigid media.

Table 7.4 Empty column and chromatography media guide

	Recommended column		
Loose media	**Tricorn**	**XK**	**HiScale**
Gel filtration			
Sephadex	•	•	o
Sepharose	•	•	o
Sephacryl	•	•	o
Superdex prep grade	•	•	o
Superose prep grade	•	•	o
Ion exchange			
Capto	•	•	•
Sepharose Fast Flow	•	•	•
Sepharose High Performance	•	•[1]	•
Sepharose XL	•	•	•
SOURCE	•	•	•
MacroCap™	•	•	•
Affinity			
Sepharose 6B/4B/CL-4B	•	•	–
Sepharose Fast Flow	•	•	–
Sepharose High Performance	•	•[1]	–
MabSelect	•	•[1]	•
Reversed phase			
SOURCE	•	–	–
Hydrophobic interaction			
Sepharose Fast Flow	•	•	•
Sepharose High Performance	•	•[1]	•
SOURCE	•	–	–
Systems			
ÄKTAmicro	•	–	–
ÄKTAprime plus	•	•	–
ÄKTApurifier 10	•	•	–
ÄKTApurifier 100	•	•	•
ÄKTA avant 25	•	•	•
ÄKTA avant 150	–	•	•
ÄKTAxpress	o[2]	o[2]	–

- • *Recommended combination*
- o *Can technically be used, but not an optimal combination*
- – *Not recommended or not applicable*
- [1] *Not recommended for XK 50*
- [2] *For optimal performance use prepacked columns where purification parameters are predefined*

For more information visit www.gelifesciences.com/protein-purification or www.gelifesciences.com/bioprocess or www.gelifesciences.com/purification_techsupport

Purification from unclarified samples

HiTrap FF crude column has been designed for purification of histidine-tagged proteins from unclarified samples, thus eliminating the time and manual labor required for sample clarification; see Figure 7.29. SpinTrap and GraviTrap columns, as well as MultiTrap plates can also be used with unclarified samples. For GraviTrap, unclarified samples may reduce the flow when run using gravity, thus potentially increasing the purification time.

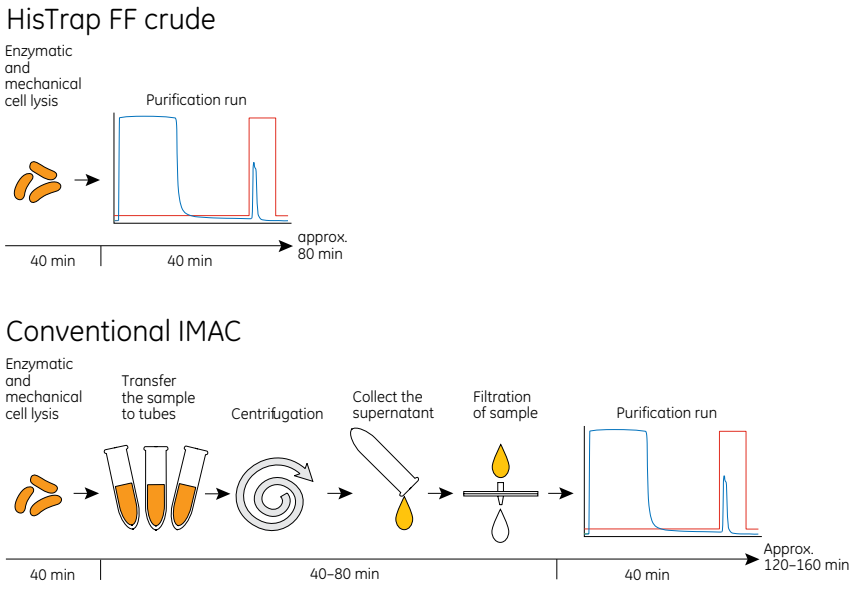


Fig 7.29. HisTrap FF crude columns save time during purification of histidine-tagged proteins compared with conventional IMAC.

Scale-up

In laboratory-scale purification, scale-up can usually be achieved by increasing the column volume with the same factor as the sample volume is increased. For example, purification of a histidine-tagged protein on HisTrap 1 ml can easily be scaled up five times on HisTrap 5 ml or 20 times on a HisPrep FF 16/10 (20 ml) prepacked column; see Figure 7.30. The five-fold scale-up step was performed using a column with the same length as the first column, whereas the 20-times scale-up was performed using a column that was both larger in diameter and longer.

When further increasing the scale of purification, it is important to keep the sample residence time (time on column) constant. This is often done by keeping the column length constant and increasing the column cross-sectional area in proportion to sample volume, while keeping the linear flow (cm/hour) constant. An elution gradient volume should be increased in proportion to the increase in column volume. For AC, IEX, and HIC, columns are often 10 to 20 cm in length independent of size. GF columns are rarely longer than 100 cm.

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

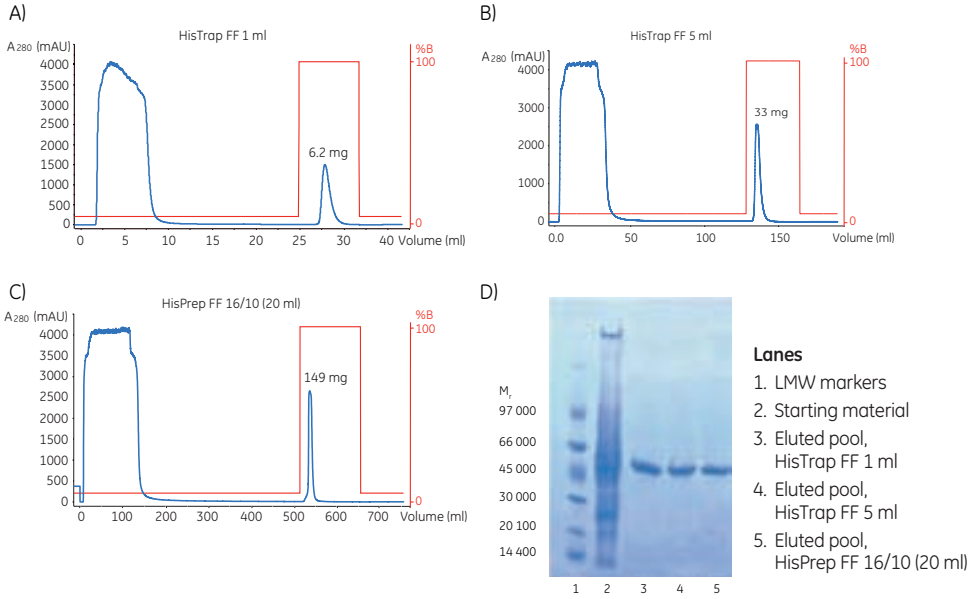


Fig 7.30. Scale-up of purification of a histidine-tagged protein from (A) HisTrap FF 1 ml via (B) HisTrap FF 5 ml to (C) HisPrep 16/10 (20 ml) prepacked column. The samples loaded contained approximately 8, 40, and 160 mg of MBP-$(His)_6$, respectively. Yield in milligrams is shown in each chromatogram. (D) SDS-PAGE (ExcelGel™ SDS Gradient 8–18) under nonreducing conditions confirms that scaling up from the 1 ml to the 20 ml column does not significantly affect the purification result.

Chapter 8
Analysis and characterization

Analytical assays are essential to follow the progress of purification. Initially, they are needed for method development for expression, sample preparation, and the different purification steps, and later for analysis of the outcome of the different steps in the protein production workflow. Finally, analyses are performed to determine the quality of the final preparation. Analytical data for the different purification steps are often compiled into a purification table (see description in Chapter 5 and Table 5.3), to allow evaluation of the purification protocol. After purification, characterization of the target protein is possible using a broad range of chemical and physical analysis methods. This chapter addresses some of the most common and convenient analysis methods used in protein purification. For further reading, refer to literature on the subject of interest.

Development of a purification protocol is facilitated by analytical information such as the identity of the protein obtained and the concentration of target protein, total protein, and specific impurities. The required information and the possibility of obtaining it depends on where one is in the purification workflow; see Table 8.1. It is important that the identity of the expressed and purified protein is established early, preferably directly after expression. If the protein has a tag for detection or affinity purification, the detection of the tag is a rather strong proof that the target protein is present. If the tag is placed on the C terminus of the protein, thus requiring full-length target protein translation to obtain the tag moiety, its presence is indicative of a complete target protein. It is good practice to secure the protein identity of the final preparation after purification to avoid wasting time studying the wrong protein.

During method development, there are several parameters that should be optimized such as yield, purity, and activity. Measurement of total protein concentration, target protein concentration, activity, and purity are usually needed for protein purification development and for the final quality check of the preparation. Analysis of size homogeneity, that is, determination of the association state of the target protein, is often important. The absence of aggregates is a requirement for many applications. Aggregates may have formed during expression, but can also be a result of protein instability that led to aggregation during purification. Some purifications require the absence or a limited concentration of a specific impurity. In such cases, an analysis protocol should be established to measure the concentration of the substance. The quality of the final protein preparation is usually determined using the same methods as for the development of the purification protocol. Once the protein is pure, additional chemical and physical analyses are available that can be used to further characterize the target protein.

Table 8.1. Protein purification workflow and analytical information

Analytical information	Expression	Sample preparation	Purification	Use/Storage	Comments
	Workflow →				
Protein identity	less common			typical use	Early check during expression and of final preparation. Detection of affinity tag is a strong proof of presence of target, but the true identity should be checked because the tag may be on the wrong protein.
Target protein concentration	typical use	typical use	typical use	typical use	For calculation of yield or recovery.
Total protein concentration	typical use	typical use	typical use	typical use	
Activity	typical use	typical use	typical use	typical use	Detection of target protein fractions during purification. Often important that the purified protein is fully native.
Purity	less common	typical use	typical use	typical use	
Concentration of specific impurities			typical use	typical use	For protein method development.
Size homogeneity	less common		typical use	typical use	Detect target protein truncations, oligomers, or aggregates.
Other homogeneity issues			less common	typical use	Analysis of post-translational modifications, or hetereogeneities thereof. Analysis of chemical modifications.
Protein stability	less common	typical use	typical use	typical use	Protein stability may be important both during purification and during subsequent use of the purified protein.

typical use
less common

Target protein identity and quantitation

The ultimate identity check of the purified protein is digestion (trypsinization) and ESI-MS analysis followed by matching against a protein sequence database. Other indirect means of checking the identity of the target protein, for example, immunodetection methods, can also be used. Some of these methods can also be used for quantitation.

Pure target protein, as in the final preparation, can be quantitated using any of the protein determination methods given later in this chapter. During development of methods for expression, sample preparation, or purification, samples to be analyzed have different degrees of complexity. The analytical methods are differently suited to function with such samples. The target protein concentration can be measured or estimated by several methods:

- Density of target protein band in SDS-PAGE
- "Color" (absorbance at specific wavelength) or fluorescence of target protein, detection tag, or affinity tag
- Immunodetection of target protein or tags; Western blotting, ELISA, immunoaffinity chromatography
- AC directed toward the target protein or the tag, followed by a simple quantitation method, for example, SDS-PAGE, absorbance, or protein determination

- Area of peaks in the absorbance at 280 nm in chromatograms
- Functional assays for the target protein (enzyme activity, receptor binding, substrate transport, etc.)

Density of target protein band in SDS-PAGE

If the target protein concentration is high enough compared with other components of the sample, it can often be sufficiently quantitated by SDS-PAGE, at least in relative estimates. A pure protein with known concentration, for example, BSA, can be included in the SDS-PAGE run and used to prepare a calibration curve. Quantitation by SDS-PAGE is often used as a simple estimation of the (relative) concentration after expression, where the presence of a new protein band of expected apparent M_r after induction is a strong indication of successful expression. As previously mentioned, enrichment of the target protein may be needed to give clear SDS-PAGE results.

Quantitation by chromatography peak integration

During purification of affinity-tagged proteins, the chromatogram obtained by the in-line UV detector can be used for quantitation. A rough estimation of the quantity of target protein can often be performed simply by integrating the target protein peak, assuming that the target protein constitutes the major part of the material corresponding to the peak. This is often the case after AC purification. The amount of target protein is estimated by determining the area of the eluted absorbance peak detected at 280 nm and using the extinction coefficient for the target protein. The extinction coefficient can be theoretically calculated from the amino acid composition of the protein, if known. Such calculations are often made using any trusted program on the Web but can easily be set up in a spreadsheet. In polishing steps, the major peak eluted during IEX, HIC, or GF can often be assumed to be relatively pure target protein and can thus be used for estimation of quantity. Note that peaks higher than approximately 1500 mAU cannot be used, because the UV absorbance detected (an instrument limitation) is not linear with concentration, and correction for this cannot be made accurately.

Alternatively, the fraction corresponding to the peak can be collected, and the total protein concentration can be determined by absorbance measurement or any other total protein determination method (see below).

Obvious weaknesses of these methods are that if the purity of the eluted peak is low, the quantity is overestimated, and if the biggest peak in a polishing purification chromatogram is an impurity rather than the target protein, the estimate is clearly not valid. For overexpressed proteins, the method generally works well. SDS-PAGE can be used to verify purity of the analyzed peak.

Detection of specific absorbance or fluorescence

The UV/visible spectrum can sometimes be unique for a specific protein, in particular if light-absorbing cofactors (flavins, heme groups, other metal ions, etc.) are present. The spectrum can be used for analyzing whether the protein is intact with respect to cofactors, coenzymes, or subunits.

In some cases, the protein or a detection or affinity tag on the protein has absorption or fluorescence properties that differ from the bulk of the sample components. Even in complex samples, these proteins can often be detected and even quantitated with sufficient accuracy by absorbance or fluorescence measurements at specific wavelengths. ÄKTA design chromatography systems can measure absorbance at multiple wavelengths; the standard absorbance measurement at 280 nm (giving approximate total protein concentration) can thus be supplemented with detection at an additional wavelength specific for the target protein. Figure 8.1 gives an example of the detection of green fluorescent protein (GFP) in an IEX purification run. The GFP variant used in this case has an absorbance maximum at 490 nm.

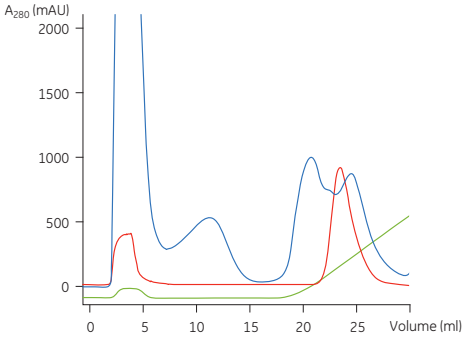


Fig 8.1. The principle of detection of the target protein by absorbance at a specific wavelength. An *E. coli* extract with a histidine-tagged GFP was applied on a Capto Q column (5 × 100 mm). The histidine-tagged GFP was specifically detected at 490 nm. Blue UV trace, absorbance at 280 nm; red, absorbance at 490 nm; green, conductivity.

Immunodetection

Immunoassays (e.g., Western blot, ELISA, immunoprecipitation, surface plasmon resonance [SPR]) can be used for quantitation if a suitable standard curve can be produced. In this case, it is not necessary to purify the tagged protein as long as a purified standard is available and the specificity of detection is sufficient. Immunoassay methods such as dot blot analysis and immunoprecipitation are often suitable for screening large numbers of samples when a simple yes/no answer is required, for example, when testing fractions from a chromatography run.

ELISA

ELISA is a frequently used immunoassay that allows the handling of many samples in parallel. The specificity depends on the antibodies used. Note that inactive target proteins may also be recognized. The GST 96-Well Detection Module from GE Healthcare provides a highly sensitive kit for ELISA for testing clarified lysates and intermediate purification fractions for the presence of GST-tagged proteins (Fig 8.2). Samples are applied directly into the wells of the plates, and GST-tagged proteins are captured by specific binding to anti-GST antibody that is immobilized on the walls of each well. After removal of unbound material by washing, the captured GST-tagged proteins are then detected with HRP/Anti-GST conjugate provided in the module. Standard curves for quantitation of tagged proteins can be constructed using purified recombinant GST, which is included as a control.

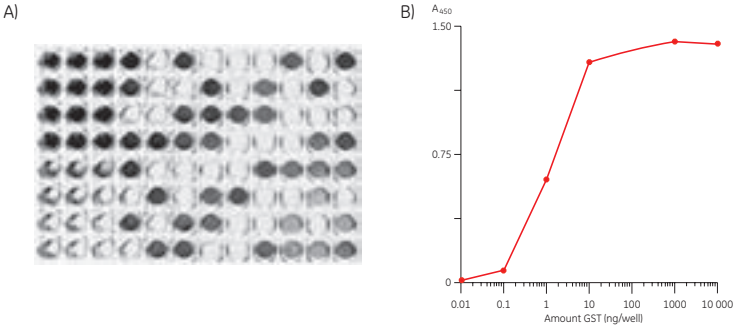


Fig 8.2. (A) Screening of bacterial lysates for GST-tagged protein expression using the GST 96-Well Detection Module. (B) Sensitive detection of recombinant GST using the GST 96-Well Detection Module.

Each tagged protein is captured uniquely. Because of this, a standard curve based on pure target protein of known concentration will give the best accuracy. Recombinant GST protein should be included as a standard control in every assay.

Surface Plasmon Resonance (SPR)

SPR analysis using Biacore™ instruments can be used to measure the concentration of a specific protein in a mixture. The measurement requires a suitable binding partner, usually an antibody directed toward the protein to be analyzed. See more about SPR later in this chapter.

AC

AC based on the affinity tag on the target protein can be used for enrichment before simple quantitation by SDS-PAGE. The specific enrichment of the target protein decreases the sample complexity and increases the thickness of its band in SDS-PAGE, so that specific detection may not be necessary. This method can be used when there is no antibody directed toward the target protein. The method has the advantage that a set of constructs of a specific target protein or a set of different target proteins can be generically quantitated or semi-quantitated, provided that they are tagged with the same affinity tag. The affinity purification can be performed in parallel using, for example, SpinTrap columns or MultiTrap plates; see Chapter 7. The example in Figure 8.3 shows quantitation of different truncated variants of a kinase using SDS-PAGE analysis after affinity enrichment on His MultiTrap HP. Quantitation, or relative quantitation from the gel, is performed by eye or by densitometric scanning, using BSA standards.

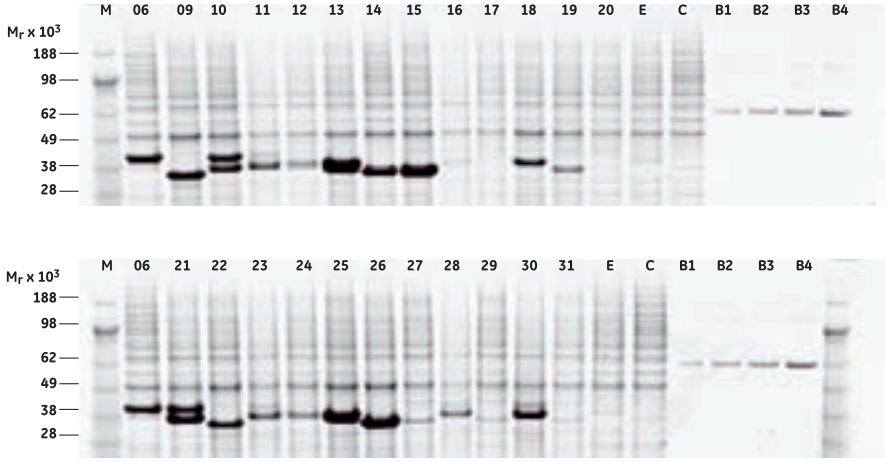


Fig 8.3. SDS-PAGE analysis of His MultiTrap HP enriched histidine-tagged truncated variants of kinase expressed in a transient insect cell system for 72 h. Lane M: marker; 06 to 31, kinase variants; E, enhanced green fluorescent protein (untagged, transfection-positive control); C, non-transfected Hi5 cells; B1 to B4, 0.5, 0.75, 1.0, and 1.5 µg BSA, respectively.

Total protein concentration

Absorbance

Measurement of UV absorbance at 280 nm is probably the most common method for determining protein concentration because it is fast, convenient, and nondestructive. Absorbance measurement can be made in 1 or 3 ml quartz or single-use plastic (low-UV-absorption type) cuvettes. For a large number of samples and if only moderate precision is required, a 96-well format can be used. It should be noted that not all proteins absorb at 280 nm. For these proteins the absorbance from the peptide bonds of the polypeptide can be used. Measurements can be made at 205 nm. A drawback with using the lower wavelength is the increased sensitivity to light scattering effects. For tips and hints on protein determination using absorbance measurements, see Appendix 4.

Protein concentration assays

Various protein determination methods are frequently used to determine the total protein concentration. These assays are destructive to the sample aliquots analyzed. The protein reacts with a reagent to generate a chromophore to be detected by absorbance measurement or a fluorescent species for fluorescence detection. An overview of common protein determination methods is given in Table 8.2.

Table 8.2. Comparison of common protein determination methods

		Compatible with			
Assay method	**Useful range (µg/ml)**	**Detergents**	**Reducing agents**	**Chelating agents**	**Comments**
NanoOrange™ assay (fluorescent when bound to protein)	0.01 to 10	No	Yes	Yes	Fluorescence detection (excitation: 470 nm, emission: 570 nm). Samples can be read for up to 6 h. Low protein-to-protein variability. No interference from nucleic acid.
FluoroProfile™ (fluorescent when bound to protein)	0.04 to 600	(Yes)	(Yes)	Yes	Fluorescence detection (excitation: 510 nm, emission: 620 nm). Low protein-to-protein variability. No interference from nucleic acid. Stable for 6 h. No interference from moderate concentration of detergents or reducing agents.
Bicinchoninic acid assay (BCA assay) (reduction of Cu^{2+}, detect complex of Cu^{+} and bicinchoninic acid)	20 to 2000	Yes	No	No	Absorbance detection at 562 nm. Low protein-to-protein variability. Samples must be read within 10 min. Temperature sensitive.
Pierce™ BCA Assay – Reducing Agent Compatible	125 to 2000	Yes	Yes	Yes	Compatible with 5 mM DTE, 35 mM β-mercaptoethanol, or 10 mM TCEP.
Bradford assay (detect complexes with Coomassie Brilliant Blue G-250)	1 to 1500	(No)	Yes	Yes	Absorbance detection at 595 nm. Protein precipitates over time. High protein-to-protein signal variability. Interference from high buffer concentration. Stable reagents (2 mo).
Pierce 660 nm assay (detect dye-metal-protein complex)	25 to 2000	Yes	Yes	Yes	Absorbance detection at 660 nm. Reaches stable end-point. Moderate protein-to-protein variability.
Lowry assay (reduction of Cu^{2+}, detect complex of Cu^{+} and Folin-Ciocalteu reagent)	1 to 1500	No	No	No	Multistep procedure. More accurate than Bradford assay. Absorbance detection at 750 nm. Reaches a stable end-point. Moderate protein-to-protein variability. Interference from carbohydrates and some buffers.
Absorbance	0.05 to 2000	Yes	Yes	Yes	Usually absorbance at 280 nm, other wavelengths possible. Detection influenced by nucleic acids and other UV-absorbing contaminants, and by light scattering from particles in the sample.

Protein determination by amino acid analysis

Amino acid analysis (AAA) is often regarded as the most accurate method for determining protein concentration. Because of the difficulties associated with obtaining reliable AAA, for most laboratories it is best to purchase the service from a dedicated facility. AAA can give relative amino acid composition of a pure protein and can be used for purity analysis of recombinant proteins by comparing the composition of amino acids obtained by analysis with the known composition. The measured content of some amino acid residues tends to be lower than the true value. The presence of groups of aliphatic amino acids, for example, Val-Val, Ile-Ile, or Val-Ile, may give underestimations of the amounts of these amino acids. Note the following information on amino acid degradation for some specific amino acids: Glutamine and asparagine convert into glutamic acid and aspartic acid, respectively. Threonine and serine are lost (5%-10% loss). Tryptophan is not recovered in standard AAA (acidic hydrolysis method). Methionine may oxidize to methionine sulfoxide, but this can be minimized by addition of β-mercaptoethanol. Some common buffers and additives may affect analysis; see Table 8.3. Various protectants can be used to minimize loss of amino acids during hydrolysis.

Table 8.3. Examples of compatibility of amino acid analysis with common buffers and additives

Substance	Compatible	Comments
Acetate	Yes	
HEPES	Yes	
Imidazole	Yes	High concentration with caution
Phosphate buffer	(Yes)	Not with low-concentration sample
Ammonium hydrogen carbonate	(Yes)	After freeze-drying
Ammonium acetate	(Yes)	After freeze-drying
Tris	(Yes)	Low Glu value
Glycine	(Yes)	With caution; not Gly
Ethylene glycol	(Yes)	< 5%
Glycerol	(Yes)	< 5%
Acetonitrile	No	
Guanidine hydrochloride	No	
Dioxane	No	

Source: Adapted from information kindly supplied by Dr. Anna Törnsten, Amino Acid Analysis Laboratory in the Department of Biochemistry, Uppsala University, Sweden. Valid for amino acid analysis based on IEX analysis after hydrolysis.

For samples with proteins of unknown amino acid composition, the amino acid content in the sample for AAA can be used to calculate the protein concentration, while accepting a possible slight underestimation because not all amino acids from the protein are recovered, as mentioned earlier. Calculate the mass (m_a) in µg of each recovered amino acid by the formula:

$$m_a = n \times M_{w,a}/1000$$

where n is the amount of amino acid in nmol, and $M_{w,a}$ is the average molecular weight for the amino acid corrected for loss of a water molecule during peptide formation. The sum of the masses of the recovered amino acids gives the mass of protein in the sample and can be used for calculation of the concentration in the sample analyzed.

For samples with a protein of known amino acid composition, the calculation of protein concentration from the amino acid amounts can be made on amino acids that are generally well recovered in AAA. For each Ala, Asx (Asn+Asp), Gly, Glx (Gln+Glu), Leu, and Lys that are well recovered, calculate the protein mass (m_p) in µg according to the formula:

$$m_p = n \times M_{w,p}/(b \times 1000)$$

where n is the amount of amino acid in nmol, $M_{w,p}$ is the average molecular weight of the protein, and b is the number of residues for the amino acid in the protein. Calculate the average protein mass and the variation from the mean. Consider discarding data for amino acids that deviate more than 5% from the mean. The selection of well-recovered amino acids can be modified based on experience with each target protein.

