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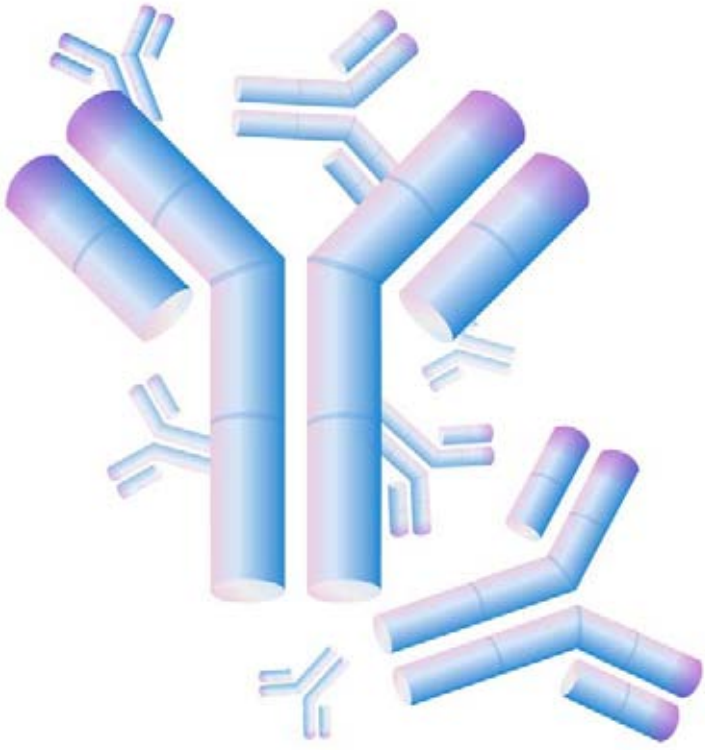

Antibody Purification

Handbook

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Antibody Purification

Handbook

Contents

Common acronyms and abbreviations

A_{280}	UV absorbance at specified wavelength (in this example, 280 nm)
AC	affinity chromatography
AIEX	anion exchange chromatography
APMSF	4-aminophenyl-methylsulfonyl fluoride
BSA	bovine serum albumin
cGMP	current good manufacturing practice
CHO	Chinese hamster ovary
CIEX	cation exchange chromatography
CIP	cleaning-in-place
CV	column volume
DNAse	deoxyribonuclease
DOC	deoxycholate
DS	desalting
EDTA	ethylene diaminetetraacetic acid
EGTA	ethylene glycol-O,O'-bis-[2-amino-ethyl]-N,N,N',N',-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
$F(ab')_2$ fragment	fragment with two antigen binding sites, obtained by pepsin digestion
Fab fragment	antigen binding fragment obtained by papain digestion
Fc fragment	crystallizable fragment obtained by papain digestion
Fv fragment	unstable fragment containing the antigen binding domain
GF	gel filtration
GST	glutathione S-transferase
HCP	host cell protein
HIC	hydrophobic interaction chromatography
HMW	high molecular weight
HSA	human serum albumin
IEX	ion exchange chromatography
IgA, IgG etc.	different classes of immunoglobulin
LC-MS	liquid chromatography –mass spectrometry
LMW	low molecular weight
MAb	monoclonal antibody
MPa	megaPascal
M_r	relative molecular weight
MS	mass spectrometry
n	native, as in nProtein A
NHS	N-hydroxysuccinimide
PBS	phosphate buffered saline
PEG	polyethylene glycol
pI	isoelectric point, the pH at which a protein has zero net surface charge
PMSF	phenylmethylsulfonyl fluoride
psi	pounds per square inch
PVDF	polyvinylidene fluoride
PVP	polyvinylpyrrolidine
r	recombinant, as in rProtein A
RNAse	ribonuclease
RPC	reversed phase chromatography
scFv	single chain Fv fragment
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCEP	tris(2-carboxyethyl) phosphine hydrochloride
Tris	tris-(hydroxymethyl)-aminomethane
v/v	volume to volume
w/v	weight to volume

Introduction

The diversity of the antibody-antigen interaction and our ability to manipulate the characteristics of the interaction has created many uses for antibodies and antibody fragments, both for immunochemical techniques within general research and for therapeutic and diagnostic applications.