Concentration determination of membrane proteins

The BCA assay can be recommended for determining the concentration of membrane proteins. Other methods are often not compatible with detergents required to keep membrane proteins in solution. Modifications of the Bradford assay have been used. Absorbance at 280 nm can be used for sufficiently pure samples, but corrections for light scattering from detergent micelles may be required. AAA (see above) can be used for quantitation of membrane proteins. Calculation based on amino acids that are well-recovered in AAA is recommended for membrane proteins when possible.

Activity

During protein protocol development, it is very useful to have a method for assessment of protein activity. This can be the catalytic activity of an enzyme, ligand binding of a receptor, or transport activity of a membrane transport protein. By measuring the activity of a sample with known total protein concentration, a specific activity can be calculated. This is a good measure of purity. By performing such measurement on samples from different purification steps, the purification factor can be calculated. The data are often used to prepare a purification table; see Chapter 5. Activity data can be used for identification of chromatography fractions containing the target protein; see Figure 8.4. Some aspects of activity measurements are briefly given below. For further reading, refer to literature on the subject.

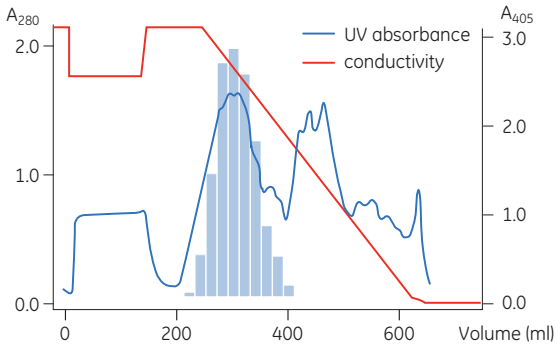


Fig 8.4. The use of target protein activity data (A_{405}) for identification of what fractions are to be collected. A tool in the Unicorn software has been used to include the fraction-wise determined activity figures (A_{405} nm) in the chromatogram.

Enzyme assays

Enzyme assays are usually unique to the enzyme in question and may be performed according to established literature procedures, but must sometimes be developed *de novo*. Assay protocols are often provided together with commercial substrates. It is important to ascertain that no interfering substances are present in the sample solution. It is sometimes necessary to perform a buffer exchange of each sample before analysis to remove interfering substances and obtain suitable conditions for the assay; see Chapter 3.

Surface plasmon resonance (SPR)

The SPR technology from Biacore is a label-free technology for monitoring molecular interactions as they happen. SPR can be used for assessing binding activity but is possibly more common as a characterization method. The SPR phenomenon occurs when polarized light, under conditions of total internal reflection, strikes an electrically conducting gold layer at the interface between media of different refractive index: the glass of a sensor surface (high refractive index) and a buffer (low refractive index).

Concentration of active/native protein is determined by monitoring the interaction of a molecule with a prepared sensor surface in the presence of a target molecule in solution (solution inhibition) or excess analyte (surface competition). Concentrations down to nanomolar range can be determined for purified molecules or for molecules in complex mixtures such as serum or food samples.

Purity

Purity can be defined as the sole presence of the target protein, that is, with no unwanted substances from the starting material or contaminants that may have been accidentally added during purification. Target protein homogeneity is defined later in this chapter.

SDS-PAGE

SDS-PAGE analysis is the dominant protein purification analysis tool, because of its simplicity and generic ability to separate and detect proteins. The method is an almost mandatory analytical procedure for protein purity control. It is used for monitoring the progress of a purification procedure (Fig 8.5) and for the identification of fractions containing the target protein.

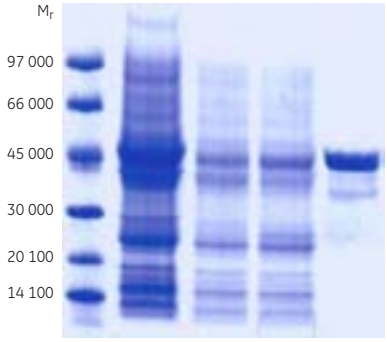


Fig 8.5. Typical results from SDS-PAGE analysis of samples collected during purification.

SDS-PAGE is based on the molecular sieving of SDS-protein complexes in the sample, migrating through a polyacrylamide gel. Samples are prepared by heating the sample in the presence of SDS to 95°C. Disulfide bonds of proteins may or may not be reduced using a reducing agent, for example, dithioerythritol (DTE) or β-mercaptoethanol. Reduction of disulfide bonds between subunits will allow their separation and thus give a pattern different from a nonreduced sample. For reduced samples, a single band in SDS-PAGE is indicative of a pure, single-subunit protein. Analysis of a protein complex or an oligomeric protein will give one band for each subunit provided that the subunits have different sizes.

It is possible that a single band can correspond to more than one protein, that is, a single sharp band in SDS-PAGE is not an absolute proof of complete purity. Further proof of purity is required, for example, amino acid composition, N-terminal sequence analysis, or MS.

Often both reduced and nonreduced samples are prepared for SDS-PAGE. This allows identification of disulfide bonds within the protein or between proteins. Disulfide bonds between proteins may cause aggregation. When purifying proteins with more than one subunit, it is recommended to also analyze size and size heterogeneity using GF. GF is performed under

native conditions, which means that intact proteins, protein complexes, or aggregates are separated according to size. GF may reveal incomplete protein complexes, lacking one or more subunits. Combining SDS-PAGE and GF can be powerful tools for quality control.

It may be useful to keep in mind that even though SDS-PAGE is a generic method that can be used for purity analysis, there are several potential problems that may occur:

- Too high an ionic strength destroys separation. Reduce by sample dilution or desalting.
- Proteins containing Asp-Pro peptide bonds (DP bonds) can be cleaved if at high temperature for too long or under mildly acidic conditions. The cleavage results in additional protein bands. Note that samples for SDS-PAGE containing Tris buffer will be acidified when heated, due to the large decrease of pK_a for Tris when heated. This will further increase the risk of DP bond cleavage. If DP bond cleavage is suspected, prepare sample without boiling, but with 60 min incubation at room temperature.
- Proteases are not always denatured by SDS. Therefore, keeping a sample with SDS at room temperature for a long time before heating may cause protein cleavage. SDS-PAGE samples should be heated to 95°C for 5 min, or if denatured by milder procedures, should be analyzed as soon as possible.
- Membrane proteins often migrate according to a lower apparent molecular weight because SDS-membrane protein complexes are more compact than SDS-complexes with water-soluble proteins.
- Some proteins, such as membrane proteins, may precipitate when heated to 95°C. This can be avoided by prolonged incubation at, for example, 60°C for 60 min or at room temperature. For membrane proteins it is recommended to try both with and without heating before becoming familiar with the samples at hand.
- Keratin from human skin may contaminate samples or sample buffer to give an M_r 60 000 band in reduced SDS-PAGE. The problem is usually evident only with sensitive staining methods, for example, silver staining. Because of size, keratin does not enter the gel if the sample is not reduced.
- Reoxidation of reduced proteins during SDS-PAGE analysis may give additional bands or smears. Alkylation by the addition of acetamide after reduction will eliminate this problem.

Western blotting

Western blotting analysis can be used for establishing the identity of the protein bands in an SDS-polyacrylamide gel and allows detection of fragments of the target protein. The method can be used for screening of suitable expression conditions (Fig 8.6) or checking whether extra bands in an SDS-polyacrylamide gel are products of protease action on the target protein. Normalization by including detection of a housekeeping protein can improve quantitation of the target protein.

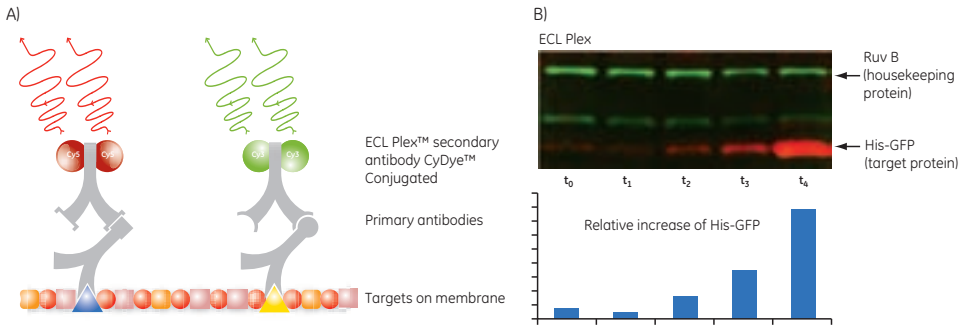


Fig 8.6. Western blotting using fluorescent secondary antibodies. Housekeeping protein can be used for normalization when analyzing multiple samples during expression screening. (A) Principle of detection. (B) Evaluation of results.

Western blotting combines electrophoretic separation with detection using specific antibodies for the analysis of the target proteins in a mixture. The sample, for example, cell culture lysate, and tissue homogenate are separated by molecular weight or isoelectric point, using PAGE. The proteins are transferred (blotted) from the gel to a membrane (nitrocellulose or PVDF) to allow antibodies to easily reach the proteins.

For radiolabeling, chemiluminescent, and chemifluorescent methods, the signal is captured using film or an imaging system. The acquired image is quantitated using image analysis software. The Enhanced Chemiluminescence (ECL) Western Blotting Detection Systems are commercial chemiluminescent reagents for Western blotting. They are at least 10-fold more sensitive than colorimetric assays. ECL, ECL Plus, and ECL Advance™ detection systems require very little antibody to achieve sufficient sensitivity, so the amount of antibody (primary and secondary) used in the protocols can be minimized.

The combination of Western blot detection and total protein staining of the SDS-polyacrylamide gel (Coomassie or silver staining) gives a powerful control of purification results. Immunodetection of the affinity tag offers a generic detection system when working with multiple target proteins that have the same affinity tag.

IEF and 2-D PAGE

Isoelectric focusing electrophoresis (IEF) is a common method for analysis of isoelectric point (pI) of proteins, and is the most common first step in two-dimensional polyacrylamide electrophoresis (2-D PAGE); see Figure 8.7. IEF allows detection of charge heterogeneities in addition to impurities. The IEF under native conditions is often limited by the low solubility of most proteins at their pI, especially at the low ionic conditions that are required for IEF. Because of this, IEF is usually performed in the presence of urea or thiourea and detergents.

2-D PAGE is usually performed with IEF in the first dimension and SDS-PAGE in the second dimension. In addition to traditional post-electrophoresis staining methods, which are the same as used for 1-D PAGE, there are some new developments in 2-D PAGE: 2-D fluorescence difference gel electrophoresis (2-D DIGE; Fig 8.8) allows accurate comparison between samples. A protein standard sample and the two samples to be compared are labeled with fluorescent dyes (CyDye DIGE Fluor minimal dyes: Cy™2, Cy3, or Cy5), mixed and analyzed together in a single run. The labeling allows independent detection of each sample. Quantitation can be performed by image analysis. 2-D PAGE and 2-D DIGE are extensively used for the characterization of complex protein mixtures in proteomics applications. The methods can also be used for analyses during the development of a purification protocol in order to facilitate optimization and to increase the understanding of the purification process; see Fig 8.9. Host cell protein impurities can be monitored throughout the expression and purification protocol development. For a detailed example of this method, see Application note 28-9794-25 from GE Healthcare.

Fig 8.7. Example of 2-D PAGE.

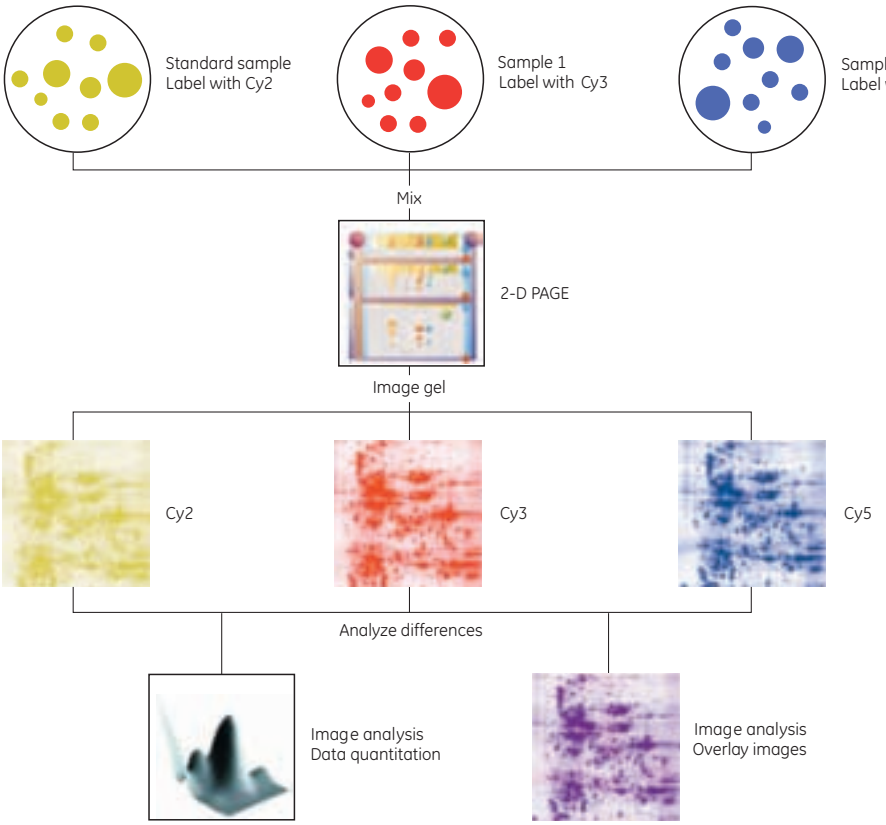


Fig 8.8. Principle of 2-D DIGE. Differently labeled samples are analyzed simultaneously in the same electrophoresis gel. DeCyder™ 2-D software allows quantitation of protein spots.

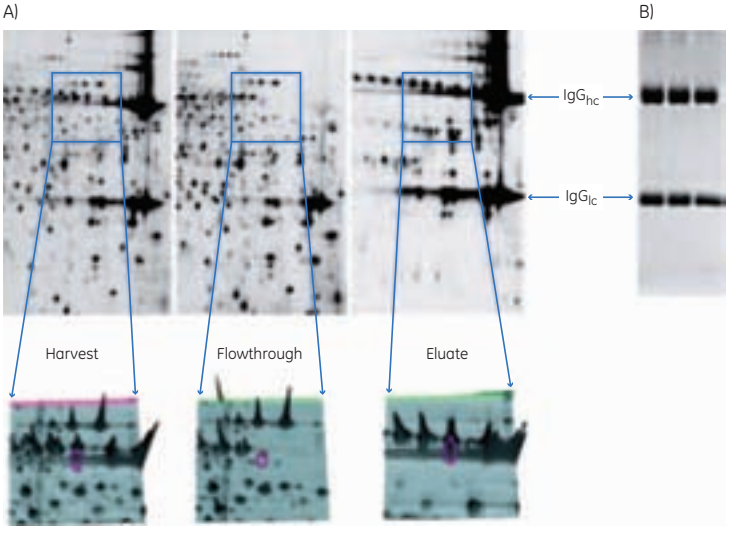


Fig 8.9. 2-D PAGE analysis of material from purification of a monoclonal antibody. Harvest, flowthrough, and eluate fractions from purification steps with MabSelect SuRe were analyzed. Spike proteins added to the samples before CyDye labeling are used to normalize samples with different spot patterns. (A) Spot maps (2-D gel images) and detailed 3-D view around a selected spot are displayed in the DeCyder 2-D software. The spot patterns of harvest, flowthrough, and eluate fractions (50 µg per fraction) are very different but complement each other. (B) Eluate fraction (5 µg) from the same sample was analyzed by 1-D SDS-PAGE stained with Coomassie for comparison. The heavy (IgG_{hc}) and light (IgG_{lc}) chains of the MAb are marked by arrows.

GF for rapid purity check

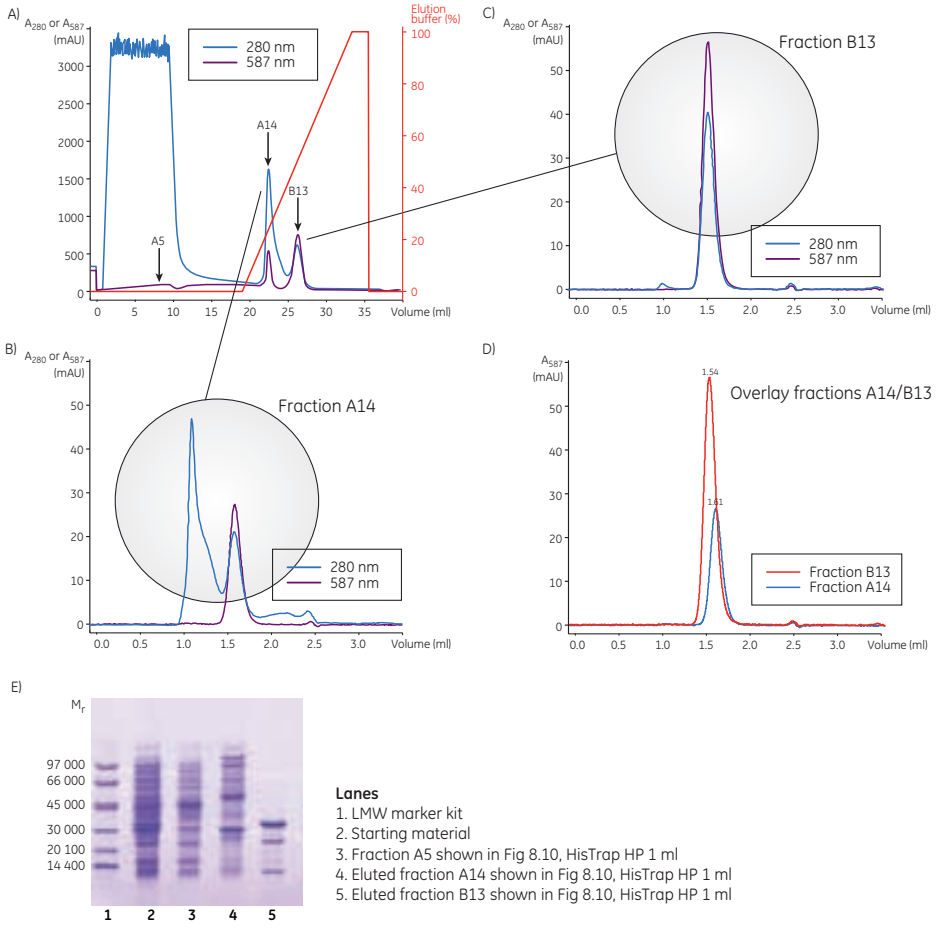
GF in short columns (15 cm) can be used for rapid assessment of protein purity. Analysis is usually performed under native conditions. Because of this, a protein composed of more than one subunit will still behave as an intact protein, giving a single peak, and any additional peaks will consist of decomposed or aggregated forms of the protein, or impurities. Figure 8.10 shows an example with purity analysis using Superdex 75 5/150 GL that allows analysis of 4 to 50 µl samples in 10 min. Other column formats for analytical GF are also available; see "Ordering information" at the end of this handbook.

IMAC purification

Column:	HisTrap HP 1 ml
Sample:	8 ml of diluted extract containing $(His)_6$-mCherry *Strep*-tag II
Binding buffer:	20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4
Flow rate:	1 ml/min
Gradient:	0% to 100% elution buffer in 15 CV
Detection:	UV 280 nm and 587 nm

Gel filtration purity analysis

Column:	Superdex 75 5/150 GL
Sample:	50 µl fractions from IMAC purification
Elution buffer:	PBS, pH 7.5
Flow rate:	0.3 ml/min
Detection:	UV 280 nm and 587 nm



Lanes
1. LMW marker kit
2. Starting material
3. Fraction A5 shown in Fig 8.10, HisTrap HP 1 ml
4. Eluted fraction A14 shown in Fig 8.10, HisTrap HP 1 ml
5. Eluted fraction B13 shown in Fig 8.10, HisTrap HP 1 ml

Fig 8.10. Example of GF purity analysis. (A) Purification by IMAC of the colored $(His)_6$-mCherry *Strep*-tag II (absorbance at 587 nm) using HisTrap HP 1 ml. (B) First peak fraction from HisTrap HP 1 ml was analyzed further by GF using Superdex 75 5/150 GL column. (C) Second peak fraction (containing target protein) from the HisTrap HP 1 ml purification was also analyzed using Superdex 75 5/150 GL column. (D) Overlay (587 nm wavelength) of the analysis on Superdex 75 5/150 GL of the two fractions from IMAC purification. A shift in retention volume (1.54 ml compared with 1.61 ml, corresponding to 0.23 min) was observed and can be explained by the presence of a truncated form of the target protein.

MALDI-MS for purity assessment

Matrix-Assisted Laser Desorption Ionization-Mass Spectrometry (MALDI-MS) can be used for purity assessment and for protein identification. When compared with Electro Spray Ionization-Mass Spectrometry (ESI-MS), the MALDI-MS ionization process is relatively tolerant to salts and small amounts of certain detergents. However, sensitivity and the quality of the data obtained are quite dependent on the purity of the sample. Best results are achieved if salts, buffers, and detergents are kept to a minimum. Table 8.4 gives tolerance levels for different sample contaminants. In general, any component present at a concentration above that of the matrix (~50 mM) may affect the ionization process. Type of matrix, spotting method, and combinatorial contaminant effects may influence the tolerance.

Table 8.4. Compatibility of MALDI-MS with different substances

Contaminant	Maximum concentration
Sodium chloride	50 mM
Phosphate	10 mM
Tris base	50 mM
Urea	1 M
Guanidine hydrochloride	1 M
Azide	0.1%
Glycerol	1%
PEG 2000	0.1%
SDS	0.01%
Triton X-100, RTX-100, NP-40	0.1%
Tween	0.1%
CHAPS	0.01%
n-Octyl-β-D-glucoside	1%
Zwittergent™	0.1%
Lauryldimethylamine oxide (LDAO)	1%

Table adapted from Current Protocols in Protein Science, Unit 16.2, supplement 4 CPPS.

Specific impurities

Some impurities may have dramatic negative effects on the use of the purified protein and thus need to be removed during purification; see Table 8.5 for some examples. A specific analysis method for the impurity will be needed to monitor purification and to obtain final proof of the removal of this impurity.

Table 8.5. Examples of purity requirements regarding specific impurities

Final application	Purity requirement
Animal experiments	Absence of endotoxin (= lipopolysaccharides, LPS); can cause fever in animals
Gene regulation studies	Absence of DNA (DNA may interfere with studies)
Enzyme activity after mutations	No endogenous, wild-type enzyme present

Size homogeneity

The size homogeneity of a purified protein can be important for the final application. GF offers size analysis under a broad range of conditions. Typically, GF is run under conditions that favor native structure and activity of the protein, but denaturing conditions can also be used. GF analysis under native conditions is an important complement to SDS-PAGE analysis. A number of prepacked columns for GF analysis are available from GE Healthcare; see "Ordering information" at the end of this handbook.

Analysis of proteins associated via noncovalent interactions

Many proteins tend to associate in solution via noncovalent interactions. GF under native conditions offers a method for analysis of the size heterogeneity of the protein, often without disrupting these interactions. Preparations of monoclonal antibodies often contain dimers or oligomers, or even aggregates. Figure 8.11 shows a typical GF analysis of a monoclonal antibody.

Column:	Superdex 200 10/300 GL
Sample:	Monoclonal antibody
Sample volume:	100 µl
Elution buffer:	20 mM Tris HCl, pH 7.5, 150 mM NaCl
Flow rate:	0.25 ml/min

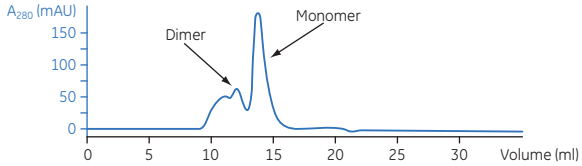
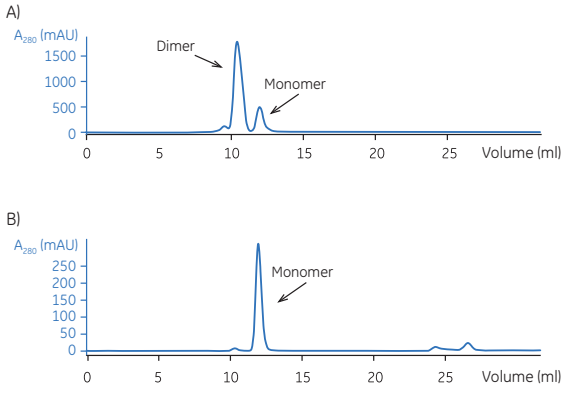


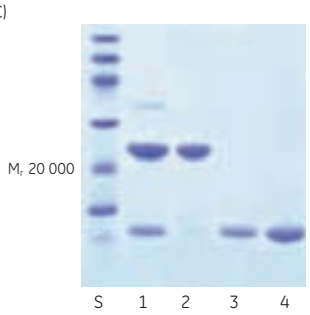
Fig 8.11. Separation of the monomer from oligomers of a monoclonal antibody on Superdex 200 10/300 GL.

Analysis of proteins associated via covalent interactions

GF analysis can be used to study disulfide bonds between proteins or protein subunits. Figure 8.12 shows a cysteine-containing protein that spontaneously forms dimers via a disulfide bridge. These dimers can be cleaved by adding the reducing agent DTE. GF analysis using Superdex 75 10/300 GL shows baseline separation between a monomer and a dimer (Fig 8.12A) and that it is possible to reduce the dimers into monomers by addition of DTE (Figs 8.12B and 8.12C). Similar studies are often performed using SDS-PAGE, which will also reveal disulfide bonds for proteins that do not fall apart during reduction, thus giving complementary information to a GF study.