The use of recombinant technology opens up the potential to create an infinite number of combinations between immunoglobulins, immunoglobulin fragments, tags and selected proteins, further manipulating these molecules to our advantage.

The purpose of this handbook is to present the most effective and most frequently used strategies for sample preparation and purification of the many different forms of antibodies and antibody fragments used in the laboratory. Advice is given on how to plan a laboratory-scale purification strategy, beginning with a consideration of the factors shown in Figure 1.

Multistep strategies for scaling up antibody purification to industrial scale are also addressed in this handbook.

Wherever possible, examples and practical protocols are included to provide a ready-to-use solution or at least a good starting point for further optimization of a specific purification.

It is hoped that this blend of general guidance and specific examples will assist the reader in a successful approach to any purification of antibodies.

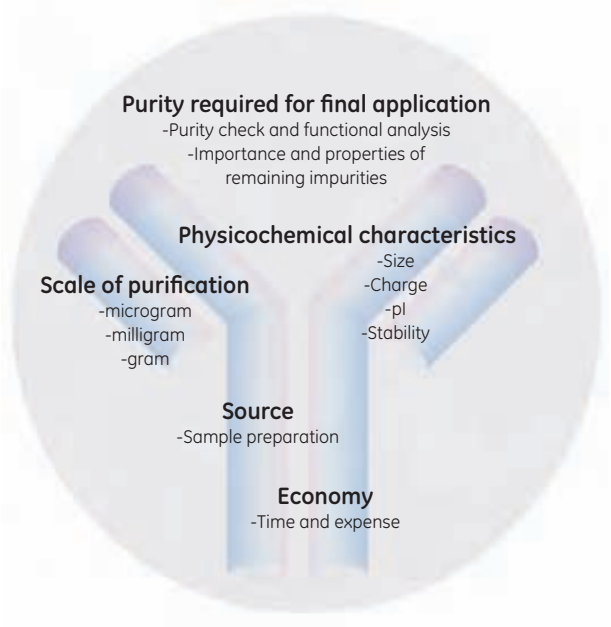


Fig 1. Factors to consider when planning purification.

Symbols and abbreviations

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

this symbol denotes advice that should be regarded as mandatory and indicates when special care should be taken.

highlights chemicals, buffers, and equipment.

outline of experimental protocol.

Chapter 1
Antibody structure, classification, and production

Antibodies are members of a family of molecules, the immunoglobulins, that constitute the humoral branch of the immune system and form approximately 20% of the plasma proteins in humans. Different populations of immunoglobulins are found on the surface of lymphocytes, in exocrine secretions and in extravascular fluids. Antibodies are host proteins produced in response to foreign molecules or other agents in the body. This response is a key mechanism used by a host organism to protect itself against the action of foreign molecules or organisms. B-lymphocytes carrying specific receptors recognize and bind the antigenic determinants of the antigen and this stimulates a process of division and differentiation, transforming the B-lymphocytes into plasma cells. It is these lymphoid or plasma cells that predominantly synthesize antibodies.