Column: Superdex 75 10/300 GL
Sample: RecCys-prot
Sample volume: 200 µl
Elution buffer: 50 mM Tris HCl, 1 mM EDTA, 150 mM NaCl, pH 7.5
Flow rate: 0.5 ml/min





Lane
S. LMW-SDS Marker Kit (17-0446-01)
1. Original dimer/monomer
2. Dimer fraction from Fig 8.12A
3. Monomer fraction from Fig 8.12A
4. Monomer peak from Fig 8.12B

Fig 8.12. (A) Dimer-monomer separation of a recombinant cysteine-containing protein (recCys-protein) on Superdex 75 10/300 GL. (B) Purification of the monomers after reduction with DTE. (C) A Coomassie stained SDS-polyacrylamide gel (samples run under nonreducing conditions). The dimer content is high, which also is reflected in (A).

Rapid check of truncated recombinant protein

Size heterogeneities can be caused by truncations of the polypeptide. Small truncations may require MS or SDS-PAGE analysis to be revealed. If the truncation is large, it can be observed using GF; see Figure 8.13. An affinity-purified recombinant protein with M_r around 17 000 often contained co-purified truncated protein with M_r around 10 000. A fraction of the purified protein was analyzed on Superdex 75 5/150 GL.

Column: Superdex 75 5/150 GL
Sample: Purified recombinant protein
Sample volume: 4 µl
Buffer: PBS, pH 7.4
Flow rate: 0.3 ml/min

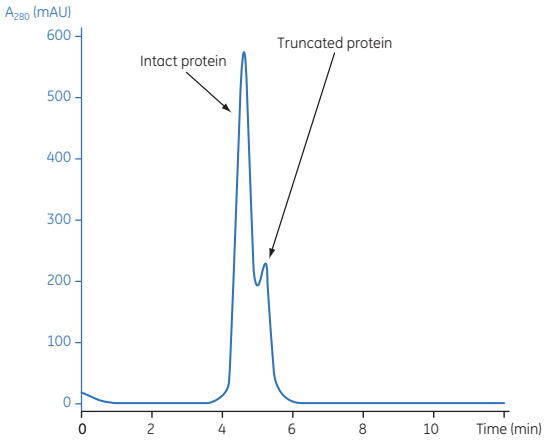


Fig 8.13. Rapid screening on Superdex 75 5/150 GL of a protein purification fraction containing truncated protein.

Other homogeneity issues

Homogeneity of the target protein in terms of charge, post-translational modification, chemical modifications, and so on, may be a requirement for the final preparation of the protein. A large number of chemical and physical analyses can be performed once the protein has been obtained in a pure form. Some examples are given below.

Analytical IEX for phosphorylated and glycosylated proteins

One of the key factors for successful protein crystallography is homogeneity; small amounts of contaminants may be far better tolerated than different forms of the same protein. Such variations may be caused by truncation, different isoforms, or post-translational modifications such as glycosylation, phosphorylation, and so on. If the variants differ in charge, it may be possible to separate them using IEX. Mono S and Mono Q high-resolution IEX media can be used for the separation of differentially phosphorylated protein kinase isoforms. To obtain enough resolution for separation of different phosphorylated forms of protein kinase, sample loading should be relatively low, and shallow gradients should be used. In the example shown in Figure 8.14, phosphorylated and nonphosphorylated forms of ZAP-70 kinase were separated. Two major peaks were eluted: a monophosphorylated kinase comprising 20% of the total ZAP-70 and a nonphosphorylated protein, eluted slightly later. The presence of a single extra charge on the protein surface made the phosphorylated kinase elute earlier than the nonphosphorylated variant. Purified nonphosphorylated ZAP-70 could be crystallized in the presence of the kinase inhibitor staurosporine. The stabilizing effect of staurosporine on another receptor tyrosine kinase was analyzed using IEX on a Mono Q column; see Figure 8.15.

Column:	Mono S HR 10/10*
Sample:	42 ml ZAP-70 kinase mixture
Start buffer:	20 mM $NaPO_4$, 5 mM DTT, 10 mM NaCl, 1 mM $MgCl_2$, pH 7.2
Elution buffer:	Start buffer + 250 mM NaCl, pH 7.2
Flow rate:	2 ml/min
Gradient:	0-250 mM NaCl over 80 CV

* *Replaced with Mono S 10/100 GL*

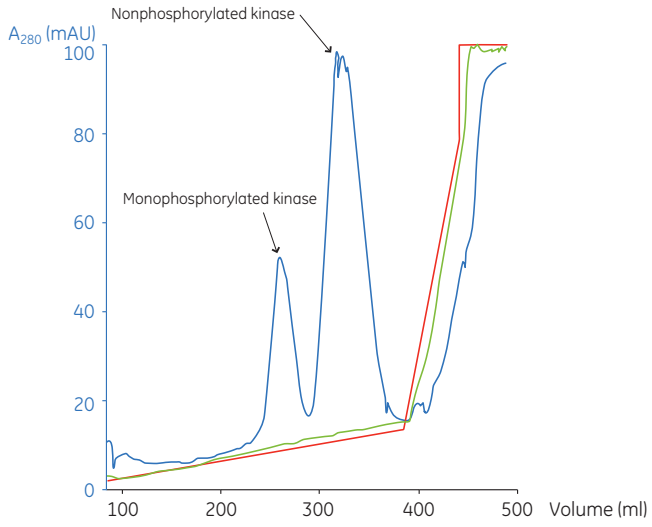


Fig 8.14. Separation of monophosphorylated and nonphosphorylated ZAP-70 kinase by CIEX with Mono S.

Column: Mono Q HR 5/5*
Sample: 1.2 mg receptor tyrosine kinase in 5.4 ml start buffer
Start buffer: 20 mM Tris-HCl, 5% v/v glycerol, 2 mM DTE, pH 8
Elution buffer: Start buffer + 1 M NaCl
Flow rate: 0.8 ml/min
Gradient: Linear 2.5% to 40% elution buffer in 37.5 CV

* *Replaced with Mono Q 5/50 GL*

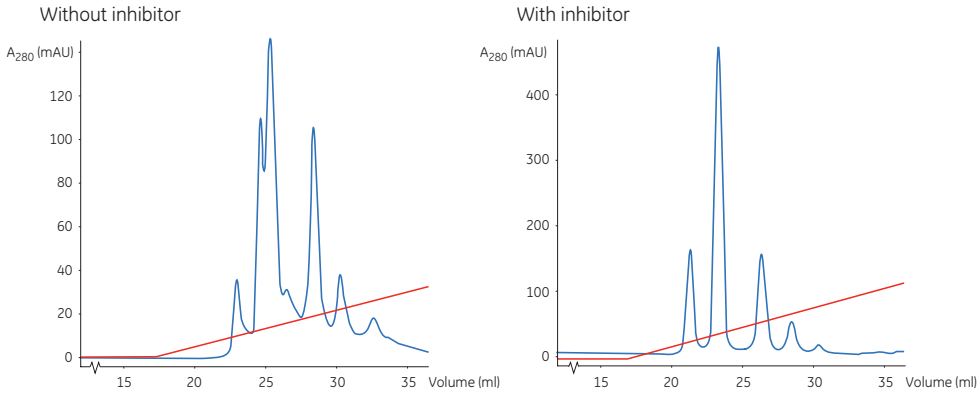


Fig 8.15. Analysis of phosphorylation isoforms of a kinase. In the chromatogram with inhibitor (at right), the peaks correspond to 0x, 1x, 2x, and 3x phosphorylated forms (1x being the predominant form). In this experiment the kinase was preincubated with inhibitor at 10 µM. *Acknowledgments: P. Ramage, B. Mathis, G. Fendrich, and R. Benoit, Novartis Institutes for Biomedical Research, Basel, Switzerland.*

Analysis of glycoforms

One of the major bottlenecks during the purification and analysis of glycoproteins is that the final product is heterogeneous in the glycan moiety. AIEX is often used to measure the concentration of carbohydrate-deficient transferrin (desialylated or asialo transferrin) to diagnose the chronic abuse of alcohol. In a healthy individual, tetra-sialo transferrin is the main isoform. N-glycan chains of transferrin differ in the degree of branching with di-, tri-, and tetra-antennary structures. Each antenna terminates with a negatively charged sialic acid. The transferrin variants can be analyzed using a Mono Q column with pH-gradient elution. The number of peaks observed corresponds to the number of different sialylated forms (Fig 8.16).

Column: Mono Q 5/50 GL
Start buffer: 20 mM piperazine, pH 9.0
Elution buffer: 20 mM piperazine, pH 4.8
Flow rate: 1 ml/min
Gradient: Linear, 0% to 100% elution buffer in 25 CV

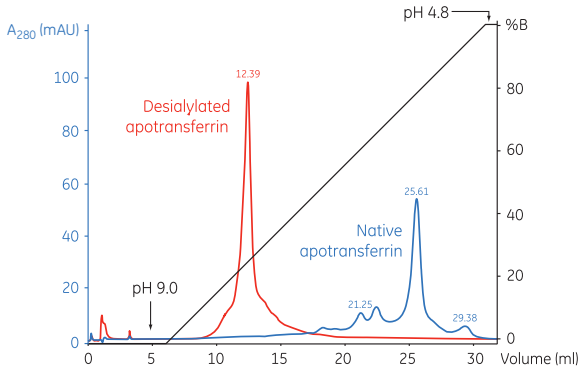


Fig 8.16. Separation of native apotransferrin (blue) and desialylated apotransferrin (red) by AIEX using a pH gradient from 9.0 to 4.8.

Protein-protein interaction analysis by GF

The possibility of size analysis under a broad range of native (and denaturing) conditions makes GF very suitable for protein-protein interaction analysis. A number of interactions can be studied:

- Formation of protein complexes
- Homo-oligomerization of a protein
- Antibody-antigen interactions
- Fab-fragment interaction with antigens
- Interaction of proteins with receptors
- Interaction of low-molecular-weight ligands with proteins (GF in desalting mode; see Chapter 3)

The principle of analysis is simple. The formation of a complex gives a new peak in the GF chromatogram corresponding to the size of the complex, and the peaks corresponding to the monomeric proteins are decreased or lost. Figure 8.17 shows the principle. As a model system, the interaction of trypsin and soy trypsin inhibitor (STI) was analyzed by a set of GF runs. Trypsin and STI were first analyzed separately, and then a 1:1 mixture was analyzed. Only one peak was eluted from the mixture of trypsin and STI with an elution volume shifted toward the void volume, indicating interaction between trypsin and STI.

Column:	Superdex 200 5/150 GL
Samples:	Trypsin 1 mg/ml (purple) Soy trypsin inhibitor 1 mg/ml (red) Trypsin 1 mg/ml and Soy trypsin inhibitor 1 mg/ml (blue)
Sample volume:	12.5 µl
Buffer:	PBS, pH 7.4
Flow rate:	0.3 ml/min

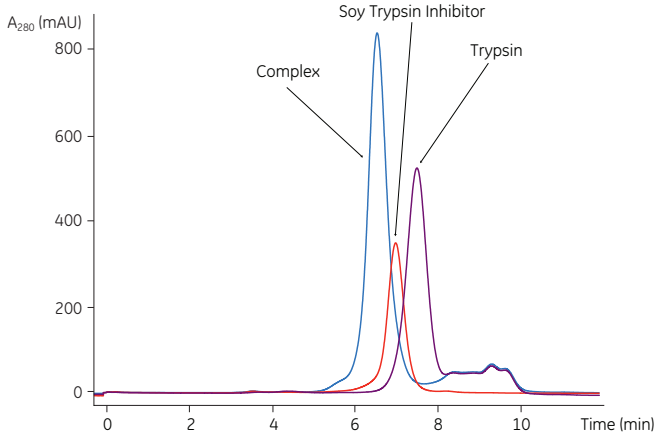


Fig 8.17. Overlayed chromatograms from analysis of complex formation between trypsin and soy trypsin inhibitor.

Specificity

The extent to which different molecules interact with a single partner immobilized on a sensor surface reveals the specificity of an interaction. Simple yes/no answers are required for a wide variety of applications:

- Search for binding partners
- Screen for inhibitor specificity
- Test for cross-reactivity
- Look for activity after purification
- Test cell culture lines for the expression of a given protein

Kinetics: rates of reaction

The kinetics of an interaction, that is, the rates of complex formation (k_a) and dissociation (k_d), can be determined from the information in an SPR sensorgram. As sample passes over a prepared sensor surface, binding occurs and is recorded by the sensorgram. If equilibrium is reached, a constant signal will be seen. Replacing sample with buffer causes the bound molecules to dissociate, and the response decreases.

Affinity: the strength of binding

The affinity of an interaction is determined from the level of binding at equilibrium (seen as a constant signal) as a function of sample concentration. Affinity can also be determined from kinetic measurements. For a simple 1:1 interaction, the equilibrium constant K_D is the ratio of the kinetic rate constants, k_d/k_a.

Protein stability

Most purification goals require that the protein is obtained in its active form. Each purification step risks reducing the protein activity. In fact, a certain loss of activity in each step cannot be avoided. The sample preparation step is an especially critical step because the sample may contain proteases that could rapidly cleave the target protein. Moreover, a high concentration of other sample components may cause those components to associate with the target protein, causing aggregation and thus loss of the target protein. Because of the importance of stabilizing the target protein during the sample preparation step, the subject of protein stabilization has been addressed previously (see Chapter 6). Protein stability should be attended to during the entire purification workflow and also for storage.

Loss of protein activity may have several causes, for example:

- Problems with the activity assay, for example, inhibitors in the sample
- Loss of tertiary and quaternary structure
- Aggregation caused by loss of structure
- Aggregation caused by decreased solubility
- Precipitation
- Proteolytic cleavage
- Nonspecific adsorption to columns, containers, or other equipment
- Loss of subunits from a protein complex
- Loss of cofactors or coenzymes
- Chemical modifications

These causes may be due to:

- The presence of harmful impurities, for example, proteases
- Changes in solution conditions during purification
- Desalting or buffer exchange
- Protein concentration
- Protein dilution
- Changes of physical condition, for example, freezing and thawing or increased temperature

Different analytical methods can be used for monitoring protein activity. As mentioned earlier, it is often useful to perform a thorough study of the target protein stability window. This can be accomplished stepwise by: 1) changing the conditions of small aliquots of the target protein by buffer exchange (see Chapter 3), changing temperature, or exposing the protein to the treatment of interest; 2) incubating (possibly at elevated temperature for accelerated study); and 3) analyzing the activity.

Other analytical methods mentioned in this chapter should be considered as well. SDS-PAGE, Western blotting, and MS can be used to detect proteolytic cleavage or other changes in size or composition. GF is a powerful method for size characterization under native conditions, and can therefore be used for analysis of amount of oligomers or aggregates in a protein preparation, or presence of subunits in protein complexes. UV/visible spectroscopy should not be underestimated as a method for detecting loss of cofactor or coenzymes. Differential scanning calorimetry (see below) is a powerful method for protein stability studies.

Differential scanning calorimetry

Differential Scanning Calorimetry (DSC) is used to study the stability of biological systems involving peptides, proteins, lipids, and nucleic acids. It can, for example, be used to screen conditions for optimum protein stability during protein purification or storage. DSC has the advantage that it does not require any incubation before analysis, which may save a significant amount of time when optimizing storage stability.

A biomolecule in aqueous solution is in equilibrium between the native (folded) conformation and its denatured (unfolded) conformation. For a protein to unfold, stabilizing forces need to be broken. During the experiment, the temperature is gradually increased while measuring the amount of energy required for the temperature increase; see Figure 8.18. At a high enough temperature. the protein is denatured. The transition midpoint, T_m, is the temperature where 50% of the protein is in its native conformation and the other 50% is denatured. The higher the T_m, the more stable the molecule.

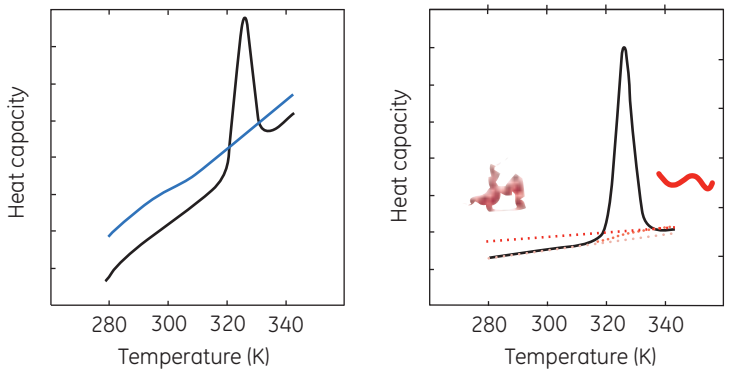


Fig 8.18. DSC data for the denaturation of 0.25 mg/ml of barnase obtained using the MicroCal™ VP-DSC instrument. The instrumental baseline recorded with buffer is indicated in blue. The panel at right shows the data after "buffer" subtraction and illustrates the process of baseline generation prior to integration of the peak.

A stability study is performed by transferring aliquots of the protein into different conditions to be tested. DSC measurements are made to determine T_m for the different conditions. The conditions that give the highest T_m give the best protein stability. Figure 8.19 shows an example of optimization of pH for an antibody. GF was used to assess the amount of aggregates formed after different storage times. DSC measurements were made at time = 0 only. GF shows that 2 weeks of storage gave a significant amount of aggregates at pH values below 5; essentially no aggregates were formed above pH 5. DSC analysis gave additional information regarding the stability above pH 5, showing that optimal stability was obtained at pH 5.7. Figure 8.20 shows the difference between the DSC diagram for a protein under conditions that stabilized or destabilized the protein from aggregation. Note the decrease in T_m and characteristic transition region for the destabilized sample.

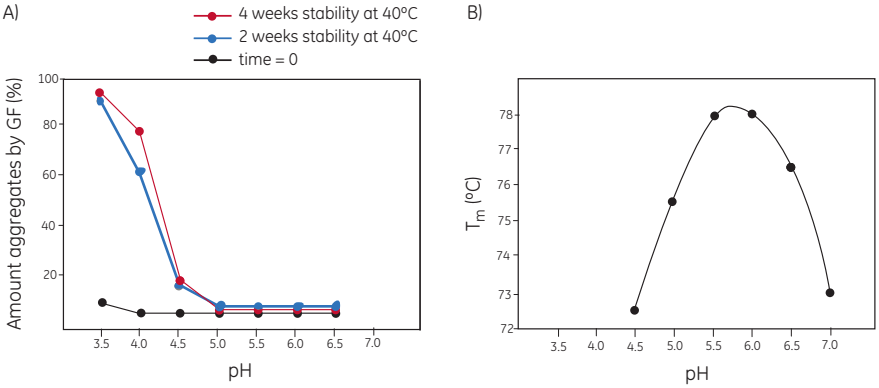


Fig 8.19. The impact of pH on the stability of an antibody determined by combining GF and DSC. (A) Amount of aggregates vs pH, as measured by GF after different times of incubation. (B) T_m vs pH.

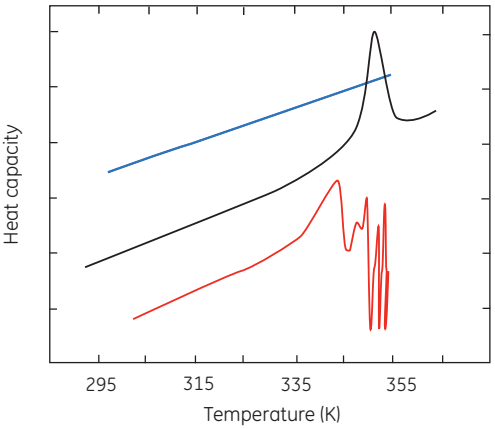


Fig 8.20. Raw DSC data for the denaturation of an aggregating protein (red). Data for the buffer baseline (blue) and the same protein (black) under different conditions where aggregation is almost completely eliminated are shown for comparison.

Chapter 9
Purification examples

The vast majority of proteins purified in laboratory scale are affinity-tagged and can therefore be purified with relative ease using AC. In most cases, this capture step is followed by a polishing step to obtain high purity. GF is generally the most powerful method for polishing because it allows removal of impurities as well as size heterogeneities of the target protein, allows the use of essentially any buffer conditions, and can usually be accomplished without optimization.

Untagged proteins obtained by overexpression or from a natural source often require several purification steps. Here, the CIPP strategy (the three-phase strategy) becomes useful. It supports selection and optimization of the methods for the different purification steps and suggests how to combine them into a complete protocol; see Chapter 4.

This chapter demonstrates purifications from simple single-step protocols to a multi-step protocol for an untagged protein. The applications were developed using ÄKTA design chromatography systems.

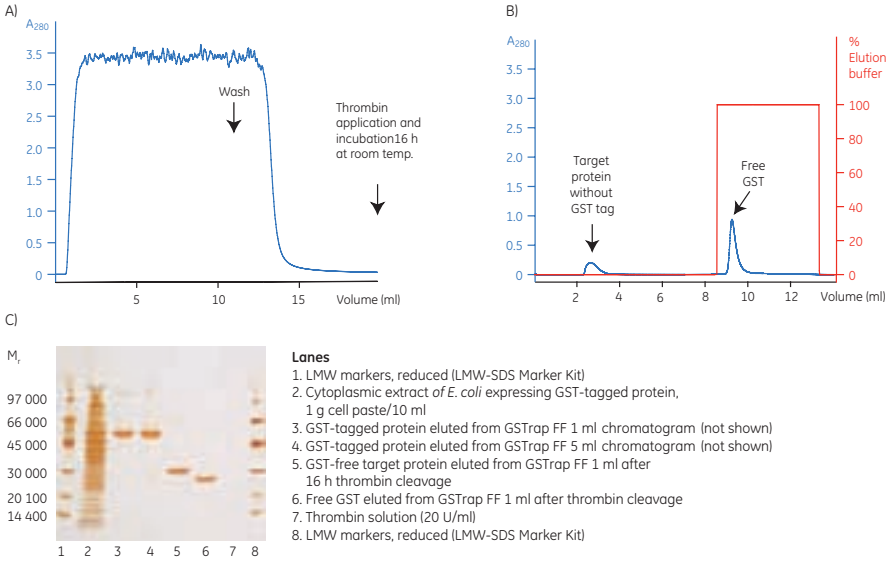
One-step purification of a GST-tagged protein and on-column tag removal

The Glutathione S-transferase (GST) tag allows AC purification on Glutathione Sepharose columns. The high selectivity of the interaction allows very high purity in a single step. The GST tag often increases the solubility of the target protein, but because it is large (M_r 26 000), it is often removed after purification, using a sequence-specific protease, for example, PreScission™ Protease. Tag removal can be performed in batch after purification or by on-column cleavage during purification with automatic removal of the cleaved-off tag from the target protein.

The high purity that is most often obtained was demonstrated by purification on GSTrap FF 1 ml with on-column tag removal using thrombin, as shown in the chromatograms in Figure 9.1. After equilibration, sample application, and washing, the column was filled by syringe with 1 ml of thrombin solution (20 U/ml in PBS), which was prepared according to the manufacturer's protocol. The column was sealed with the supplied connectors and incubated for 16 h at room temperature. After incubation, the target protein but not the GST tag was washed out using PBS, and after that the bound GST (tag) was eluted with elution buffer. The cleavage reaction yield was 100% as judged from the SDS-PAGE analysis (lane 5), as no non-cleaved GST-tagged protein can be detected in the bound material eluted from the column (lane 6). The amount of the site-specific protease required and the time for the cleavage reaction depend on the properties and the amount of the specific GST-tagged protein. For best results, this should be checked for each individual cleavage. Purity after five-fold scale-up on GSTrap FF 5 ml without tag removal is demonstrated in lane 4 of the gel.

Column: GSTrap FF 1 ml
Sample: Cytoplasmic extract from *E. coli* expressing GST-tagged protein
Sample volume : 10 ml
Binding buffer: PBS, pH 7.3
Elution buffer: 50 mM Tris-HCl, pH 8.0, with 10 mM reduced glutathione
Flow rate: 1 ml/min
Procedure: 4 CV binding buffer, 10 ml sample, 10 CV binding buffer, and filling the column with 1 ml thrombin solution using a syringe. Incubation 16 h at room temperature

Procedure: 8 CV binding buffer (elution of cleaved target protein)
5 CV elution buffer (elution of free GST and non-cleaved GST-tagged proteins)
5 CV binding buffer



Lanes
1. LMW markers, reduced (LMW-SDS Marker Kit)
2. Cytoplasmic extract *of E. coli* expressing GST-tagged protein, 1 g cell paste/10 ml
3. GST-tagged protein eluted from GSTrap FF 1 ml chromatogram (not shown)
4. GST-tagged protein eluted from GSTrap FF 5 ml chromatogram (not shown)
5. GST-free target protein eluted from GSTrap FF 1 ml after 16 h thrombin cleavage
6. Free GST eluted from GSTrap FF 1 ml after thrombin cleavage
7. Thrombin solution (20 U/ml)
8. LMW markers, reduced (LMW-SDS Marker Kit)

Fig 9.1. Purification and on-column tag removal of a GST-tagged protein using GSTrap FF columns. (A) Sample application, washing, and start of thrombin incubation. (B) Elution of cleaved target protein (without the GST tag) and free GST. (C) SDS-PAGE with silver staining.

Two-step purification of an MBP-tagged protein

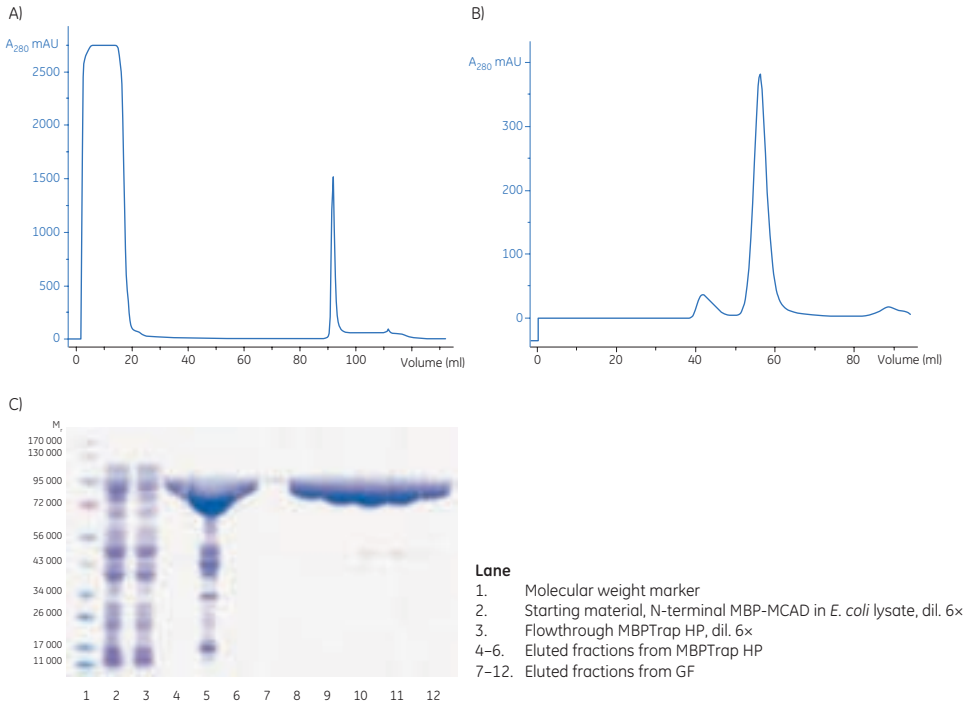
Two-step purification by combining AC for capture and GF for polishing is probably the most useful way of achieving high-purity tagged proteins. Most purifications can be performed with no or a minimum of optimization, by simply following the instructions for the different products.

This example shows a simplified purification of a protein involved in a metabolic disease, medium-chain acyl-CoA dehydrogenase (MCAD). This homotetramer was purified for stability, folding, and kinetic studies. The protein was tagged with maltose binding protein (MBP). Purification on MBPTrap HP 5 ml (Fig 9.2) eliminated a concentration step used in an earlier purification procedure that required concentration prior to the final GF step. The highly concentrated target protein was eluted from the MBPTrap HP column in a small volume (Fig 9.2A) and loaded on a HiLoad 16/60 Superdex 200 pg column. Consequently, the earlier concentration step prior to final GF could be avoided and significant time saved.

The purity of the eluted fractions from MBPTrap HP and GF was determined by SDS-PAGE analysis. Some impurities were detected after the affinity step (Lanes 4-6). This may be due to the presence of truncated variants having intact N-terminal MBP tag or possibly *E. coli* proteins associated with the target protein, but was not further investigated. Note that the sample load in the SDS-PAGE analysis was large and therefore could reveal this rather small amount of impurities. GF efficiently removed all impurities detectable in the Coomassie stained gel. The final yield was approximately 8.4 mg.

Column:	MBPTrap HP 5 ml
Sample:	N-terminal MBP-MCAD in *E. coli* lysate, M_r ~85 500
Sample volume:	15 ml
Binding buffer:	20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.4
Elution buffer:	10 mM maltose in binding buffer
Flow rate:	5 ml/min (0.5 ml/min during sample loading)

Column:	HiLoad 16/60 Superdex 200 pg
Sample:	Eluted fraction from MBPTrap HP 5 ml
Sample volume:	2 ml
Buffer:	20 mM HEPES, 200 mM NaCl, pH 7.0
Flow rate:	0.4 ml/min



Lane

1. Molecular weight marker
2. Starting material, N-terminal MBP-MCAD in *E. coli* lysate, dil. 6×
3. Flowthrough MBPTrap HP, dil. 6×
4–6. Eluted fractions from MBPTrap HP
7–12. Eluted fractions from GF

Fig 9.2. Two-step purification of an MBP-tagged protein. (A) Purification of MBP-MCAD with MBPTrap HP, (B) polishing purification on Superdex 200 pg in XK 16/20 of material eluted from the MBPTrap column, (C) SDS-PAGE analysis (Coomassie stained, reducing conditions). *Acknowledgments: Dr. E. M. Maier, Dr. von Haunersches Kinderspital, Munich, Germany.*

Two-step purification of a mouse monoclonal IgG$_1$

In laboratory-scale purification, monoclonal antibodies are often purified by the following convenient two-step protocol based on AC using Protein A Sepharose or Protein G Sepharose, followed by GF.

A mouse monoclonal IgG_1 from cell culture supernatant was captured using HiTrap Protein G HP; this step was followed by a polishing step using GF on HiLoad 16/60 Superdex 200 pg (Fig 9.3). Both steps were performed on an ÄKTAprime plus chromatography system. The cell culture supernatant was filtered through a 0.45 µm filter before application. Step elution from the AC column allowed collection of the material in a small volume (2 ml) that could be directly applied to the 120 ml GF column without concentration of the protein. Purity was controlled by SDS-PAGE under reducing conditions, which showed that the antibody was highly pure already after the first affinity step. The GF step further improved target quality by separating the dimers and monomers of the antibody. Note that both dimers and monomers run as heavy and light chains in SDS-PAGE under the reducing conditions.

Column: HiTrap Protein G HP 1 ml
Sample: Cell culture supernatant containing mouse monoclonal IgG_1
Sample volume: 10 ml
Binding buffer: 20 mM potassium phosphate, pH 7.0
Elution buffer (B): 100 mM glycine-HCl, pH 2.7
Flow rate: 1 ml/min

Column: HiLoad 16/60 Superdex 200 pg
Sample: Pooled fractions from the capture step
Sample volume: 2 ml
Buffer: Phosphate buffered saline (PBS), pH 7.4
Flow rate: 1 ml/min

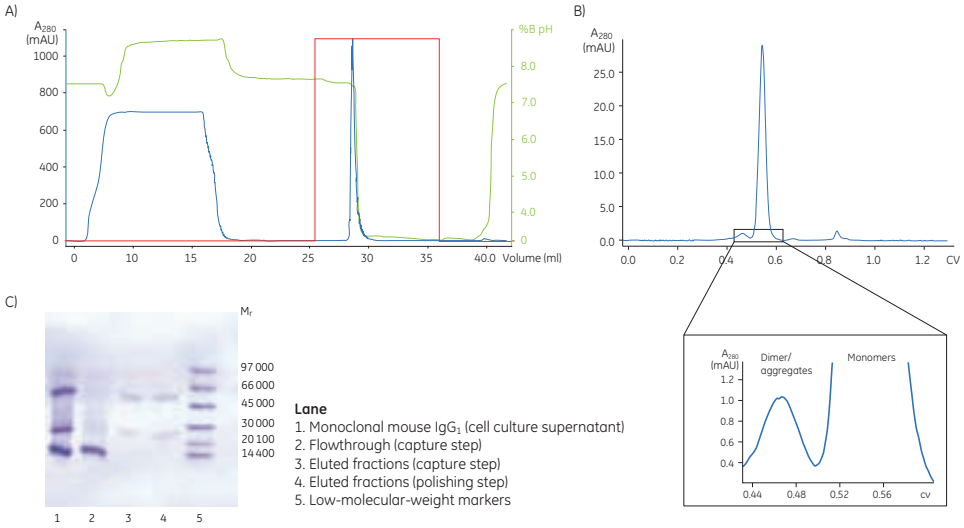


Fig 9.3. Two-step purification of a mouse IgG_1. (A) Capture of mouse monoclonal IgG_1 using HiTrap Protein G HP. The curves shown are absorbance (blue), pH (green), and conductivity (red). (B) Polishing by GF. (C) Purity analysis by SDS-PAGE, reducing conditions.

Three-step purification of a protein without an affinity tag

This example demonstrates one of the most common purification strategies for untagged proteins: IEX for capture, HIC for intermediate purification, and GF for the polishing step. The objective of this purification was to obtain highly purified Deacetoxycephalosporin C synthase (DAOCS), an oxygen-sensitive enzyme for crystallization and structure determination. A more detailed description of this work can be found in GE Healthcare Application Note 18-1128-91.

Sample preparation

Cells were suspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, 0.2 M benzamidine-HCl, 0.2 mM PMSF, pH 7.5), and lysed using ultrasonication. Streptomycin sulfate (1%) and polyethyleneimine (0.1%) were added to precipitate DNA, and the extract was clarified by centrifugation. The sample was kept on ice at all times to avoid proteolysis and denaturation.

Capture

The isoelectric point was calculated to be 4.8, which led to the selection of AIEX for the capture step. HiTrap IEX Selection Kit was used to screen for optimal chromatography media for optimal purity. Q Sepharose XL, well suited for capture, was chosen. Optimization of the capture step allowed the use of a step elution at high flow rate to speed up the purification (Fig 9.4). This was particularly advantageous when working with this potentially unstable protein.

Column: HiPrep Q XL 16/10
Sample: Clarified *E. coli* extract
Sample volume: 40 ml
Binding buffer: 50 mM Tris-HCl, 1 mM EDTA, pH 7.5; 2 mM DTT, 0.2 M benzamidine-HCl, 0.2 mM PMSF
Elution buffer: Binding buffer + 1.0 M NaCl
Flow rate: 10 ml/min (300 cm/h)

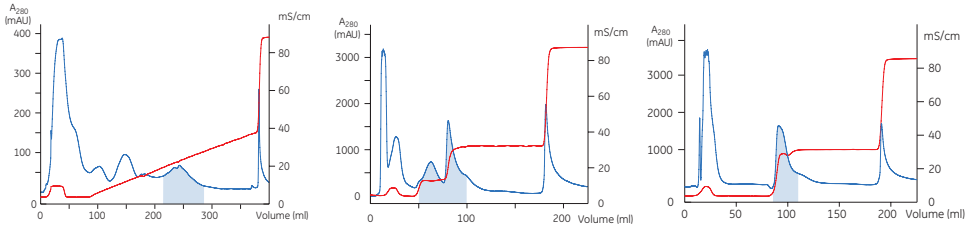


Fig 9.4. Capture using IEX and optimization of purification conditions. The elution position of DAOCS (shaded) was determined by identifying fractions that showed a protein band in SDS-PAGE with the expected apparent M_r.

Intermediate purification

HIC was selected for the intermediate purification step because the method is complementary to IEX and compatible with high-salt samples eluted from IEX columns, thus requiring minimal sample conditioning. Hydrophobic properties are difficult to predict, and it is always recommended to screen different media. The RESOURCE HIC Test Kit was used for screening chromatography media, and SOURCE 15ISO was selected on the basis of the resolution achieved. In this intermediate step, shown in Figure 9.5, the maximum possible speed for separation was sacrificed in order to achieve higher resolution and to allow significant reduction of impurities.

Column: SOURCE 15ISO, packed in HR column 16/10
Sample: DAOCS pool from HiPrep Q XL 16/10
Sample volume: 40 ml
Binding buffer: 1.6 M ammonium sulfate, 10% glycerol, 50 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, 0.2 mM benzamidine-HCl, 0.2 mM PMSF, pH 7.5
Elution buffer (B): 50 mM Tris-HCl, 10% glycerol, 1 mM EDTA, 2 mM DTT, 0.2 mM benzamidine-HCl, 0.2 mM PMSF, pH 7.5
Flow rate: 5 ml/min (150 cm/h)
Gradient: 0–16% in 4 CV, 16–24% B in 8 CV, 24–35% B in 4 CV, 100% B in 4 CV

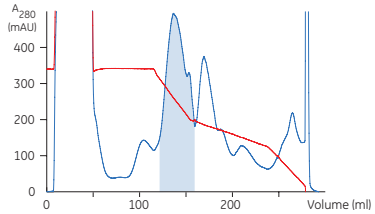


Fig 9.5. Intermediate purification using HIC.

Polishing

The main goal of the polishing step was to remove aggregates and minor contaminants and to transfer the purified sample into a buffer suitable for use in further structural studies. Superdex 75 prep grade was selected because the molecular weight of DAOCS (M_r 34 500) is within the optimal separation range for this chromatography medium (Fig 9.6).

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

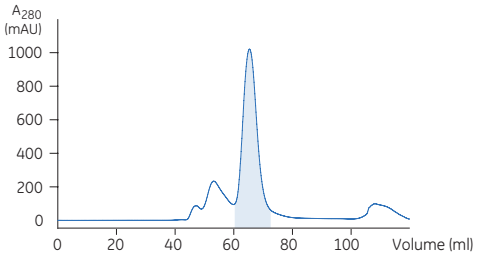
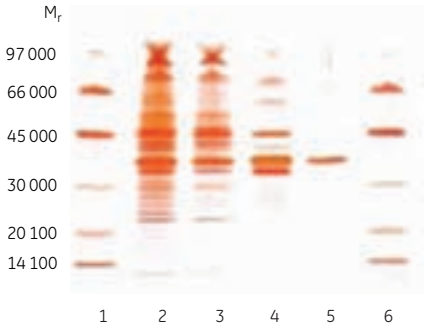


Fig 9.6. Polishing using GF.

The purity of the target protein after each of the three purification steps was analyzed by SDS-PAGE; see Figure 9.7. The purity increased with each purification step, and the final purity (Lane 5) was very high. The prepared protein was used successfully in X-ray diffraction studies.



Lane
1, 6. LMW Marker Kit
2. Cell homogenate
3. DAOCS pool from Q Sepharose XL
4. DAOCS pool from SOURCE 15ISO
5. DAOCS pool from Superdex 75 pg

Fig 9.7. Purity analysis of the target protein by SDS-PAGE with silver staining. Separation was performed using ExcelGel SDS Gradient 8-18.

Unattended multistep purification and tag removal

Unattended multistep purification using ÄKTAxpress is based on the principle that the overexpressed affinity-tagged protein will be the dominant protein after the first affinity purification step. The largest peak, containing the target protein, is automatically collected and reloaded on the next column for the subsequent purification step, and so on with up to four purification steps. An ÄKTAxpress module can process up to four samples in each cycle, and up to twelve modules can be connected and handled in parallel using one computer.

A histidine-tagged protein was purified in four steps by the combination of IMAC-Desalting (DS)-IEX-GF using ÄKTAxpress (Fig 9.8). On-column tag cleavage using a histidine-tagged TEV protease was included in the IMAC step. Because of the histidine tag on the protease, it bound to the column together with uncleaved target protein and the cleaved-off histidine tag. The purified protein was analyzed by SDS-PAGE (Fig 9.9).

Sample:	APC234, M_r 32 500 (Cleaved product: M_r 30 100)
Columns:	HisTrap HP, 5 ml; HiPrep 26/10 Desalting; RESOURCE Q, 6 ml; HiLoad 16/60 Superdex 75 pg
Cleavage conditions:	200 units of AcTEV™ Protease (Invitrogen)/mg protein, 8 h incubation time at room temperature

Buffers

IMAC binding buffer:	50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.5
IMAC cleavage buffer:	50 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole, pH 7.5
IMAC elution buffer:	50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.5
DS and IEX binding buffer:	50 mM Tris-HCl, pH 8.0
IEX elution buffer:	50 mM Tris-HCl, 1 M NaCl, pH 8.0
GF buffer:	50 mM Tris-HCl, 150 mM NaCl, pH 7.5

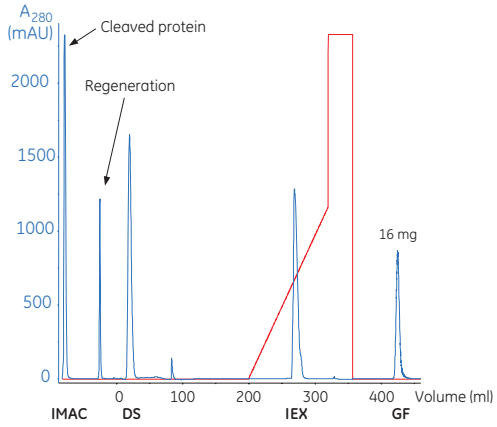
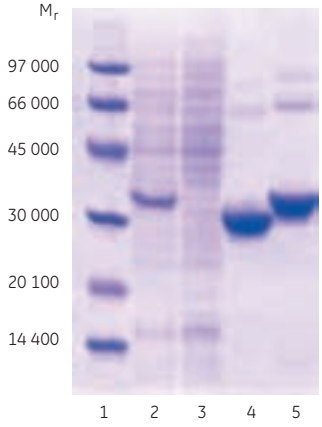


Fig 9.8. Unattended multistep purification of a histidine-tagged protein on ÄKTAxpress chromatography system.



Lane

1. Low Molecular Weight marker
2. Starting sample
3. Flowthrough
4. Purified cleaved APC234
5. Reference: uncleaved APC234

Fig 9.9. SDS-PAGE analysis.

Micropurification

Many proteins are present at extremely low amounts in their natural environment and frequently must be purified from small amounts of tissue from various model organisms. Such micropurification requires the use of small, high-resolution columns and a chromatography system that can take full advantage of such columns.

PEP-19 (Purkinje cell protein 4; PCP 4) is a brain protein with M_r 6800 that belongs to a family of proteins involved in calmodulin-dependent signal transduction. PEP-19 has been shown to be expressed in decreased amounts in an animal model of Parkinson's disease. In order to allow further characterization of the protein, biologically active PEP-19 was purified from mouse brain.

Mouse brain tissue was homogenized in 200 µl of 20 mM Tris-HCl, pH 8.0 supplemented with 2 µl of Nuclease Mix and 2 µl of Protease Inhibitor Mix using a combination of Sample Grinding Kit and sonication. The sample was clarified by centrifugation (14 000 rpm, 4°C, 30 min).

The micropurification was performed using ÄKTAmicro system with the fraction collector Frac-950 and the Microfraction Collection Kit (Fig 9.10). The first purification step was performed by AIEX using Mono Q PC 1.6/5. The sample, 150 µl of clarified supernatant, was diluted with 350 µl of distilled water before loading on the column. The eluate was collected in 96-well microplates in 100 µl fractions. Optimization work showed that the best separation was obtained using a shallow linear gradient from 0 to 150 mM NaCl in 40 CV, and a low flow rate of 0.1 ml/min. Each fraction from the Mono Q run was analyzed by tandem MS, which revealed the position of PEP-19 in the chromatogram.

The second and final purification step consisted of GF on a Superdex Peptide PC 3.2/30 column. The PEP-19-fraction was concentrated to 10 µl and loaded on the GF column. A peak was obtained at a retention volume of 1.05 ml as detected by absorbance at 215 nm. There was no absorbance at 280 nm at the same elution position, which agrees with the fact that PEP-19 does not have aromatic amino acid residues. Fractions of 40 µl were collected and analyzed by MS.

Fractions were reduced and digested by trypsin before MS analyses. Separation was performed on nanoRPC using Ettan™ MDLC coupled to a Finnigan™ LTQ™ linear ion trap mass spectrometer (Thermo Electron Corp.). Samples were first loaded on a ZORBAX™ 300SB-C18 trap column (300 µm i.d. × 5 mm, Agilent Technologies) for sample concentration and buffer conditioning. Separation was performed on a ZORBAX 300SB-C18 RPC column, 75 µm i.d. × 150 mm, using a 90 min gradient of A: 0.1% formic acid and B: 84% acetonitrile and 0.1% formic acid. Five different tryptic peptides from PEP-19 were obtained with coverage of 77% of the protein sequence.

AIEX

Column:	Mono Q PC 1.6/5
Sample:	Mouse brain extract corresponding to 15 mg brain tissue
Sample volume:	150 µl
Binding buffer:	20 mM Tris-HCl, pH 8.0
Elution buffer (B):	20 mM Tris-HCl, 1 M NaCl, pH 8.0
Flow rate:	0.1 ml/min
Gradient:	0% to 15% B in 4 ml, 100% B in 0.5 ml
Fraction size:	100 µl

GF

Column:	Superdex Peptide PC 3.2/30
Sample:	Fraction "Target" from Mono Q PC 1.6/5 (Fig 9.10A) containing PEP-19 concentrated to a volume of 10 µl
Sample volume:	10 µl
Buffer:	20 mM Tris-HCl, 100 mM NaCl, pH 8.0
Flow rate:	0.025 ml/min
Fraction size:	40 µl

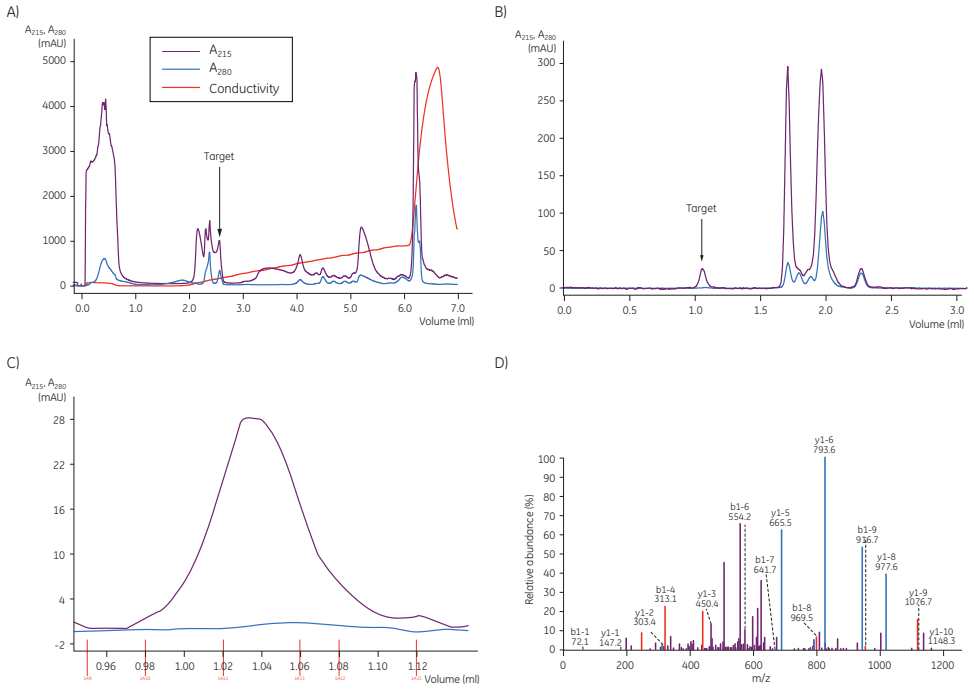


Fig 9.10. Micropurification of PEP-19, a mouse brain protein. (A) Capture by AIEX. (B) Polishing by GF. (C) Enlarged view of the GF chromatogram highlighting the PEP-19 peak. (D) MS/MS spectrum of one tryptic peptide (AAVAIQSQFRK) from purified and digested PEP-19.

Two-step purification of membrane proteins

Affinity-tagged membrane proteins can often be purified using the same methods as for water-soluble proteins, with an important difference. Membrane proteins must be solubilized from the biomembrane using a detergent, and a detergent must be present in all solutions during purification to keep proteins in solution. A common problem, however, is that most membrane proteins are rather unstable in detergent-solubilized form, and they tend to denature and aggregate. This is often the major obstacle in purification of these proteins, and it needs to be addressed by screening for optimal conditions for purification. The following section will briefly address condition screening. One of the most powerful and generic purification protocols for membrane proteins is exemplified in this section: the combination of IMAC and GF for purification of histidine-tagged membrane proteins.

Cells expressing a histidine-tagged putative transferase membrane protein were lysed using chemical and freeze-thaw methods. The membranes in the lysate (no membrane preparation) were solubilized with Fos-choline-12 (FC12), a rather strong detergent, and applied without clarification on a HisTrap FF crude 1 ml column (Fig 9.11). The column was washed and eluted in the presence of n-dodecyl-β-D-maltoside (DDM), which is a milder detergent. The same detergent was used for the GF step, performed on a HiLoad 16/60 Superdex 200 pg column.

The polishing step by GF ascertained that the final preparation did not contain any traces of impurities or aggregates of the target protein, and that the protein was obtained in a suitable buffer free from imidazole.

IMAC

Column:	HisTrap FF crude 1 ml
Sample:	Unclarified *E. coli* cell lysate with 1% FC12 (detergent)
Sample volume:	5 ml
Binding buffer:	20 mM sodium phosphate, 500 mM sodium chloride, 20 mM imidazole, 0.5 mM TCEP, 1% FC12, pH 7.4
Wash buffer:	20 mM sodium phosphate, 500 mM sodium chloride, 40 mM imidazole, 0.5 mM TCEP, 0.03% DDM, 1% FC12, pH 7.4
Elution buffer:	Wash buffer with 500 mM imidazole

GF

Column:	HiLoad 16/10 Superdex 200 pg
Sample:	Pooled fractions selected from affinity purification step
Buffer:	20 mM Tris-HCl, 50 mM NaCl, 0.5 mM TCEP, 0.03% DDM, pH 8.0
Flow rate:	0.3 ml/min

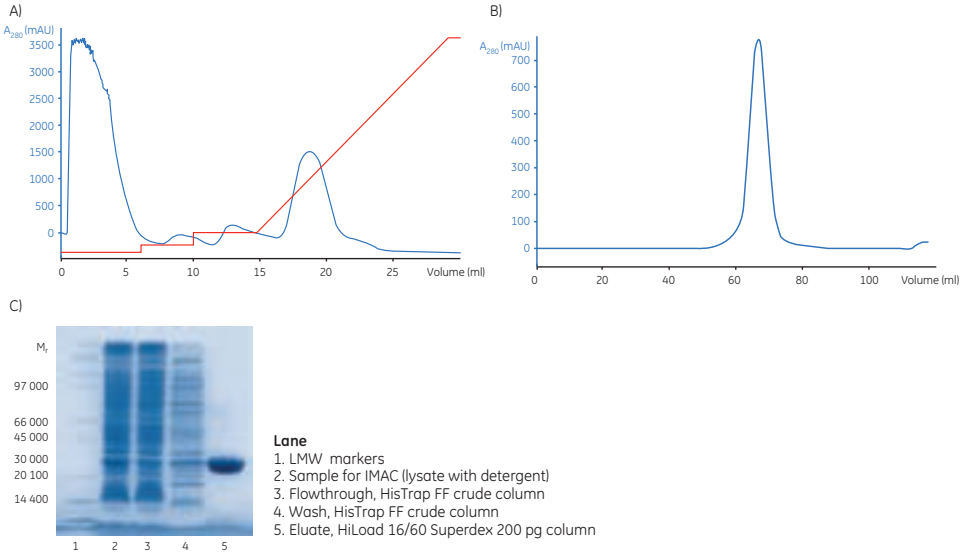


Fig 9.11. Two-step purification of a membrane protein. (A) Capture of the histidine-tagged target protein from unclarified sample on HisTrap FF crude. (B) Polishing by GF to remove any traces of impurities or aggregated target protein. (C) SDS-PAGE (Coomassie stained gel) analysis for purity check after the purification steps.