Native sources

Immunoglobulins

All immunoglobulins, independent of their specificity, have a common structure with four polypeptide chains: two identical heavy (H) chains, each carrying covalently attached oligosaccharide groups; and two identical, nonglycosylated light (L) chains. A disulfide bond joins a heavy chain and a light chain together. The heavy chains are also joined to each other by disulfide bonds. These disulfide bonds are located in a flexible region of the heavy chain known as the *hinge*, a region of approximately 12 amino acids that is exposed to enzymatic or chemical cleavage. Each globular region formed by the folding of the polypeptide chains as a result of the disulfide bonding is termed a *domain*. All four polypeptide chains contain constant (C) and variable (V) regions, found at the carboxyl and amino terminal portions, respectively. Heavy and light chains have a single V region. Heavy chains contain three C regions while light chains possess a single C region. The V regions of both heavy and light chains combine to form two identical antigen binding sites (the parts of the antibody which bind the antigen). Effector functions of antibodies, such as placental transport or antigen-dependent cellular toxicity, are mediated by structural determinants within the Fc region of the immunoglobulin. Figure 1.1 illustrates the basic H2L2 structure of a typical immunoglobulin.

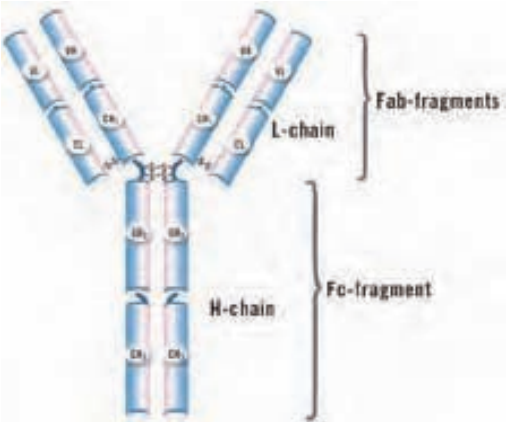


Fig 1.1. Basic H2L2 structure of a typical immunoglobulin.

Immunoglobulins are divided into five major classes according to their H chain components: IgG (γ), IgA (α), IgM (μ), IgD (δ) and IgE (ε). There are two types of light chain, κ and λ. Individual molecules may contain κ or λ chains but never both. In man, the ratio of immunoglobulins containing κ or λ light chains is about 60:40, whereas in mouse the ratio is 95:5. Figure 1.2 and Table 1.1 provide a summary of human and mouse antibody classes and their physicochemical characteristics.

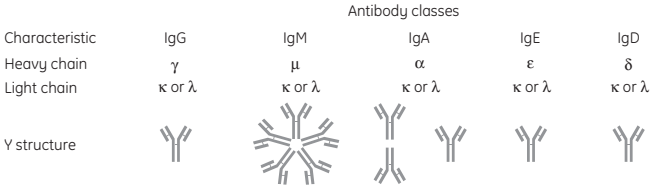


Fig 1.2. The five major classes of immunoglobulin.

1. Antibodies of classes G, D, and E are of monomeric type H2L2.
2. IgA in serum is mainly monomeric, but in secretions, such as saliva and tears, IgA is found as a dimer held together by the secretory piece and the J-polypeptide chain (H2L2)-SC-J-(H2L2). The dimer has four antigen binding sites.
3. IgM is composed of five monomeric units $(H2L2)_5$ and has 10 antigen binding sites.
4. IgG and IgA are further divided into subclasses that result from minor differences in the amino acid sequence within each class. In humans, the four IgG subclasses IgG_1, IgG_2, IgG_3, and IgG_4 have g_1, g_2, g_3, and g_4 heavy chains, respectively. Mouse IgG has four IgG subclasses: IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3, with heavy chains g_1, g_{2a}, g_{2b}, and g_3. These heavy chains have virtually the same size and similar electrophoretic properties, but their amino acid sequences differ considerably. Human IgA has two subclasses; IgA_1 and IgA_2, while mouse IgA has only one subclass.