Screening of conditions for membrane protein purification

The importance of screening conditions for membrane protein purification was mentioned in the previous section. In a short period of time, purification by IMAC in the presence of different detergents followed by analytical GF with the same detergents and under various buffer conditions was used to analyze the protein association state under a wide range of conditions. The selected conditions were subsequently used for scaling up the purification using the two-step (IMAC+GF) procedure described in the previous section.

A series of histidine-tagged membrane proteins were expressed overnight in *E. coli*, each in 0.5 l of TB medium at 20°C induced with 0.2 mM IPTG. The cells were lysed by resuspension in 2.5 ml 20 mM Tris, 50 mM NaCl, 1 mM DTT, 1 mg/ml lysozyme, 10 U DNase per gram of cells and passed three times through an EmulsiFlex™ system (Avestin Inc.). The membranes were prepared by centrifugation (100 000 × g, for 45 min) and resuspended in 5 ml of 20 mM sodium phosphate buffer, 300 mM NaCl, 1 mM DTT, 20 mM imidazole, pH 7.5 (IMAC binding buffer) per gram of cells, and frozen in 5 ml batches using liquid nitrogen.

Aliquots of 5 ml membrane suspensions were thawed carefully and were solubilized by addition of a 10% stock solution of detergent to a final concentration of 1% detergent. For each target protein, up to four detergents were tested. The following detergents were used in the examples given here: Octaethylene glycol monododecyl ether ($C_{12}E_8$), lauryldimethylamine oxide (LDAO), n-Dodecyl-β-D-maltoside (DDM), cyclohexyl-n-hexyl-β-D-maltoside (CYMAL-6), n-Octyl-β-D-glucoside (OG), n-Decyl-β-D-maltoside (DM), n-Nonyl-β-D-maltoside (NM), and Fos-choline 12 (FC12).

Solubilization occurred over 90 min with mild agitation in a cold room, and samples were clarified by centrifugation (100 000 × g, for 15 min).

IMAC purification was performed by incubation of 5 ml of solubilized material with 130 µl of Ni Sepharose 6 Fast Flow chromatography medium with end-over-end mixing for 30 min at 4°C. The medium was then sedimented in a 5 mm i.d. column for gravity elution. The column was washed with 20 column volumes of binding buffer with 40 mM imidazole and eluted with binding buffer including 150 mM NaCl, 500 mM imidazole, and the same detergent as used for the solubilization.

Analytical GF was performed using a Superdex 200 5/150 GL column equilibrated with a selection of buffers: 20 mM Na-acetate (pH 5.2), 20 mM HEPES (pH 7.5), or 20 mM Tris-HCl (pH 8.5) all with 300 mM NaCl, 1 mM DTT, and with the same detergent as used for solubilization and purification. Typical screening results are shown in Figure 9.12.

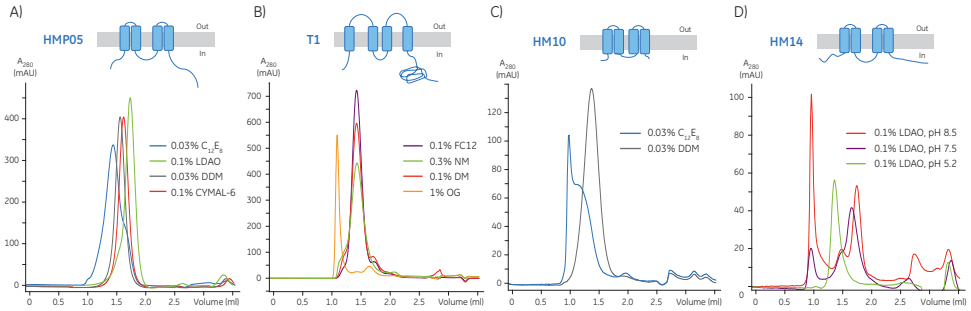


Fig 9.12. Effects of detergents and buffers on protein stability and size homogeneity as analyzed by GF. *Acknowledgment: Dr. Per Moberg, Dept. of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm Sweden.*

HMP05 (Fig 9.12A) shows a tailing peak in $C_{12}E_8$ and a leading peak in LDAO, while both DDM and CYMAL-6 gave symmetrical peaks. This may indicate that HMP05 is monomeric in LDAO, possibly with a fraction of dimers. The earlier elution positions with DDM and CYMAL-6 indicate dimers.

T1 (Fig 9.12B) was completely aggregated in OG, whereas in DM, NM, and FC12 it appeared homogeneous, possibly monomeric.

HM10 (Fig 9.12C) was aggregated in the presence of $C_{12}E_8$, whereas the protein was eluted in a well-defined symmetric peak in DDM.

HM14 (Fig 9.12D) was partially aggregated in LDAO at both pH 7.5 and pH 8.5. Non-aggregated material gave raise to a number of peaks. The protein was monodisperse at pH 5.2. Notice that the protein was exposed to the different pH values for the time period of the chromatography run, about 10 min.

Matrix-assisted refolding of proteins expressed as inclusion bodies

Inclusion bodies are insoluble aggregates of denatured or partly denatured protein sometimes formed in bacterial expression systems. The aggregates may be caused by erroneous folding due to too high expression levels; also, because the protein comes from another species than

used for expression, it may not be fully compatible with the folding machinery of the host cells. The protein in inclusion bodies is usually inactive. Inclusion body formation may be advantageous when desiring high expression levels, and it can allow toxic proteins to be overexpressed. Preparation of active protein from inclusion bodies requires solubilization and refolding. The general success rate for protein refolding with high yields is often disappointing. In fact, protein refolding often requires extensive screening of suitable conditions. The examples of refolding given here aim at describing a tool for efficient screening and scale-up of refolding conditions.

Isolation and solubilization of inclusion bodies were addressed in Chapter 6 with respect to sample preparation. Inclusion body preparation can be performed using multiple centrifugation steps, and the obtained inclusion bodies can be stored frozen until use. Solubilization is achieved by adding a strong denaturant such as guanidine hydrochloride or urea. A reducing agent is often included to remove any erroneously formed S-S-bonds between the denatured protein molecules. Thorough solubilization is a prerequisite for successful refolding.

Because of the rather good purity of the target protein that can be obtained by the isolation of the inclusion bodies, purification can sometimes be omitted before refolding, but purification of the solubilized protein before refolding often improves the success rate.

Refolding is initiated by decreasing the concentration of or completely removing the denaturant. This can be accomplished by dilution, dialysis, or chromatography methods, also known as matrix-assisted refolding.

In matrix-assisted refolding, the denatured protein is bound to a chromatography medium, and the concentration of denaturant is reduced to affect refolding while the protein is attached to the medium.

Refolding

The simplicity of dilution and dialysis is probably the main reason for these methods being the most popular. In laboratory-scale applications, these methods are inexpensive. Drawbacks are the long time and large buffer volumes required, and the low final protein concentrations after dilution refolding. Matrix-assisted refolding is much faster compared with dilution and dialysis refolding. A much higher protein concentration can be used, and the final protein concentration can be a thousand times higher than after dilution refolding. It seems clear that binding of the protein on a chromatography medium during refolding reduces protein aggregation, which is highly detrimental to refolding in solution. Whether the solid support may catalyze the folding of the protein is a topic of discussion. The high protein concentration used means that buffer consumption is small. Another advantage with matrix-assisted refolding is that refolding can be combined with purification in the same step. There are, however, some obvious limitations to matrix-assisted refolding. It may require an affinity tag, usually a histidine tag, and binding to the matrix must be compatible with the solubilization conditions.

IMAC is the most common method for on-column refolding, with GF in second place (no binding needed). Several of the other chromatography methods for protein purification have also been used for refolding, for example, IEX and HIC.

As mentioned earlier, the most important mechanism for matrix-assisted refolding is the binding of the solubilized protein on the chromatography medium. GF is an exception, where the matrix seems to reduce aggregation without binding of the protein. Binding hinders aggregation while allowing folding of the protein. IMAC is an especially useful method because the histidine tag makes the method generic for all proteins with this tag. The tag is generally placed at the N- or C-terminus of the protein, which means that the protein can move freely to allow folding of the polypeptide chain. The affinity between the histidine tag and the immobilized metal ions is stable under most denaturing conditions. Other binding methods such as IEX require multipoint attachments that may reduce movement during folding.

Figure 9.13 gives an example of on-column refolding using IMAC. The work started with solubilization of inclusion bodies. The sample was loaded in the presence of denaturant. Some contaminants passed through the column. Refolding was performed by running a 20 CV gradient from 6 M urea to 0 M over 60 min. The protein was eluted with an imidazole gradient. This method thus allows combining refolding with purification.

Sample:	Clarified homogenate of *E. coli* expressing a histidine-tagged protein
Column:	HisTrap FF (1 ml chelating column charged with Ni^{2+})
Flow rate:	0.5 ml/min during the refolding gradient; 1.0 ml/min during rest of the method
Binding buffer:	6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM β-mercaptoethanol, pH 8.0
Wash buffer:	6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM β-mercaptoethanol, pH 8.0
Refolding buffer:	20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM β-mercaptoethanol, pH 8.0
Elution buffer:	20 mM Tris-HCl, 0.5 M NaCl, 500 mM imidazole, 1 mM β-mercaptoethanol. pH 8.0

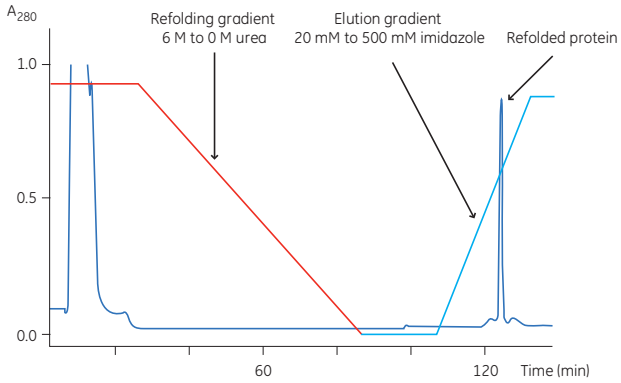


Fig 9.13. On-column refolding using a urea gradient. Purified refolded protein was further analyzed by GF. A major peak is observed at the native molecular weight and also a minor peak at a higher molecular weight, indicating a successful refolding of the protein.

Screening and scaling up matrix-assisted protein refolding

As mentioned earlier, successful refolding usually requires screening of refolding conditions. A powerful workflow for refolding divided in two parts was used for screening and scale-up of refolding of several proteins (Fig 9.14). The first part involved parallel screening based on His MultiTrap FF, 96-well filter plates containing IMAC medium, His MultiTrap FF. The best conditions were selected and used for scale-up on a HisTrap FF 1 ml column.

The development work begins with the inclusion body preparation. In addition to obtaining inclusion bodies, it is often possible to prepare minor amounts of soluble native target protein. This material is important as reference material when analyzing refolding yields. The screening starts with solubilization of the target protein, and the solubilized protein may or may not be purified before application to His MultiTrap. Refolding of the bound protein takes place in the His MultiTrap plate by application of multiple refolding buffers in the different wells. The protein is eluted from the plate and analyzed for native protein. Multiple rounds of screening are possible. Finally, the best refolding conditions are selected and used for scale-up.

A large number of refolding conditions should be tested. The refolding reaction is often slow; it can take from minutes to hours. This makes it very useful to perform screening in a parallel format, and if possible, also the analysis.

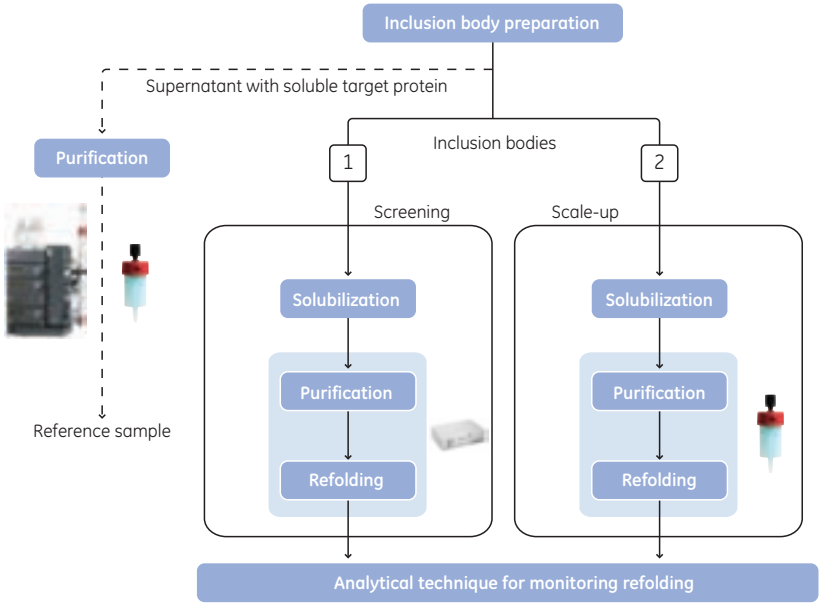


Fig 9.14. Workflow for screening and scale-up of matrix-assisted refolding.

Conditions and additives

Key condition parameters for refolding are buffer substance, pH, and salt concentration. In addition, there are a large number of substances that may be added to the refolding reaction to support protein refolding. Some of these additives are listed in Table 9.1.

Table 9.1. Additives used in refolding

		Effects	
	Additives	**Protein structure**	**Intra- and intermolecular interactions**
Denaturants	Urea	Destabilized	Disrupted
	Guanidine-HCl		
	Strong detergent		
Aggregation suppressors	Arginine-HCl	Neutral	Reduced
	Urea (low conc.)		
	Guanidine-HCl (low conc.)		
	Mild detergents	Neutral	Reduced
	PEGs		
	Proline		
	Cyclodextrins		
Folding enhancers	Sugars	Stabilized	Enhanced
	Polyols		
	Ammonium sulfate		
	Magnesium chloride		
	Glycine		
	Alanine		
Reducing agents	DTT	Reduced S-S bridges	
	DTE		
	TCEP		
	GSH		

The effects of additives on proteins can be divided in two: stabilization or destabilization of protein structure, and reduced or enhanced intra- and intermolecular interactions. In refolding, additives are often grouped into aggregation suppressors and folding enhancers.

The aggregation suppressors reduce interactions, often hydrophobic interactions, but do not have significant effects on native protein. Low concentrations of urea or guanidine-HCl can be used in the refolding solution to reduce protein-protein interactions, but if the concentrations are too high the protein will stay unfolded. Arginine is a common additive that increases solubility of folding intermediates without destabilizing native proteins. Other aggregation suppressors, such as nonionic detergents and polyethylene glycols, are believed to interact with hydrophobic surfaces.

Proteins and their interactions are stabilized by folding enhancers. The mechanisms are not completely understood; thermodynamics theory needs to be part of the discussion. Sugars and polyols are typical folding enhancers. A low concentration of salts can stabilize by nonspecific electrostatic interactions with the protein. Salts used for salting-out of proteins can, at high concentrations, stabilize proteins as well.

Generally, additives are required at high concentrations (100 mM to 2 M) due to their weak interactions with proteins and their sometimes indirect action on proteins in aqueous solution. Finally, reducing agents at critically balanced concentrations are important additives used to hinder erroneous disulfide bonds from forming during refolding.

Figure 9.15 shows a strategy for screening in parallel format using His MultiTrap plates. Refolding parameters are screened in several steps, essentially in order of importance. First, pH and buffer substance are screened. The conditions giving the best refolding yield (often two to three conditions) are selected for the next screening round. Next, NaCl concentrations are tested. Finally, the best conditions from this step are used for setting up a screen of different additives. The additives can be aggregation suppressors, folding enhancers, and reducing agents, as discussed earlier. The preferred alternative to the mentioned strategy is to apply a Design of Experiment (DoE) approach. This allows reduction of the number of experiments that are needed during screening and takes into consideration the effects of combining factors (conditions or additives) on the outcome.

Fig 9.15. Strategy for screening conditions and additives for refolding.

His MultiTrap FF contains 50 µl of Ni Sepharose Fast Flow medium per well. The volume of the wells is large enough to handle at least 500 µl of solution. The runs are performed using a plate centrifuge or a vacuum manifold and can be done manually or using automation.

In the following studies, the fresh plate was first opened, and storage solution was exchanged for buffer. In all steps, solutions were removed from the wells by centrifugation for 5 min at 500 × g. In the second step, the plate was equilibrated with the solution used for the protein solubilization. After equilibration, 50 µg of solubilized protein (100 µl) was applied to each well, the plate was incubated for 2 min, and it was then washed with solubilization solution.

Refolding was accomplished by applying refolding solution and incubating at room temperature for 60 min. Each refolding condition was tested in triplicate. A reference sample containing native protein was included in triplicate and handled as the solubilized samples but the solubilization solution was exchanged for a buffer without denaturant. Elution was performed by applying a buffer with 500 mM imidazole.

The activity of native protein eluted from each well was analyzed and compared with nondenatured starting material.

Below is an example of screening refolding conditions for ferredoxin NADP+ reductase, a monomeric protein (Fig 9.16). Each diagram shows the yield at multiple conditions from a screening step. The best conditions in each step were selected and combined with the different settings of the new parameters of the next screening step. We can see a gradual general improvement of the refolding yield from step to step.

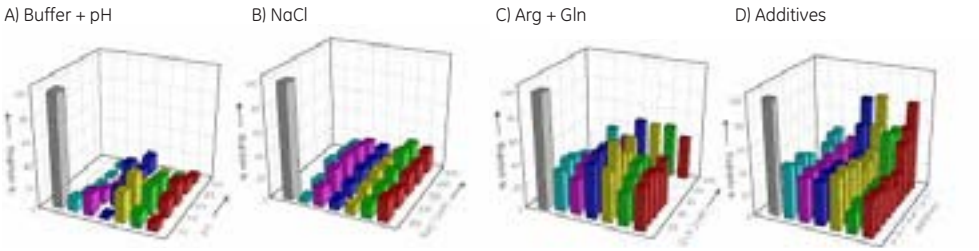


Fig 9.16. Screening of multiple refolding conditions for ferredoxin NADP+ reductase using His MultiTrap FF. The screening was done stepwise; for each screening step (A-D), a few best conditions were selected to be combined with a new parameter in the next screening step. *Acknowledgment: Dashivets, T.* et al.*, Rapid Matrix-Assisted Refolding of Histidine-Tagged Proteins*, Chembiochem. ***10(5)****, 869-876 (2009) (Figure S1 in the Supporting Information). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.*

Scale-up of the optimal refolding conditions for another histidine-tagged protein, GLK, was done using a HisTrap FF column packed with the same chromatography medium as used in His MultiTrap FF (Fig 9.17). The HisTrap FF column was equilibrated with the denaturing buffer used for solubilization of the protein to be denatured. The solubilized sample was loaded on the column, and the column was washed with denaturing buffer. The column was filled with refolding buffer, and the flow was stopped during a 60-min incubation. The column was washed with the refolding buffer, and a gradient of imidazole in equilibration buffer was used to elute the refolded protein.

The described matrix-assisted refolding method supported refolding of proteins with more than one subunit. Citrate synthase and glukokinase (homodimeric proteins) and β-galactosidase (homotetrameric protein; four identical subunits with M_r of 116 000) have been refolded with good yields.

Column:	HisTrap FF 1 ml
Sample:	Histidine-tagged GLK
Denaturing buffer:	40 mM sodium phosphate, 300 mM NaCl, 6 M Gua-HCl, pH 8.0
Equilibration buffer:	40 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, pH 8.0
Refolding buffer:	40 mM sodium phosphate, 200 mM NaCl, 50 mM Arg-HCl, 50 mM Gln, 5 mM TCEP, pH 8.0
Refolding pause:	1 h
Gradient elution:	500 mM imidazole in equilibration buffer
Flow rate:	0.5 ml/min

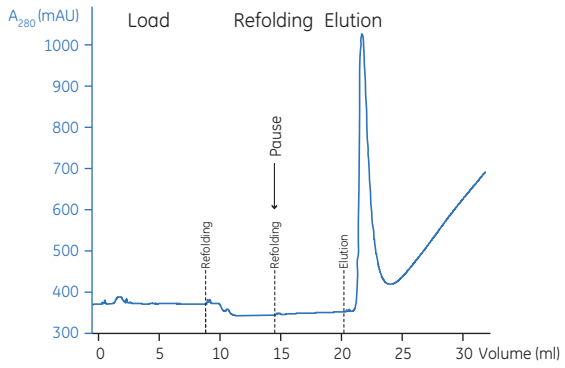


Fig 9.17. Scale-up of refolding of glucokinase on HisTrap FF 1 ml column under selected conditions.

Chapter 10
Large-scale purification

Key concerns in large-scale purification differ to some extent from those typical at laboratory scale. The emphasis in large-scale purification is on the development of robust and cost-effective protocols with a minimum number of unit operations in order to improve overall process economy, all without ever compromising the quality (purity) of the desired product. When going from laboratory scale to production scale, buffer consumption will be an issue, and choice of chromatography media must be thoroughly considered based on economics, security of supply, and adherence to regulatory requirements.

It is recommended to consider the scale-up perspective already at the research stage during the development of new protein drugs so as to avoid problems at later stages, for example, scalability of separation methods, packability of chromatography media, and process economy. The CIPP strategy described in Chapter 4 is valid also for process development for large-scale purification. This chapter gives only a brief introduction of process development and large-scale purification. For further reading, refer to the *Handbook of Process Chromatography: Development, Manufacturing, Validation and Economics* by Lars Hagel, Günter Jaschies, and Gail Sofer, 2nd ed., 2008, Academic Press.

Process development

Development of a biopharmaceutical drug requires, from the outset, laboratory-scale purification to obtain material for research and throughout the discovery and development phases. When the first purification protocol is being developed, future process development needs to be considered by avoiding the use of methods that cannot be efficiently scaled up. Regulatory requirements also change when moving from a purification process for the explorative discovery environment to a purification process for the highly regulated production environment.

Process development involves choosing and optimizing chromatography methods and noting how they are optimized with respect not only to basic performance but also to robustness, simplicity, costs, capacity, and so on. Similarly, the choice of proper chromatography media should be made in terms of chemical and physical stability, functionality, reproducibility, and so on. A thorough knowledge of the purification parameter space is needed to ensure a robust process. It is recommended to explore a wide range of chromatography conditions early, to increase process understanding and increase the likelihood of developing a robust purification process. Figure 10.1 shows a conceptual workflow for process development involving parallel screening, allowing a broad range of chromatography conditions to be explored using PreDictor prefilled 96-well filter plates. Alternatively, the initial screening process can be performed using small columns. After initial screening, media and conditions are selected for further optimization on HiTrap or HiScreen columns prior to scaling up.

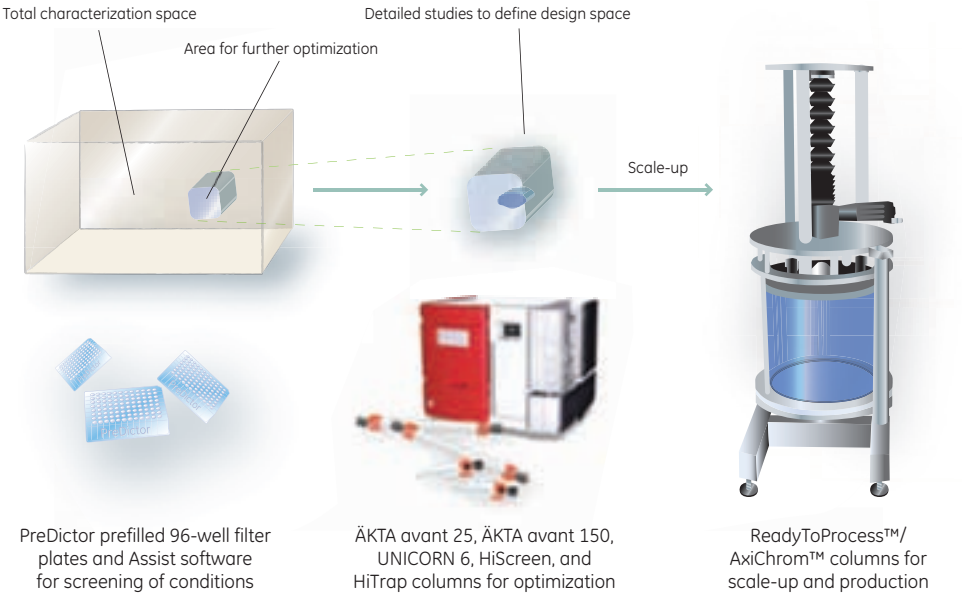


Fig 10.1. Conceptual workflow for process development.

High-throughput process development

Time-to-clinic and time-to-market are key factors for successful biopharmaceutical development. The number of projects and the amount of R&D spending both increase, but the number of new products decreases. Efficient development of the manufacturing process is a crucial component of the overall project plan. However, screening optimal process conditions can be time-consuming and tedious. In addition, steadily increasing demands from regulatory authorities for better understanding and control of manufacturing processes put even more pressure on development work.

The challenge to the process developer in the biopharmaceutical industry is to increase the project throughput and shorten the development time, and at the same time increase process knowledge and design a robust process. Employing high-throughput tools for process development helps address these challenges. High-throughput process development (HTPD) shortens development time at the same time as it increases the amount of information available during early process development, by working in parallel. As a result, a large number of experimental conditions can be evaluated simultaneously. This allows a large experimental space to be characterized and supports the definition of a well-established process design space where process parameters that need to be monitored and controlled are understood.

PreDictor 96-well filter plates are prefilled with GE Healthcare BioProcess chromatography media to support high-throughput process development by allowing parallel screening of chromatography conditions, either in a manual or in an automated workflow. Assist software supports the PreDictor workflow from setup of experimental design to data evaluation. For further information, refer to the handbook *High-throughput Process Development with PreDictor Plates*, Code No. 28-9403-58.

Practical considerations in scale-up

It is important to define the parameters during process development to obtain an efficient process with high productivity and to know how changes influence the process and the final product. Established conditions are used as the basis for scale-up. Scale factors between 10 and 100 per step are recommended. There are a number of chromatography parameters that have to be maintained to ensure conformity in performance between laboratory scale and final production scale:

- Residence time of sample on column (important)
- Maintenance of gradient slope (gradient volume/media volume)
- Sample concentration and composition
- Ratio of sample volume/media volume

Scale-up is obtained by increasing:

- Column diameter
- Volumetric flow rate
- Sample volume proportionally
- Gradient volume proportionally

Increasing the bed volume by increasing the column diameter and increasing volumetric flow, sample load volume, and gradient volume accordingly, will ensure the same performance and cycle time as in laboratory scale during method development.

In IEX, HIC, and AC, the highest productivity is normally obtained with short (10 to 20 cm) and wide columns. In some cases, if the ideal column is not available, it might be an advantage to slightly increase the bed height using a column with a diameter slightly smaller than calculated.

Chapter 11
Chromatography principles and considerations

This chapter introduces some of the key concepts in chromatography and addresses two considerations that are important in most purification situations—peak broadening and backpressure.

Chromatographic purification

In column chromatography purification, the protein of interest may be separated from contaminants by binding to the stationary phase (the chromatography medium), followed by selective elution or, alternatively, by binding impurities, allowing the target protein to pass through the column without being retained. Alternatively, purifications may be performed under conditions that delay sample components (without binding), thereby giving different elution positions. GF is one example of this latter method.

Binding of protein allows protein fractionation during the elution step. Elution is achieved by changing the conditions in the eluent, for example, by increasing salt concentration, changing pH, or adding substances that compete with the target protein for the ligands on the chromatography medium.

There are three possible elution methods:

- Gradient elution: The eluent composition is changed continuously toward conditions favoring protein dissociation from the chromatography medium. Elution position will differ between substances depending on their affinity.
- Stepwise elution: The eluent composition is changed stepwise, at one or several occasions. Several substances may be eluted in each step.
- Isocratic elution: The composition of the eluent is selected to give weak or no interactions between sample components and chromatography medium, and the conditions are kept unchanged during the entire purification (binding and elution). The target protein passes through the column slower or faster than impurities.

Gradient elution allows separation of components with a wider range of properties compared with isocratic elution, and peak tailing is reduced. The steeper the gradient, the closer the proteins will be eluted. Gradients are in most cases linear, but can in some cases be convex, concave, or with a custom-programmed shape in order to optimize the resolution within certain parts of the gradient.

Stepwise elution is a faster alternative to gradient elution; it uses discrete steps instead of a continuous gradient. The buffer consumption is lower, and simpler equipment, for example, manual formats, can be used. Therefore, stepwise elution is often preferred for routine and large-scale purifications. Optimization may be required to achieve satisfactory results. Often optimization of elution conditions is performed by gradient elution, and suitable conditions are selected and then used for step elution.

Isocratic purification can give peaks with a sharp leading edge and a long tail , especially at high loads or strong retention. The use of too strong an eluent will lead to early elution and loss in resolution. The advantage with isocratic elution is simplicity and minimal requirements for equipment and handling. This approach may be suitable for cases where components with very similar properties are separated, because high selectivities can be obtained after proper optimization.

Resolution

Definition

The resolution of a chromatographic purification is a combination of the distance between the peaks eluted from the column (selectivity) and the ability of the column to produce narrow, symmetrical peaks (efficiency). These factors are influenced by practical factors such as matrix properties, binding and elution conditions, column packing, flow rates, and system peak broadening. Resolution (R_s) is defined as the distance between peak maxima compared with the average base width (W_b) of the two peaks. R_s can be determined from a chromatogram, as shown in Figure 11.1.

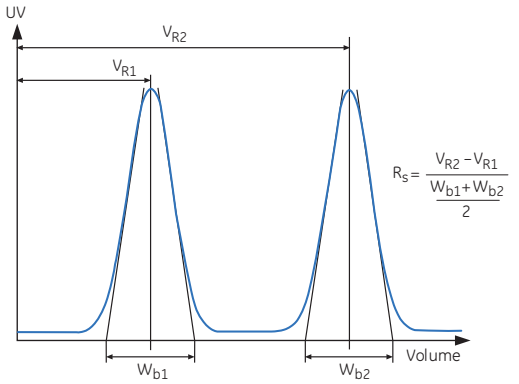


Fig 11.1. Determination of the resolution (R_s) between two peaks.

Elution volumes and peak widths are measured with the same units to give a dimensionless resolution value. R_s gives a measure of the relative separation between two peaks and can be used to determine if further optimization of the chromatography procedure is necessary. If R_s = 1.0 (Figure 11.2), then 98% purity has been achieved at 98% of peak recovery, provided the peaks are symmetrical and approximately equal in size. Baseline resolution requires that R_s > 1.5. At this value, the peak purity is 100% (theoretically 99.9%).

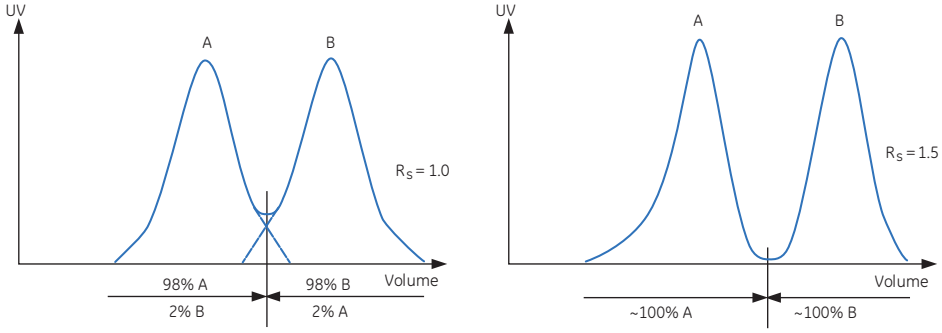


Fig 11.2. Separation results with different resolutions.

A single, well-resolved peak is not necessarily a pure substance, but may represent several components that could not be separated under the chosen elution conditions. Further purification may be required using an alternative chromatography method; see Chapter 2.

Efficiency

Column efficiency (the ability to elute narrow, symmetrical peaks from a packed bed) relates to the peak broadening that occurs on the column and is frequently stated in terms of the number of theoretical plates (see Appendix 3 for determination of column efficiency). The key factors for efficiency are bead size, column dimensions, packing of the column, and flow rate. High efficiency also requires small peak broadening, in the column and in the chromatography system; see the section on peak broadening below. Good column packing gives high efficiency. This is especially true for GF columns. Uneven packing, a too tightly or too loosely packed bed, or a bed containing air bubbles will lead to uneven passage of sample (and buffer) through the column, peak broadening, and hence loss of resolution. Figure 11.3 illustrates the parameters that contribute to good column efficiency. The particle size is a significant factor for efficiency. The smallest particles will give the most efficient mass transfer, which results in the narrowest peaks under suitable elution conditions and with a well-packed column.

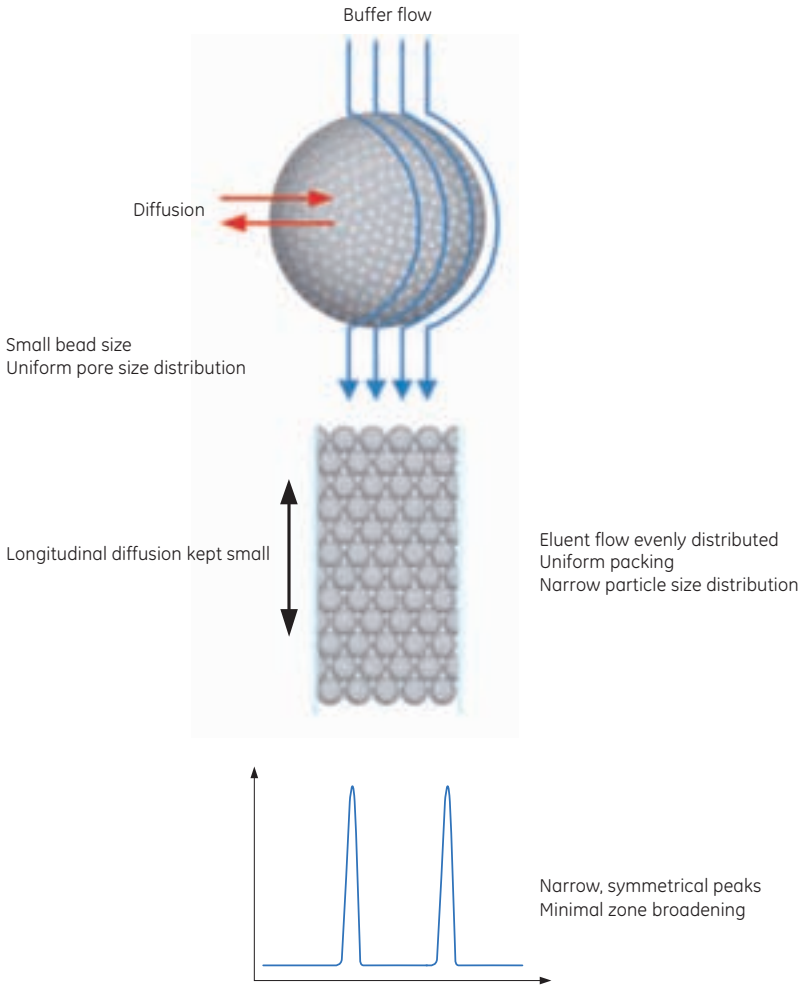


Fig 11.3. Factors that affect column efficiency.

Although efficiency can be improved by decreasing the particle size of the matrix, using smaller particles creates an increase in backpressure so that flow rates need to be decreased, thus lengthening the run time. Hence it is preferable to match the chromatography medium with the requirements for the purification (speed, resolution, purity, etc.).

The viscosity of large volumes of highly concentrated sample may reduce resolution when running columns packed with small particles. Samples may need to be diluted, or larger particles may need to be used.

Selectivity

Selectivity (the degree of separation between peaks) has a great impact on resolution and is a more important factor than high efficiency in determining resolution (Fig 11.4).

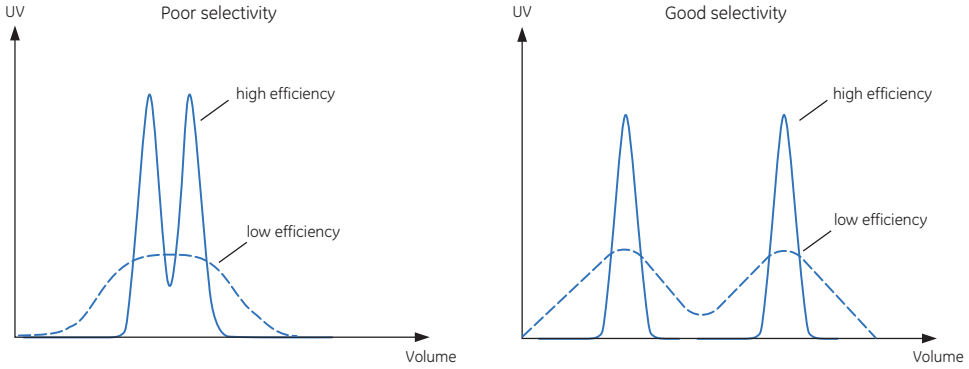


Fig 11.4. Schematic view of the effect of selectivity and efficiency on resolution.

Selectivity depends on several factors: the nature of the ligand (for AC, IEX, HIC, etc.) and its degree of substitution and distribution on the matrix (IEX and HIC); and the characteristics of the matrix, especially the pore structure (GF). The selectivity obviously also depends on the target protein, the impurities, and conditions used for purification, including mode of elution. Establishing a balance between these properties leads to a well-resolved, highly selective separation.

Peak broadening

Resolution greatly depends on the selectivity and efficiency of the chromatography medium. In addition to this, the column hardware and the chromatography system give a broadening of the peaks that are eluted. One of the main causes of peak broadening is longitudinal diffusion (diffusion in the column direction) of the sample. Peak broadening can be minimized if the distances available for longitudinal diffusion are minimized, for example, by keeping columns as short as possible while still obtaining enough selectivity. The combination of chromatography medium and column hardware should be carefully selected; see Table 7.4 in Chapter 7. The use of prepacked columns is recommended, but column packing by the user is also possible; see Appendix 3.

The design of the chromatography system and the suitable combination of column and system is important to avoid extensive peak broadening. All components in the flow path of the chromatography system will contribute to peak broadening to a different degree, for example, tubing/capillaries, cells for in-line detection, valves, and so on. The length of the tubing should be as short as possible. The tubing diameter will also greatly affect peak broadening. Figure 11.5 shows an example of how different tubing diameters in an ÄKTA design system affect the resolution obtained using a Superdex 200 5/150 GL column, that is, more narrow tubing allows for better resolution and selectivity. Chromatography systems for high-resolution chromatography should thus have short and narrow tubing. To obtain the best resolution, it may be necessary to optimize the flow path and only connect the necessary components, for example, column valves, and in-line cells for pH or conductivity measurements.

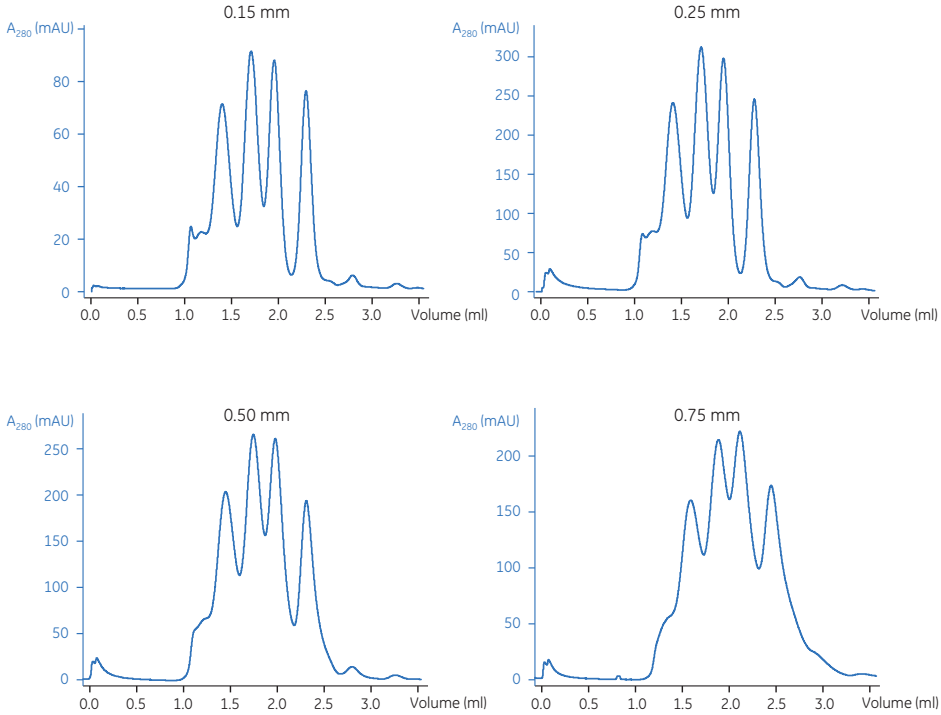


Fig 11.5. Effect of tubing inner diameter (0.15 to 0.75 mm) on resolution. Column: Superdex 200 5/150 GL (column volume: 3 ml); flow rate: 0.3 ml/min.

Importance of a clean column for resolution

Most biological samples will contain substances, for example, lipids or particles from the source material, that tend to be bound nonspecifically to the chromatography medium. Such bound material will change the properties so that the resolution is decreased because of broad and tailing peaks or shifts in retention; see Fig 11.6. A dirty medium may also show decreased binding capacity and increased pressure. Cleaning usually restores the performance.

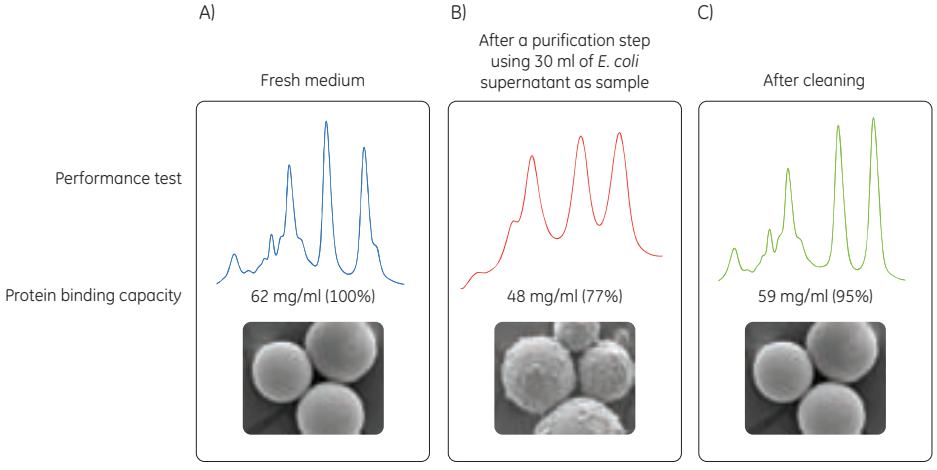


Fig 11.6. Effects of a dirty medium on resolution and binding capacity. Performance of (A) a column packed with fresh medium; (B) a column after use in capture purification; and (C) the column in Fig 11.6B after cleaning.

Effects of sample volume on resolution in GF

The volume of the sample does not affect resolution in purification methods based on binding of sample components, because binding concentrates the target protein(s) in the upper part of the column. In GF the sample is not bound, and there is therefore no concentration of the sample. Because of this, sample volume that is too large will give a decrease in resolution. A rule of thumb is to keep the sample volume smaller than 0.5% of the volume of an analytical GF column and 4% of the volume of a preparative GF column. This can be contrasted with buffer exchange, where a sample volume of up to 30% of the column volume is acceptable, in order to increase the capacity, because of the group separation between low-M_r substances and macromolecules (e.g., proteins).

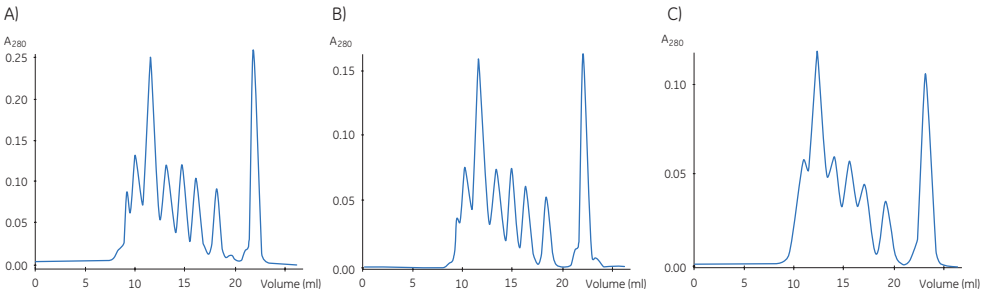


Fig 11.7. Effect of sample volume on resolution in GF on Superdex 200 10/300 GL (Column volume 24 ml). (A) 25 µl sample (0.1% of CV); (B) 250 µl sample (1% of CV); (C) 1000 µl sample (4% of CV).

Handling backpressure

When the measured pressure in a chromatography system reaches a set maximum pressure, it is usually programmed to stop the run or decrease the flow rate, to avoid compressing the bed or breaking the column or parts of the system. An unplanned interruption is obviously unwanted and can often be avoided by taking proper action before starting the purification.

The pressure measured by the system is caused by resistance of the flow of eluent through the column and system components and capillaries. The term backpressure is often used. Increased backpressure can have a number of causes related to the sample, the eluent, the column, and the flow path of the chromatography system.

Sample

In order to obtain trouble-free purification, it is strongly recommended that the samples applied to chromatography columns are thoroughly clarified (see Chapter 6). HisTrap FF crude IMAC columns are exceptions to this rule. These columns accept unclarified cell lysates; see Chapter 7.

Even a well-clarified sample, however, will contain some small amounts of particulate matter that in time will reduce the performance of the column and system, unless cleaning-in-place (CIP) is performed on a regular basis. It should also be noted that a clarified sample, for example, an *E. coli* extract, that is kept for some time or is freeze-thawed, will become opalescent because of precipitate formation. If this happens, the sample should be reclarified before loading on the column. For this purpose, filtration through a 0.22 µm or a 0.45 µm syringe filter is often sufficient.

Samples can also give high pressure due to high viscosity, for example, caused by the presence of nucleic acids or by working in a cold room. Thorough mechanical cell disruption can sometimes avoid this, but it is often useful to add nucleases, for example, Benzonase, to cleave the nucleic acids. Alternatively, nucleic acids can be precipitated using polycationic substances such as polyamines, for example, polyethyleneimine.

Some chromatography systems allow automatic reduction of the flow rate if the pressure increases too much during sample loading; see Figure 11.8. The reduction in flow is balanced to keep the pressure at the maximum allowed pressure until the sample loading has reached its end. When the pressure is going down during the wash, the flow rate is automatically up-regulated to the original setting.

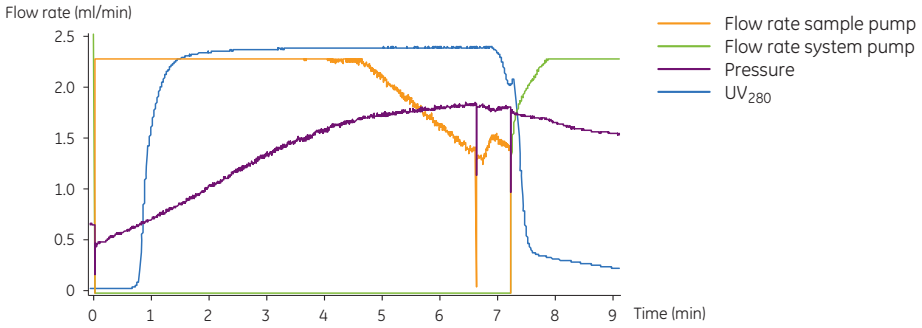


Fig 11.8. Pressure-controlled sample loading available in some chromatography systems.

Eluent

In chromatography, the pressure across column and system depends on the flow, the viscosity of samples and eluents, and the resistance met when passing through the column and system. The viscosity of the eluent depends on its composition and temperature. Table 11.1 gives some examples of pure substances in water to give a general view of the effects of concentrations and temperature on viscosity.

As a rule of thumb: The viscosity of water at 20°C is 1.0 cP but is nearly doubled at 0°C. A typical buffer for protein purification has just slightly higher viscosity than water and will also be doubled when cooling to 0°C. Higher viscosity of the eluent will give higher backpressure in the chromatography system at a given flow rate with a linear relation; see Figure 11.9.
A chromatography column that has a maximum flow rate of 4 ml/min at room temperature using standard buffer should not be run at a flow rate higher than half of this, that is, 2 ml/min when working in the cold room. Some additives are used at high concentrations, for example, glycerol, urea, or guanidine hydrochloride, and can give very high viscosities; see Table 11.1.

The maximum flow rate in the cold room is about half of the maximum flow rate at room temperature.

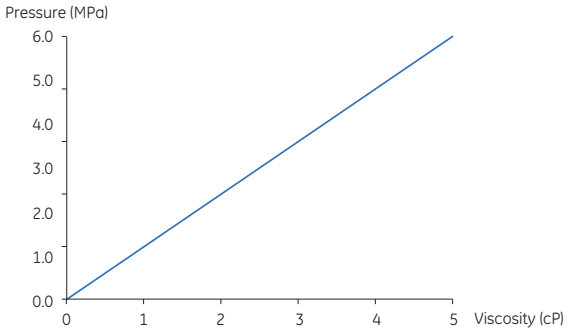


Fig 11.9. Effect of viscosity on backpressure in a capillary. Data correspond to solvent passing with a flow of 10 ml/min through 1 m tubing with an inner diameter of 0.5 mm.

Table 11.1. Viscosity of some substances in water

	Viscosity (cP)		
Composition	**0°C**	**10°C**	**20°C**
Water (Ref. 1)	1.792	1.308	1.005
5% NaCl (0.856 M) (Ref. 2)			1.097
25% NaCl (4.28 M) (Ref. 2)			2.4
10% glycerol (Ref. 3)	2.44	1.74	1.31
50% glycerol (Ref. 2)	14.6		6.00
8 M urea (Ref. 4)			1.7 (at 25°C)
6 M guanidine hydrochloride (Ref. 4)			1.6 (at 25°C)

Column

Full control of column pressure is important when performing purification using stand-alone pumps or chromatography systems. Pressure that is too high can compress the bed or break the column. A column has two pressure limits that must not be passed: the *column hardware pressure limit* and the *maximum pressure over the packed bed*. The pressure denoted p_1 (precolumn pressure) is the pressure at the top of the column. The pressure at this position is caused by the backpressure of the chromatography medium and all system components of the flow path connected to the bottom of the column, downstream; see Figure 11.10. This is the highest pressure that the column hardware is exposed to and should be kept lower than the *column hardware pressure limit*. If this pressure becomes too high, the column may break or start to leak. The pressure drop over the packed bed is $\Delta p=p_1-p_2$. The pressure p_2 at the bottom of the column can only be measured in the ÄKTA avant 25 and ÄKTA avant 150. This pressure drop is caused by the flow resistance of the chromatography medium and should be kept lower than the maximum pressure over the packed bed. If this pressure becomes too high, the bed may start to compress, giving a gap, and possibly cause a sudden further increase in backpressure that may break the column.

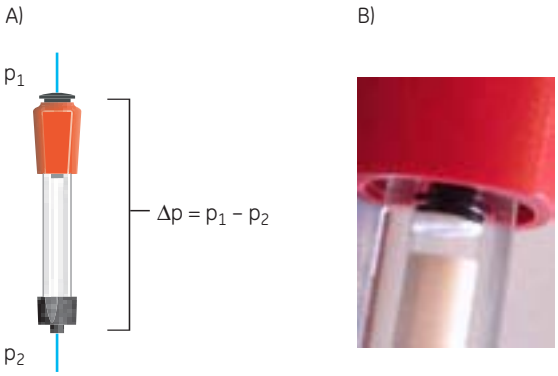


Fig 11.10. Column pressures. (A) Pressure p_1 is the highest pressure that the column hardware is exposed to; p_2 is the pressure at the bottom of the column that is caused by the backpressure from the system components connected downstream of the column. Δp is the pressure drop across the chromatography bed. (B) Gap caused by too high a pressure drop across the chromatography bed.