Table 1.1a. Physicochemical properties of human immunoglobulins

Immunoglobulin	Heavy chain	Light chain	Sedimentation coefficient	Relative molecular weight (M_r)	M_r, heavy chain	Carbohydrate content (%)	$A_{280\ nm}$	pI
IgG_1	λ_1	κ, λ	7S	146 000	50 000	2–3	13.8	5.0–9.5
IgG_2	λ_1	κ, λ	7S	146 000	50 000	2–3		5.0–8.5
IgG_3	λ_1	κ, λ	7S	170 000	60 000	2–3		8.2–9.0
IgG_4	λ_1	κ, λ	7S	146 000	50 000	2–3		5.0–6.0
IgM	μ	κ, λ	19S	900 000	68 000	12	12.5	5.1–7.8
IgA_1	α_1	κ, λ	7S	160 000	56 000	7–11	13.4	5.2–6.6
IgA_2	α_2	κ, λ	7S	160 000	52 000	7–11		5.2–6.6
IgD	δ	κ, λ	7S	184 000	68 000	12	17.0	–
IgE	ε	κ, λ	8S	190 000	72 000	12	15.3	–

Table 1.1b. Physicochemical properties of mouse immunoglobulins

Immunoglobulin	Heavy chain	Light chain	Sedimentation coefficient	Relative molecular weight (M_r)	M_r, heavy chain	Carbohydrate content (%)	pI
IgG_1	λ_1	κ, λ	7S	150 000	50 000	2–3	7.0–8.5
IgG_{2a}	λ_{2a}	κ, λ	7S	150 000	50 000	2–3	6.5–7.5
IgG_{2b}	λ_{2b}	κ, λ	7S	150 000	50 000	2–3	5.5–7.0
IgG_3	λ_3	κ, λ	7S	150 000	50 000	2–3	–
IgM	μ	κ, λ	19S	900 000	80 000	12	4.5–7.0
IgA	α	κ, λ	7S	170 000	70 000	7–11	4.0–7.0
IgD	δ	κ, λ	7S	180 000	68 000	12–14	–
IgE	ε	κ, λ	8S	190 000	80 000	12	–

IgY immunoglobulin

The use of avian antibodies, IgY, has several major advantages. Avian species produce an elevated antibody response to highly conserved, weakly immunogenic mammalian antigens. Because of the phylogenetic distance between birds and mammals, IgY can be used to provide a source of highly specific antibodies against mammalian antigens with minimum cross-reactivity.

The antibodies are most commonly produced in eggs, which are more easily collected than blood samples. A few eggs per week can provide the same amount of immunoglobulin as repeated bleeding of an immunized rabbit.

Antibody fragments

Partial enzymatic digestion of immunoglobulins generates biologically active antibody fragments that can be used to elucidate antibody structure or as specific reagents. These fragments can also be produced using recombinant technology.

Fragmentation of immunoglobulins has created the potential for new applications. For example, chimeric, non-immunogenic 'humanized' mouse Fab, Fab', and $F(ab')_2$ fragments are of great interest in tumor therapy since they penetrate tumors more rapidly and are also cleared from the circulation more rapidly than full-size antibodies.

Figure 1.3 shows the fragments created by enzymatic cleavage.

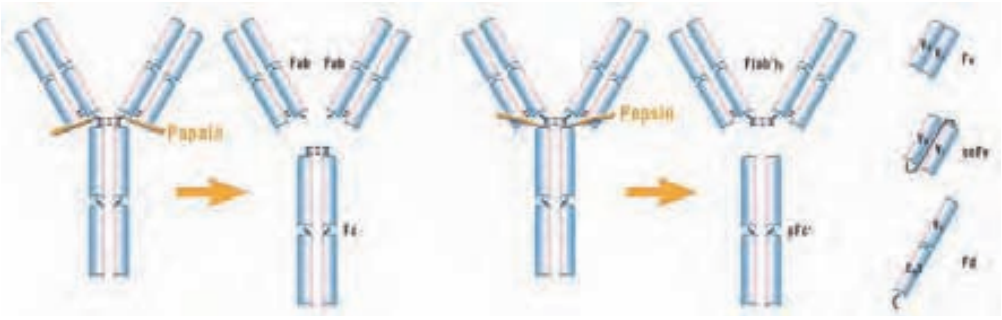


Fig 1.3. Antibody fragments are created by enzymatic cleavage.