The pressure drop across a column can be described by the equation below:

$\Delta p = \eta L v / (\theta d_p^2)$

where Δp is the pressure drop across the column, η the eluent viscosity, L the column length, v the linear flow rate, d_p the bead diameter, and θ a dimensionless constant. From this equation we can learn that the pressure drop depends inversely on the square of the particle size (diameter). Small beads give high backpressure and large beads give low backpressure. As was mentioned earlier, small beads gives better resolution than large beads. Thus, selection of chromatography bead size has to be a compromise between resolution and backpressure. The selection of a medium giving high backpressure will require a chromatography system that can handle the high pressure (and the resolution). Refer to the discussion of compatibility of chromatography media and systems in Chapter 7.

Small chromatography beads give higher backpressure than large beads.

Particles from samples or eluents may be accumulated on the top filter of the column or in the chromatography medium. Proper clarification and immediate use of samples (see above), as well as filtration of eluents, will reduce the risk of column deterioration. Using an in-line filter for the eluents is strongly recommended. Particles collected on the column top filter may for some columns be removed by exchanging the top filter or performing cleaning-in-place (CIP) of the column with the flow backward. Take these actions only if recommended in the product instructions. CIP should be performed regularly and according to the manufacturer's instructions.

Chromatography system

In purifications using columns and chromatography systems that are compatible with each other, there should be no backpressure problems due to the chromatography system. Chromatography systems designed for analysis or micropurification contain narrow tubing in order to give optimum performance with high-resolution columns. Narrow tubing gives higher backpressure than wider tubing. A system with narrow tubing will therefore contribute significantly to the total backpressure during a purification run. When using a column designed for purifications at low pressure with such a chromatography system, there is a risk that the pressure in the column rises above the maximum limit. In these cases it may be necessary to adjust the flow path of the chromatography system to reduce the backpressure, thus allowing the purification to take place.

The pressure reading in most chromatography systems is made in the pumps (just after the pumps); see Figure 11.11. This pressure reading corresponds to the pressure drop of the entire flow path of the system including the connected column. This pressure value is an overestimate of the pressure at the top of the column, because the flow path before the column may provide a significant contribution to the total pressure. To obtain the pressure drop across the column, the system backpressure contribution with the same eluent and flow (and temperature) must be determined for the system without the column. The column can be exchanged for a short, wide capillary for this measurement. The pressure drop over the column is obtained by subtracting the pressure reading without the column from the value with the column connected. In most purifications using standard chromatography systems, the described exact determination is not necessary because the system backpressure is low and the obtained pressures are much lower than maximum column pressure. Analytical and microscale chromatography systems containing narrow capillaries give higher system backpressures, and the column pressure drop calculation may be necessary to allow running the purification at the recommended flow for the column.

In ÄKTA avant chromatography systems, additional pressure sensors have been included in the column valve to allow pressure measurements just before and after the column, giving the true p_1 and p_2 pressure values, allowing the calculation of the pressure drop, $\Delta p = p_1 - p_2$.

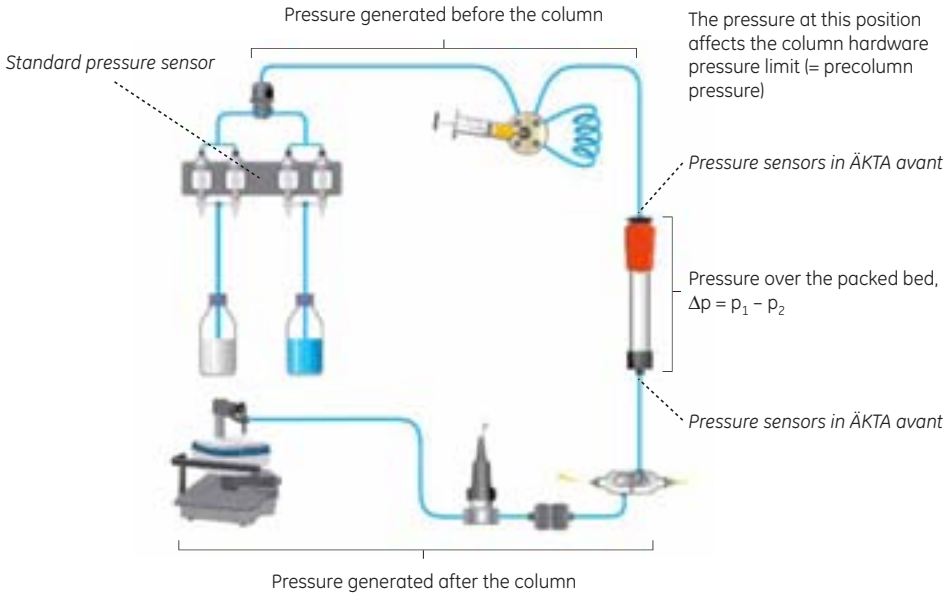


Fig 11.11. Principle of pressure readings in chromatography systems.

References

1. N.E. Dorsey, *Properties of Ordinary Water-Substance,* p. 184. Reinhold Pub. Corp., New York (1940).
2. Specific Gravity and Viscosity of Liquids. http://www.csgnetwork.com/sgvisc.html Accessed September 1, 2010.
3. Viscosity of Aqueous Glycerine Solutions. http://www.dow.com/glycerine/resources/table18.htm Accessed September 1, 2010.
4. Kawahara, K. and Tanford, C. *Viscosity and Density of Aqueous Solutions of Urea and Guanidine Hydrochloride. J Biol Chem* **241**, 3228-3232 (1966).

Appendices
Appendix 1

Biological buffers

Nonvolatile buffers for anion exchange chromatography

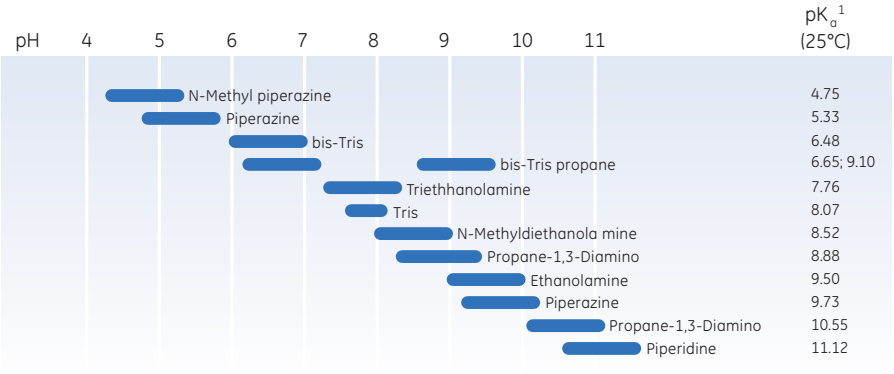


Fig A1.1. pH range and pK_a data for various nonvolatile buffers for AIEX.

Table A1.1. Physical data for various nonvolatile buffers for AIEX

pH interval	Substance	Conc. (mM)	Counter ion	pK_a (25°C)[1]	d(pK_a)/dT (°C)
4.3–5.3	N-Methylpiperazine	20	Cl^-	4.75	-0.015
4.8–5.8	Piperazine	20	Cl^- or $HCOO^-$	5.33	-0.015
5.5–6.5	L-Histidine	20	Cl^-	6.04	
6.0–7.0	bis-Tris	20	Cl^-	6.48	-0.017
6.2–7.2; 8.6–9.6	bis-Tris propane	20	Cl^-	6.65; 9.10	
7.3–8.3	Triethanolamine	20	Cl^- or CH_3COO^-	7.76	-0.020
7.6–8.6	Tris	20	Cl^-	8.07	-0.028
8.0–9.0	N-Methyldiethanolamine	20	SO_4^{2-}	8.52	-0.028
8.0–9.0	N-Methyldiethanolamine	50	Cl^- or CH_3COO^-	8.52	-0.028
8.4–9.4	Diethanolamine	20 at pH 8.4 50 at pH 8.8	Cl^-	8.88	-0.025
8.4–9.4	Propane-1,3-diamino	20	Cl^-	8.88	-0.031
9.0–10.0	Ethanolamine	20	Cl^-	9.50	-0.029
9.2–10.2	Piperazine	20	Cl^-	9.73	-0.026
10.0–11.0	Propane-1,3-diamino	20	Cl^-	10.55	-0.026
10.6–11.6	Piperidine	20	Cl^-	11.12	-0.031

[1] David R. Lide, ed., *CRC Handbook of Chemistry and Physics* (83rd edition), CRC Press (2002)

Nonvolatile buffers for cation exchange chromatography

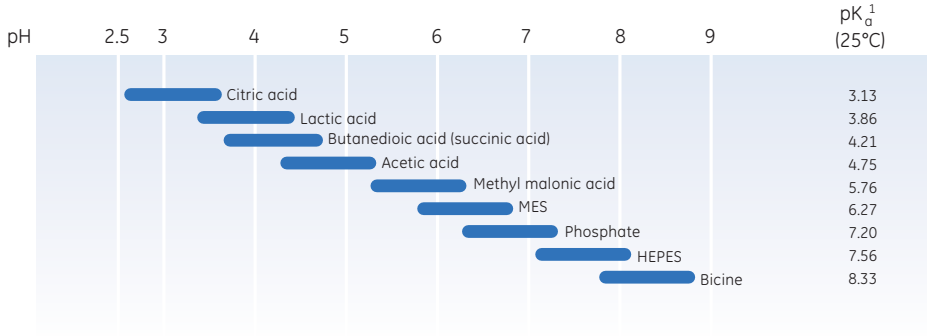


Fig A1.2. pH range and pK_a data for various nonvolatile buffers for CIEX.

Table A1.2. Physical data for various nonvolatile buffers for CIEX

pH interval	Substance	Conc. (mM)	Counter ion	pK_a (25 °C)[1]	d(pK_a)/dT (°C)
1.4–2.4	Maleic acid	20	Na^+	1.92	
2.6–3.6	Methyl malonic acid	20	Na^+ or Li^+	3.07	
2.6–3.6	Citric acid	20	Na^+	3.13	-0.0024
3.3–4.3	Lactic acid	50	Na^+	3.86	
3.3–4.3	Formic acid	50	Na^+ or Li^+	3.75	+0.0002
3.7–4.7; 5.1–6.1	Succinic acid	50	Na^+	4.21; 5.64	-0.0018
4.3–5.3	Acetic acid	50	Na^+ or Li^+	4.75	+0.0002
5.2–6.2	Methyl malonic acid	50	Na^+ or Li^+	5.76	
5.6–6.6	MES	50	Na^+ or Li^+	6.27	-0.0110
6.7–7.7	Phosphate	50	Na^+	7.20	-0.0028
7.0–8.0	HEPES	50	Na^+ or Li^+	7.56	-0.0140
7.8–8.8	Bicine	50	Na^+	8.33	-0.0180

[1] David R. Lide, ed., *CRC Handbook of Chemistry and Physics* (83rd edition), CRC Press (2002)

Volatile buffer systems

Table A1.3. Physical data for various volatile buffers

pH range	Buffer system	Counter ion	pK_a values for buffering ions[1]
3.3–4.3	Formic acid	H^+	3.75
3.3–4.3; 4.8–5.8	Pyridine/formic acid	$HCOO^-$	3.75; 5.25
3.3–4.3; 9.3–10.3	Trimethylamine/formic acid	$HCOO^-$	4.75; 9.81
4.3–5.8	Pyridine/acetic acid	CH_3COO^-	4.75; 5.25
4.3–5.3; 9.3–10.3	Trimethylamine/acetic acid	CH_3COO^-	4.75; 9.81
3.3–4.3; 8.8–9.8	Ammonia/formic acid	$HCOO^-$	3.75; 9.25
4.3–5.3; 8.8–9.8	Ammonia/acetic acid	CH_3COO^-	4.75; 9.25
5.9–6.9; 9.3–10.3	Trimethylamine/carbonate	CO_3^{2-}	6.35; 9.81
5.9–6.9; 8.8–9.8	Ammonium bicarbonate	HCO_3^-	6.35; 9.25
5.9–6.9; 8.8–9.8	Ammonium carbonate/ammonia	CO_3^{2-}	6.35; 9.25
5.9–6.9; 8.8–9.8	Ammonium carbonate	CO_3^{2-}	6.35; 9.25
4.3–5.3: 7.2–8.2	N-Ethylmorpholine/acetate	$HCOO^-$	4.75; 7.72

[1] David R. Lide, ed., *CRC Handbook of Chemistry and Physics* (83rd edition), CRC Press (2002)

Appendix 2
Ammonium sulfate precipitation

In order to maintain protein activity, it is often desirable to perform precipitation at low temperature. Table A2.1 gives the amounts of solid ammonium sulfate to be added to obtain a given percentage saturation at 0°C. The amount to be added varies with temperature. A protocol for ammonium sulfate precipitation at 20°C can be found in *Recombinant Protein Handbook: Principles and Methods* (Code No. 18-1142-75).

1. Clarify the extract and keep on ice.
2. Add 1 part ice-cold 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Add solid ammonium sulfate while stirring. Stir gently for 30 min. (Use Table A2.1 to calculate how much ammonium sulfate to add to obtain the chosen percent saturation [such that it will not precipitate the target protein].)
4. Centrifuge for 20 min at 10 000 to 50 000 × g at a maximum of 4°C.
5. Collect the supernatant (contains the target protein).
6. Add solid ammonium sulfate while stirring. Stir gently for 30 min. (Calculate how much ammonium sulfate to be added to obtain the chosen percent saturation [such that it will precipitate the target protein].)
7. Centrifuge for 20 min at 10 000 to 50 000 g at a maximum of 4°C.
8. Wash the pellet twice by resuspension in an equal volume of ammonium sulfate solution of the same concentration (i.e., a solution that will not redissolve the precipitated protein or cause further precipitation).
9. Centrifuge again.
10. Collect the pellet and dissolve it in a small volume of chromatography buffer.

Ammonium sulfate precipitation can also be accomplished by adding saturated solutions at, for example, 20°C. If required, ammonium sulfate may be removed by desalting on a Sephadex G-25 column, for example, HiTrap Desalting 5 ml (accepts up to 1.5 ml sample) or HiPrep Desalting 53 ml (accepts up to 15 ml sample). Up to 3 to 4 columns can be connected in series to increase capacity. Alternatively, HIC is a convenient following purification step because it requires the presence of ammonium sulfate.


Screening of concentration of ammonium sulfate that begins to precipitate the target protein should preferably be established by following the above procedure in small scale.


If desired, the protocol could be reduced to a single ammonium sulfate addition step, by adjusting the percentage saturation to precipitate the target molecule or to precipitate contaminants.


If the pellet is not solid, increase centrifugation g-value or time, or reduce the presence of DNA by adding nucleases during the extraction.


The quantity of ammonium sulfate required to reach a given degrees of saturation varies according to temperature. See Dawson, R.M.C., *et al., Data for Biochemical Research* (3rd ed.), p. 537. Oxford Science Publications, Clarendon Press, Oxford (1986).

Table A2.1. Amount ammonium sulfate to be added to obtain a certain percentage saturation at 0°C

Initial concentration of ammonium sulfate (percentage saturation at 0°C)	Percentage saturation at 0°C																	
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (g) to be added to 1 L of solution																	
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	239	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	134	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

Data adopted from Englard, S. and Seifter S. Precipitation techniques. Methods Enzymol. **182**, *285–300 (1990)*

Appendix 3
Column packing and preparation

Efficient column packing is essential for a good separation, especially when using gradient elution. A poorly packed column gives rise to poor and uneven flow, peak broadening, and loss of resolution. Prepacked columns from GE Healthcare will ensure reproducible results and the highest performance.

Use small prepacked columns or 96-well filter plates (MultiTrap platform) for chromatography media screening and method optimization to increase efficiency in method development.

If column packing is required, the following guidelines will apply at all scales of operation:

- When using a binding technique, use short, wide columns (typically 5 to 20 cm bed height) for rapid purification, even with low linear flow.
- The amount of chromatography medium required will depend on the binding capacity of the medium and the amount of sample. The binding capacity is always significantly influenced by the nature of the sample as well as the medium itself and must be determined empirically. Estimate the amount of chromatography medium required to bind the sample of interest and use five times this amount to pack the column. The required amount can be reduced if resolution is satisfactory.
- Once separation parameters have been determined, scale up a purification by increasing the diameter of the column to increase column volume. Avoid increasing the length of the column as this will alter separation conditions.

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

HiScale columns offer axial compression of the gel beds, making them well-suited for packing rigid chromatography media. See HiScale packing instructions for more information.

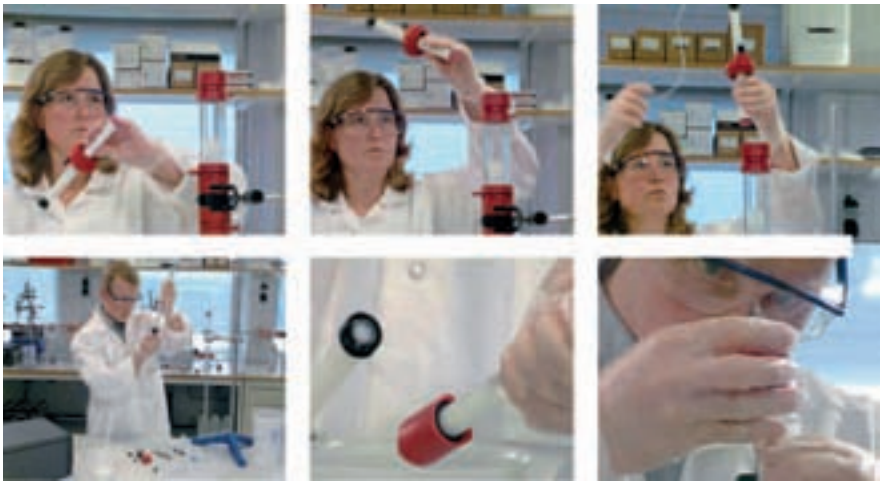


Fig A3.1. "Column Packing — The Movie" provides a step-by-step demonstration of column packing.

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 that 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.

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 in the instruction manual.
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. Fill the column with buffer immediately.
7. Mount the column top piece and connect to a pump.
8. Open the column outlet and set the pump to the desired flow rate (for example, 15 ml/min in an XK 16/20 column).

When the slurry volume is greater than the total volume of the column, connect a second glass column to act as a reservoir or a packing 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 has been 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 a convex surface at the top.
11. Insert the adapter into the column at an angle, ensuring that no air is trapped under the net.
12. Slide the adapter slowly down the column (the outlet of the adapter should be open) until the mark is reached. Lock the adapter in position.
13. Connect the column to the pump and begin equilibration. Reposition the adapter if necessary.

The chromatography medium must be thoroughly washed to remove the storage solution, usually 20% ethanol. Residual ethanol may interfere with subsequent procedures.

Always follow the specific storage instructions supplied with the product.

Column selection

Tricorn, XK, and HiScale columns are compatible with the high flow rates allowed with modern chromatography 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 applications using gravity flow. For a complete listing of available columns, refer to www.gelifesciences.com/protein-purification.

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

	Column size			
	i.d. (mm)	**Length (cm)**	**Bed volume (ml)**	**Bed height (cm)**
Tricorn 5/20	5	2	0.31–0.55	1.6–2.8
Tricorn 5/50	5	5	0.90–1.14	4.6–5.8
Tricorn 10/20	10	2	1.26–2.20	1.6–2.8
Tricorn 10/50	10	5	3.61–4.56	4.6–5.8
Tricorn 10/100	10	10	7.54–8.48	9.6–10.8
HiScale 16/20	16	20	0-40	0-20
HiScale 16/40	16	40	16-80	8-40
HiScale 26/20	26	20	0-106	0-20
HiScale 26/40	26	40	69-212	13-40
HiScale 50/20	50	20	0-393	0-20
HiScale 50/40	50	40	297-785	14-40
XK 16/20	16	20	5–31	2.5–15.0
XK 16/40	16	40	45–70	22.5–35
XK 26/20	26	18	5.3–66	1–12.5
XK 26/40	26	40	122–186	23–35
XK 50/20	50	18	0–274	0–14
XK 50/30	50	30	265–559	13.5–28.5
Empty Disposable PD-10[2]	15	7.4	8.3	4.8–5.0

[1] *All column specifications apply when one adapter is used (not applicable for PD-10)*

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

Determination of column efficiency—control of packing quality

Column efficiency is expressed as the number of theoretical plates per meter of chromatography bed (N) or as H (height equivalent to a theoretical plate, HETP), which is the bed length (L) divided by the plate number. Since column efficiency is related to the peak broadening that can occur on a column, it can be calculated from the expression:

$$N = 5.54 \times \left(\frac{V_R}{W_h}\right)^2$$

V_R = volume eluted from the start of sample application to the peak maximum

w_h = peak width measured as the width of the recorded peak at half of the peak height

H is calculated from the expression:

$$H = \frac{L}{N}$$

L = height of packed bed

Measurements of V_R and w_h can be made in distance (mm) or volume (ml) but both parameters must be expressed in the same unit.

The asymmetry factor, A_s, is calculated from the expression:

$A_s = b/a$

where a = first half peak width at 10% peak height, and b = second half peak width at 10% peak height.

Column performance should be checked at regular intervals by injecting acetone to determine column efficiency (N) and peak shape (asymmetry factor, A_s). Because the observed value for N depends on experimental factors such as flow rate and sample loading, comparisons must be made under identical conditions. Efficiency is measured under isocratic conditions by injecting a substance that can be detected in the flowthrough but that does not interact with the chromatography medium, for example, acetone or NaCl, depending on the medium, and measuring the eluted peak as shown in Figure A3.2.

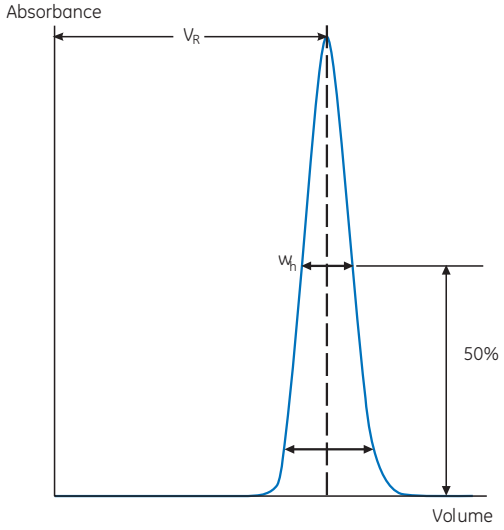


Fig A3.2. Measurement of efficiency and asymmetry.

Appendix 4
Protein determination by absorbance measurements

Proteins generally have UV absorbance maxima at 190 nm caused by the peptide bonds and at 280 nm caused by the aromatic amino acids Trp and Tyr. Protein structure (secondary, tertiary, and quaternary structure) and solution conditions (e.g., pH and ionic strength) may affect the absorbance spectrum. Coenzymes and cofactors may introduce additional absorbance at other wavelengths.

The absorbance is proportional to the protein concentration, c:

$A = c \times E \times l$

where A = absorbance, E = absorbance coefficient, and l = path length of the cuvette in cm. E varies greatly for different proteins. Absorbance at 280 nm (A_{280}) is typically used for concentration determination.

Some proteins, however, lack Trp and Tyr amino acid residues and therefore do not give absorbance at 280 nm. For these proteins, measurement can be made at 205 nm (absorbance from peptide bonds). This value is used instead of the maximum at 190 nm because various technical limitations may be introduced at this low wavelength. In addition to peptide bonds some amino acid side chains also contribute to absorbance at 205 nm. For a detailed protocol, refer to *Current Protocols in Protein Science*, Unit 3.1 (Spectrophotometric Determination of Protein Concentration), John Wiley and Sons, Inc. (date varies, as it is updated regularly).

Absorbance measurements

Absorbance should be measured within the linear range of the absorbance photometer. Keeping the values between 0.2 and 1.0 is recommended, but absorbance values of up to 1.5 to 2 can sometimes be used (see the manual for the instrument used). If higher values are obtained, dilute the sample. A quartz cuvette, or a plastic cuvette or multiwell plates made for UV measurement, should be used. Remember to correct for the pathlength of the cuvette. Fill enough sample to cover the light path.

1. Prepare the instrument. Warming up may take some time.
2. Measure A_{280} for the buffer used (blank).
3. Measure A_{280} for the protein sample. If the value is above the limit for the instrument, dilute the sample.
4. For correction of light scattering contribution at A_{280}, also measure A_{330} for the protein sample and correct as described on the following page.

Protein concentration calculations

The protein concentration, c, in mg/ml is calculated by:

$c = A_{280}/(E_{280,1\ mg/ml}) \times l)$

The absorbance coefficient ($E_{280,1\ mg/ml}$) corresponds to the A_{280} of a 1 mg/ml solution of the protein and varies between proteins. $E_{280,1\ mg/ml}$ can be determined 1) by measuring the absorbance of the protein in a solution of known concentration or 2) by the theoretical calculation:

$E_{280,\ 1\ mg/ml} = (5500n_{Trp} + 1490n_{Tyr} + 125n_{S\text{-}S})/M$

where n_{Trp}, n_{Tyr} and $n_{S\text{-}S}$ are the number of Trp and Tyr residues, $n_{S\text{-}S}$ is the number of disulfide bonds (S-S bonds) in the protein sequence, and M is the molecular weight of the protein. Coenzymes and cofactors may also contribute. Examples of values for $E_{280,\ 1\ mg/ml}$ include 0.67 for BSA, 1.37 for IgG, and 2.64 for lysozyme.

Light scattering correction of the A_{280} value can be made by:

$A_{280} = A_{280}$ (measured) – $1.929 \times A_{330}$ (measured)

Nucleic acids have absorbance at 280 nm (maximum at 260 nm). If the presence of nucleic acids is suspected, the protein concentration can be estimated (with less accuracy) according to Christian, W. and Warburg, O. *Biochemische Zeitung* **310**, 384 (1941):

$C\ (mg/ml) = 1.55 \times A_{280} - 0.76 \times A_{260}$

The constants 1.55 and 0.76 refer to a specific protein used by Christian and Warburg. For best accuracy, the factors should be determined for the target protein at hand. Refer to the NanoVue Plus User Manual, Code No. 28-9574-75 AD from GE Healthcare.

Considerations

- Make sure that plastic cuvettes or multiwell plates are suitable for UV absorbance measurements.
- The cuvette surface should be kept clean on the outside.
- The sample should be free from particles or opalescence. Remove particles by centrifugation or filtration.
- Adding cold solutions into the cuvette may cause fogging of the cuvette surface.
- Warming of a solution in the cuvette may cause air bubbles.

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 rate (cm/h). However, flow is usually measured in volumetric flow rate (ml/min). To convert between linear flow and volumetric flow rate use one of the formulas below:

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

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

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

where

Y = linear flow in cm/h
d = column inner diameter in cm

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

Y = linear flow = 150 cm/h
d = inner diameter of the column = 1.6 cm

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

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

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

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

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

where

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

Example:
What is the linear flow in a Tricorn 5/50 column (i.d. 0.5 cm) when the volumetric flow rate is 1 ml/min?

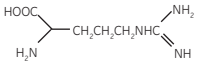
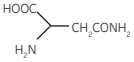
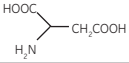
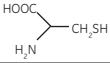
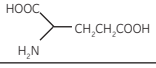
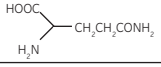
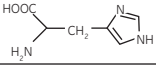
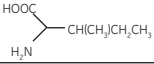
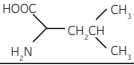
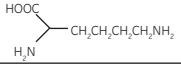
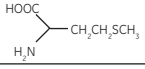
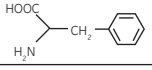
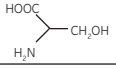
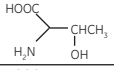
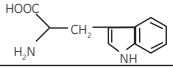
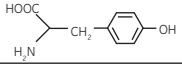
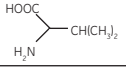
Z = Volumetric flow rate = 1 ml/min
d = column inner diameter = 0.5 cm

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

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

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 acid data

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	■		

Product index*

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

Related literature

	Code no.
Handbooks	
Affinity Chromatography: Principles and Methods	18-1022-29
Antibody Purification: Principles and Methods	18-1037-46
Gel Filtration: Principles and Methods	18-1022-18
Hydrophobic Interaction and Reversed Phase Chromatography: Principles and Methods	11-0012-69
Ion Exchange Chromatography and Chromatofocusing: Principles and Methods	11-0004-21
Recombinant Protein Purification Handbook: Principles and Methods	18-1142-75
GST Gene Fusion System	18-1157-58
Purifying Challenging Proteins	28-9095-31
Cell Separation Media: Methodology and applications	18-1115-69
High-throughput Process Development with PreDictor Plates: Principles and Methods	28-9403-58
Isolation of mononuclear cells: Methodology and applications	18-1152-69
Microcarrier Cell Culture: Principles and Methods	18-1140-62
2-D Electrophoresis using immobilized pH gradients: Principles and Methods	80-6429-60
Nucleic Acid Sample Preparation for Downstream Analyses: Principles and Methods	28-9624-00
Electrophoresis in Practice; Westermeier, R., Wiley-VCH Verlag GmbH, Weinheim (2001)	18-1124-59
Selection guides	
Selection guides can be a great help in planning purifications, for the novice as well as the more experienced worker.	
Prepacked chromatography columns for ÄKTA design systems	28-9317-78
Ni Sepharose and IMAC Sepharose	28-4070-92
Glutathione Sepharose—total solutions for preparation of GST-tagged proteins	28-9168-33
Affinity chromatography columns and media	18-1121-86
Gel filtration columns and media	18-1124-19
Ion exchange columns and media	18-1127-31
Sample preparation for analysis of proteins, peptides, and carbohydrates—desalting, buffer exchange, cleanup, concentration	18-1128-62
Protein and nucleic acid sample prep—get it right from the start	28-9320-93
Note: Interactive selection guides available at www.gelifesciences.com/protein-purification	
Brochures	
Years of experience in every column	28-9090-94
Pure simplicity for tagged proteins	28-9353-64
CDs	
Column Packing CD—The Movie	18-1165-33
Data files and application notes	
Refer to www.gelifesciences.com/protein-purification	

Ordering information

Affinity chromatography

Column	Quantity	Code no.
HiTrap NHS-activated HP	5 × 1 ml	17-0716-01
HiTrap NHS-activated HP	1 × 5 ml	17-0717-01
NHS HP SpinTrap	5 ml medium and 24 empty spin columns	28-9031-28
NHS Mag Sepharose	1 × 500 µl	28-9440-09
NHS Mag Sepharose	4 × 500 µl	28-9514-80
HiTrap rProtein A FF	2 × 1 ml	17-5079-02
HiTrap rProtein A FF	5 × 1 ml	17-5079-01
HiTrap rProtein A FF	1 × 5 ml	17-5080-01
HiTrap rProtein A FF	5 × 5 ml	17-5080-02
HiTrap Protein A HP	2 × 1 ml	17-0402-03
HiTrap Protein A HP	5 × 1 ml	17-0402-01
HiTrap Protein A HP	1 × 5 ml	17-0403-01
HiTrap Protein A HP	5 × 5 ml	17-0403-03
Protein A HP SpinTrap	16 spin columns	28-9031-32
Protein A HP MultiTrap	4 × 96-well plates	28-9031-33
Protein A Mag Sepharose	1 × 500 µl	28-9440-06
Protein A Mag Sepharose	4 × 500 µl	28-9513-78
Protein A Mag Sepharose Xtra	2 × 1 ml	28-9670-56
Protein A Mag Sepharose Xtra	5 × 1 ml	28-9670-62
HiTrap Protein G HP	2 × 1 ml	17-0404-03
HiTrap Protein G HP	5 × 1 ml	17-0404-01
HiTrap Protein G HP	1 × 5 ml	17-0405-01
HiTrap Protein G HP	5 × 5 ml	17-0405-03
Protein G HP SpinTrap	16 spin columns	28-9031-34
Protein G HP MultiTrap	4 × 96-well plates	28-9031-35
Protein G Mag Sepharose	1 × 500 µl	28-9440-08
Protein G Mag Sepharose	4 × 500 µl	28-9513-79
Protein G Mag Sepharose Xtra	2 × 1 ml	28-9670-66
Protein G Mag Sepharose Xtra	5 × 1 ml	28-9670-70
HiTrap MabSelect	5 × 1 ml	28-4082-53
HiTrap MabSelect	1 × 5 ml	28-4082-55
HiTrap MabSelect	5 × 5 ml	28-4082-56
HiTrap MabSelect Xtra	5 × 1 ml	28-4082-58
HiTrap MabSelect Xtra	1 × 5 ml	28-4082-60
HiTrap MabSelect Xtra	5 × 5 ml	28-4082-61
HiTrap MabSelect SuRe	5 × 1 ml	11-0034-93
HiTrap MabSelect SuRe	1 × 5 ml	11-0034-94
HiTrap MabSelect SuRe	5 × 5 ml	11-0034-95

Column	Quantity	Code no.
HiScreen MabSelect	1 × 4.7 ml	28-9269-73
HiScreen MabSelect Xtra	1 × 4.7 ml	28-9269-76
HiScreen MabSelect SuRe	1 × 4.7 ml	28-9269-77
HiTrap Albumin & IgG Depletion	2 × 1 ml	28-9466-03
Albumin & IgG Depletion SpinTrap	10 spin columns	28-9480-20
HiTrap IgM Purification HP	5 × 1 ml	17-5110-01
HiTrap IgY Purification HP	1 × 5 ml	17-5111-01
HiTrap Heparin HP	5 × 1 ml	17-0406-01
HiTrap Heparin HP	1 × 5 ml	17-0407-01
HiTrap Heparin HP	5 × 5 ml	17-0407-03
HiPrep Heparin FF 16/10	1 × 20 ml	28-9365-49
HiTrap Blue HP	5 × 1 ml	17-0412-01
HiTrap Blue HP	1 × 5 ml	17-0413-01
HiScreen Blue FF	1 × 4.7 ml	28-9782-43
HisTrap HP	5 × 1 ml	17-5247-01
HisTrap HP	100 × 1 ml*	17-5247-05
HisTrap HP	1 × 5 ml	17-5248-01
HisTrap HP	5 × 5 ml	17-5248-02
HisTrap HP	100 × 5 ml*	17-5248-05
HisTrap FF	5 × 1 ml	17-5319-01
HisTrap FF	100 × 1 ml*	17-5319-02
HisTrap FF	5 × 5 ml	17-5255-01
HisTrap FF	100 × 5 ml*	17-5255-02
HiScreen Ni FF	1 × 4.7 ml	28-9782-44
HisPrep FF 16/10	1 × 20 ml	28-9365-51
HisTrap FF crude	5 × 1 ml	11-0004-58
HisTrap FF crude	100 × 1 ml*	11-0004-59
HisTrap FF crude	5 × 5 ml	17-5286-01
HisTrap FF crude	100 × 5 ml*	17-5286-02
HiTrap IMAC HP	5 × 1 ml	17-0920-03
HiTrap IMAC HP	5 × 5 ml	17-0920-05
HiTrap IMAC FF	5 × 1 ml	17-0921-02
HiTrap IMAC FF	5 × 5 ml	17-0921-04
HiScreen IMAC FF	1 × 4.7 ml	28-9505-17
HiPrep IMAC FF 16/10	1 × 20 ml	28-9365-52
HiTrap Chelating HP	5 × 1 ml	17-0408-01
HiTrap Chelating HP	1 × 5 ml	17-0409-01
HiTrap Chelating HP	5 × 5 ml	17-0409-03
HiTrap Chelating HP	100 × 5 ml*	17-0409-05
HiTrap Streptavidin HP	5 × 1 ml	17-5112-01
HiTrap Benzamidine FF (high sub)	2 × 1 ml	17-5143-02
HiTrap Benzamidine FF (high sub)	5 × 1 ml	17-5143-01
HiTrap Benzamidine FF (high sub)	1 × 5 ml	17-5144-01

* *Special pack size delivered on specific customer order*

Column	Quantity	Code no.
GSTrap HP	5 × 1 ml	17-5281-01
GSTrap HP	100 × 1 ml*	17-5281-05
GSTrap HP	1 × 5 ml	17-5282-01
GSTrap HP	5 × 5 ml	17-5282-02
GSTrap HP	100 × 5 ml*	17-5282-05
GSTrap FF	2 × 1 ml	17-5130-02
GSTrap FF	5 × 1 ml	17-5130-01
GSTrap FF	100 × 1 ml*	17-5130-05
GSTrap FF	1 × 5 ml	17-5131-01
GSTrap FF	5 × 5 ml	17-5131-02
GSTrap FF	100 × 5 ml*	17-5131-05
GSTrap 4B	5 × 1 ml	28-4017-45
GSTrap 4B	100 × 1 ml*	28-4017-46
GSTrap 4B	1 × 5 ml	28-4017-47
GSTrap 4B	5 × 5 ml	28-4017-48
GSTrap 4B	100 × 5 ml*	28-4017-49
GSTPrep FF 16/10	1 × 20 ml	28-9365-50
StrepTrap HP	5 × 1 ml	28-9075-46
StrepTrap HP	1 × 5 ml	28-9075-47
StrepTrap HP	5 × 5 ml	28-9075-48
MBPTrap HP	5 × 1 ml	28-9187-78
MBPTrap HP	1 × 5 ml	28-9187-79
MBPTrap HP	5 × 5 ml	28-9187-80

Ion exchange chromatography

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
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
Mono Q PC 1.6/5	1 × 0.10 ml	17-0671-01
Mono Q 5/50 GL	1 × 1 ml	17-5166-01
Mono Q 4.6/100 PE	1 × 1.7 ml	17-5179-01
Mono Q 10/100 GL	1 × 8 ml	17-5167-01
Mono Q HR 16/10	1 × 20 ml	17-0506-01
Mono S PC 1.6/5	1 × 0.10 ml	17-0672-01
Mono S 5/50 GL	1 × 1 ml	17-5168-01
Mono S 4.6/100 PE	1 × 1.7 ml	17-5180-01
Mono S 10/100 GL	1 × 8 ml	17-5169-01
Mono S HR 16/10	1 × 20 ml	17-0507-01
Mono P 5/50 GL	1 × 1 ml	17-5170-01
Mono P 5/200 GL	1 × 4 ml	17-5171-01
SOURCE 15Q 4.6/100 PE	1 × 1.7 ml	17-5181-01
SOURCE 15S 4.6/100 PE	1 × 1.7 ml	17-5182-01

* *Special pack size delivered on specific customer order*

Column	Quantity	Code no.
RESOURCE Q, 1 ml	1 × 1 ml	17-1177-01
RESOURCE Q, 6 ml	1 × 6 ml	17-1179-01
RESOURCE S, 1 ml	1 × 1 ml	17-1178-01
RESOURCE S, 6 ml	1 × 6 ml	17-1180-01
HiTrap IEX Selection Kit	7 × 1 ml	17-6002-33
HiTrap SP HP	5 × 1 ml	17-1151-01
HiTrap SP HP	5 × 5 ml	17-1152-01
HiScreen SP HP	1 × 4.7 ml	28-9505-15
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 Q HP	5 × 1 ml	17-1153-01
HiTrap Q HP	5 × 5 ml	17-1154-01
HiScreen Q HP	1 × 4.7 ml	28-9505-11
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 DEAE FF	5 × 1 ml	17-5055-01
HiTrap DEAE FF	5 × 5 ml	17-5154-01
HiScreen Capto DEAE	1 × 4.7 ml	28-9269-82
HiPrep DEAE FF 16/10	1 × 20 ml	28-9365-41
HiScreen Q HP	1 × 4.7 ml	28-9505-11
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 CM FF	5 × 1 ml	17-5056-01
HiTrap CM FF	5 × 5 ml	17-5155-01
HiPrep CM FF 16/10	1 × 20 ml	28-9365-42
HiTrap Q FF	5 × 1 ml	17-5053-01
HiTrap Q FF	5 × 5 ml	17-5156-01
HiScreen Q FF	1 × 4.7 ml	28-9505-10
HiPrep Q FF 16/10	1 × 20 ml	28-9365-43
HiTrap SP FF	5 × 1 ml	17-5054-01
HiTrap SP FF	5 × 5 ml	17-5157-01
HiScreen SP FF	1 × 4.7 ml	28-9505-13
HiPrep SP FF 16/10	1 × 20 ml	28-9365-44
HiTrap ANX FF (high sub)	5 × 1 ml	17-5162-01
HiTrap ANX FF (high sub)	5 × 5 ml	17-5163-01
HiTrap Q XL	5 × 1 ml	17-5158-01
HiTrap Q XL	5 × 5 ml	17-5159-01
HiPrep Q XL 16/10	1 × 20 ml	28-9365-38
HiTrap SP XL	5 × 1 ml	17-5160-01
HiTrap SP XL	5 × 5 ml	17-5161-01
HiPrep SP XL 16/10	1 × 20 ml	28-9365-40
HiTrap Capto IEX Selection Kit	5 × 1 ml	28-9343-88
HiTrap Capto Q	5 × 1 ml	11-0013-02

Column	Quantity	Code no.
HiTrap Capto Q	5 × 5 ml	11-0013-03
HiScreen Capto Q	1 × 4.7 ml	28-9269-78
HiTrap Capto S	5 × 1 ml	17-5441-22
HiTrap Capto S	5 × 5 ml	17-5441-23
HiScreen Capto S	1 × 4.7 ml	28-9269-79
HiTrap Capto MMC	5 × 1 ml	11-0032-73
HiTrap Capto MMC	5 × 5 ml	11-0032-75
HiScreen Capto MMC	1 × 4.7 ml	28-9269-80
HiTrap Capto adhere	5 × 1 ml	28-4058-44
HiTrap Capto adhere	5 × 5 ml	28-4058-46
HiScreen Capto adhere	1 × 4.7 ml	28-9269-81
HiTrap Capto DEAE	5 × 1 ml	28-9165-37
HiTrap Capto DEAE	5 × 5 ml	28-9165-40
HiScreen Capto DEAE	1 × 4.7 ml	28-9269-82

Gel filtration

Superdex Peptide PC 3.2/30	1 × 2.4 ml	17-1458-01
Superdex Peptide 10/300 GL	1 × 24 ml	17-5176-01
Superdex 75 PC 3.2/30	1 × 2.4 ml	17-0771-01
Superdex 75 5/150 GL	1 × 3 ml	28-9205-04
Superdex 75 10/300 GL	1 × 24 ml	17-5174-01
Superdex 200 PC 3.2/30	1 × 2.4 ml	17-1089-01
Superdex 200 5/150 GL	1 × 3 ml	28-9065-61
Superdex 200 10/300 GL	1 × 24 ml	17-5175-01
HiLoad 16/60 Superdex 30 pg	1 × 120 ml	17-1139-01
HiLoad 26/60 Superdex 30 pg	1 × 320 ml	17-1140-01
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
Superose 6 PC 3.2/30	1 × 2.4 ml	17-0673-01
Superose 6 10/300 GL	1 × 24 ml	17-5172-01
Superose 12 PC 3.2/30	1 × 2.4 ml	17-0674-01
Superose 12 10/300 GL	1 × 24 ml	17-5173-01
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
HiPrep 16/60 Sephacryl S-400 HR	1 × 120 ml	28-9356-04
HiPrep 26/60 Sephacryl S-400 HR	1 × 320 ml	28-9356-05
HiPrep 16/60 Sephacryl S-500 HR	1 × 120 ml	28-9356-06
HiPrep 26/60 Sephacryl S-500 HR	1 × 320 ml	28-9356-07

Desalting, buffer exchange, and concentration

Column	Quantity	Code no.
HiTrap Desalting	5 × 5 ml	17-1408-01
HiTrap Desalting	100 × 5 ml*	11-0003-29
HiPrep 26/10 Desalting	1 × 53 ml	17-5087-01
HiPrep 26/10 Desalting	4 × 53 ml	17-5087-02
PD-10 Desalting columns	30	17-0851-01
PD SpinTrap G-25	50	28-9180-04
PD MultiTrap G-25	4 × 96-well filter plates	28-9180-06
PD MiniTrap G-25	50	28-9180-07
PD MidiTrap G-25	50	28-9180-08
PD MiniTrap G-10	50	28-9180-10
PD MidiTrap G-10	50	28-9180-11
Vivaspin 500 MWCO 3000	25	28-9322-18
Vivaspin 500 MWCO 5000	25	28-9322-23
Vivaspin 500 MWCO 10 000	25	28-9322-25
Vivaspin 500 MWCO 30 000	25	28-9322-35
Vivaspin 500 MWCO 50 000	25	28-9322-36
Vivaspin 500 MWCO 100 000	25	28-9322-37
Vivaspin 2 MWCO 3000	25	28-9322-40
Vivaspin 2 MWCO 5000	25	28-9322-45
Vivaspin 2 MWCO 10 000	25	28-9322-47
Vivaspin 2 MWCO 30 000	25	28-9322-48
Vivaspin 2 MWCO 50 000	25	28-9322-57
Vivaspin 2 MWCO 100 000	25	28-9322-58
Vivaspin 6 MWCO 3000	25	28-9322-93
Vivaspin 6 MWCO 5000	25	28-9322-94
Vivaspin 6 MWCO 10 000	25	28-9322-96
Vivaspin 6 MWCO 30 000	25	28-9323-17
Vivaspin 6 MWCO 50 000	25	28-9323-18
Vivaspin 6 MWCO 100 000	25	28-9323-19
Vivaspin 20 MWCO 3000	12	28-9323-58
Vivaspin 20 MWCO 5000	12	28-9323-59
Vivaspin 20 MWCO 10 000	12	28-9323-60
Vivaspin 20 MWCO 30 000	12	28-9323-61
Vivaspin 20 MWCO 50 000	12	28-9323-62
Vivaspin 20 MWCO 100 000	12	28-9323-63

Reversed phase chromatography

SOURCE 5RPC ST 4.6/150	1 × 2.5 ml	17-5116-01
SOURCE 15RPC ST 4.6/100	1 × 1.7 ml	17-5068-01
RESOURCE RPC, 1 ml	1 × 1 ml	17-1181-01
RESOURCE RPC, 3 ml	1 × 3 ml	17-1182-01
µRPC C2/C18 ST 4.6/100	1 × 1.7 ml	17-5057-01

* *Special pack size delivered on specific customer order*

Hydrophobic interaction chromatography

Column	Quantity	Code no.
HiTrap HIC Selection Kit	7 × 1 ml	28-4110-07
HiTrap Phenyl FF (low sub)	5 × 1 ml	17-1353-01
HiTrap Phenyl FF (low sub)	5 × 5 ml	17-5194-01
HiScreen Phenyl FF (low sub)	1 × 4.7 ml	28-9269-89
HiPrep Phenyl FF (low sub) 16/10	1 × 20 ml	28-9365-46
HiTrap Phenyl FF (high sub)	5 × 1 ml	17-1355-01
HiTrap Phenyl FF (high sub)	5 × 5 ml	17-5193-01
HiScreen Phenyl FF (high sub)	1 × 4.7 ml	28-9269-88
HiPrep Phenyl FF (high sub) 16/10	1 × 20 ml	28-9365-45
HiTrap Phenyl HP	5 × 1 ml	17-1351-01
HiTrap Phenyl HP	5 × 5 ml	17-5195-01
HiScreen Phenyl HP	1 × 4.7 ml	28-9505-16
HiLoad 16/10 Phenyl Sepharose HP	1 × 20 ml	17-1085-01
HiLoad 26/10 Phenyl Sepharose HP	1 × 53 ml	17-1086-01
HiTrap Butyl HP	5 × 1 ml	28-4110-01
HiTrap Butyl HP	5 × 5 ml	28-4110-05
HiScreen Butyl HP	1 × 4.7 ml	28-9782-42
HiTrap Butyl FF	5 × 1 ml	17-1357-01
HiTrap Butyl FF	5 × 5 ml	17-5197-01
HiScreen Butyl FF	1 × 4.7 ml	28-9269-84
HiPrep Butyl FF 16/10	1 × 20 ml	28-9365-47
HiTrap Butyl-S FF	5 × 1 ml	17-0978-13
HiTrap Butyl-S FF	5 × 5 ml	17-0978-14
HiScreen Butyl-S FF	1 × 4.7 ml	28-9269-85
HiTrap Octyl FF	5 × 1 ml	17-1359-01
HiTrap Octyl FF	5 × 5 ml	17-5196-01
HiScreen Octyl FF	1 × 4.7 ml	28-9269-86
HiPrep Octyl FF 16/10	1 × 20 ml	28-9365-48
SOURCE 15PHE 4.6/100 PE	1 × 1.7 ml	17-5186-01
RESOURCE ETH	1 × 1 ml	17-1184-01
RESOURCE ISO	1 × 1 ml	17-1185-01
RESOURCE PHE	1 × 1 ml	17-1186-01
RESOURCE HIC Test Kit	3 × 1 ml	17-1187-01

Empty columns

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	1	28-4064-17

Tricorn columns are delivered with a column tube, adapter unit, end cap, a filter kit containing adapter and bottom filters and O-rings, two stop plugs, two fingertight fittings, adapter lock and filter holder, and two M6 connectors for connection to FPLC System, if required

Column	Quantity	Code no.
XK 16/20 column	1	18-8773-01
XK 26/20 column	1	18-1000-72
XK 50/20 column	1	18-1000-71

XK columns are delivered with one AK adapter, 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

HiScale 16/20	1	28-9644-41
HiScale 16/40	1	28-9644-24
HiScale 26/20	1	28-9645-14
HiScale 26/40	1	28-9645-13
HiScale 50/20	1	28-9644-45
HiScale 50/40	1	28-9644-44

HiScale columns are delivered with two adapters, PEEK tubing (1.0 mm i.d. for HiScale 16 and HiScale 26 columns, 2.0 mm i.d. for HiScale 50 columns), Valco fittings, protective tube, 20 µm support snap-on filter rings, and instructions

HR 16/5 column	1	18-1000-98
HR 16/10 column	1	19-7403-01
HR 16/50 column	1	18-1460-01

HR columns are delivered with a column tube, adapter unit, end cap, a filter kit containing adapter and bottom filters and O-rings and M6 male fittings for connection to FPLC System

Empty PD-10 Desalting columns	50	17-0435-01

Complete information on the range of empty columns is available at www.gelifesciences.com/protein-purification

Accessories and spare parts

LabMate PD-10 Buffer Reservoir	10	18-3216-03
Packing Connector XK 16	1	18-1153-44
Packing Connector XK 26	1	18-1153-45
Tricorn packing equipment 10/100†	1	18-1153-25
Tricorn packing connector 10-10‡	1	18-1153-23
1/16" male/Luer female§	2	18-1112-51
Tubing connector flangeless/M6 female§	2	18-1003-68
Tubing connector flangeless/M6 male§	2	18-1017-98
Union 1/16" female/M6 male	6	18-1112-57
Union M6 female /1/16" male	5	18-3858-01
Union Luerlock female/M6 female	2	18-1027-12
HiTrap/HiPrep, 1/16" male connector for ÄKTA design	8	28-4010-81
Stop plug female, 1/16"¶	5	11-0004-64
Fingertight stop plug, 1/16"**	5	11-0003-55

† *Tricorn packing equipment 10/100 includes Tricorn packing connector 10-10, Tricorn 10/100 glass tube, bottom unit, and stop plug*
‡ *Connects extra glass column to a Tricorn 10 column to act as a packing reservoir for efficient packing*
§ *One connector included in each HiTrap package*
¶ *Two, five, or seven female stop plugs included in HiTrap packages, depending on products*
** *One fingertight stop plug is connected to the top of each HiTrap column*

For a complete listing refer to www.gelifesciences.com/protein-purification

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