The most common types of antibody fragments are:

Fab and Fc fragments: papain digestion creates two Fab (antigen binding) fragments and one Fc (crystallizable) fragment.

F(ab')$_2$ fragment: pepsin digestion creates a fragment containing two antigen binding sites and comprises two Fab units and the hinge.

Fv fragment: an unstable fragment able to bind to an antigen. An Fv fragment has two V regions, V_L and V_H.

Single chain Fv fragment (scFv): scFv is a stable variant of Fv, commonly produced by recombinant technology, in which a peptide linker connects the two V regions.

Fd fragment: the N-terminal half of the H chain.

Polyclonal antibodies

Most frequently, a host will produce a large number of antibodies that recognize independent *epitopes* (the antibody binding site) on the antigen. Each specific antibody is produced by a different clone of plasma cells. Serum is a very good source of polyclonal antibodies. These antibodies are commonly used as reagents in immunochemical techniques, using crude serum as the source. Further purification may be required, either to isolate the group of polyclonal antibodies or to isolate a specific antibody from the group.

Monoclonal antibodies

Monoclonal antibodies (MAbs) are highly specific antibodies produced from hybridoma cells. These hybridoma cells are created by isolating plasma cell precursors, which are then fused with immortal cells. The hybridoma cells can be single-cell cloned and expanded as individual clones that secrete only one antibody type, a monoclonal antibody. The high specificity of a monoclonal antibody is a significant advantage, particularly in therapeutic applications. Monoclonal antibodies are frequently used in the form of tissue culture supernatants harvested from the hybridoma culture, or as crude extracts produced from hybridoma cells grown as tumors in syngeneic mice.

Production of monoclonal antibodies using hybridoma technology has been successful for the production of mouse monoclonal antibodies, but this has meant that therapeutic applications have always been associated with the risk of immunogenic reactions (only human antibodies are nonimmunogenic to humans). The development of genetically engineered antibodies and antibody fragments seems likely to overcome the problem of the high immunogenicity of mouse MAbs (see Genetically engineered sources on the next page).

Genetically engineered sources

Recombinant technology is used increasingly for the manipulation and production of antibodies and their fragments.

For antibodies to be most effective when used as a therapeutic agent they should have a long serum half-life, low immunogenicity, a high affinity for the antigen, and be able to neutralize the activity of the antigen. These are all features that can be enhanced by genetic manipulation. To reduce immunogenicity, mouse-human chimeric antibodies have been produced, containing some human constant region sequences along with the mouse V regions (Fig 1.4). Another approach to reducing immunogenicity is to produce humanized monoclonal antibodies that contain human sequences. Antibody phage libraries and breeding transgenic mice that contain parts of the human immune system provide alternative sources of therapeutic antibodies with a fully human sequence. For a comprehensive review of the state of production of human antibodies with low immunogenecity from transgenic mice, see reference 1.

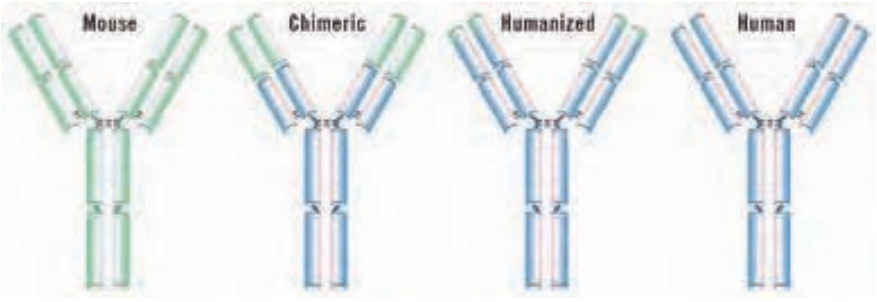


Fig 1.4. Various modifications of both native and recombinant antibodies are now possible.

Antibody fragments

Figure 1.4 illustrates various modifications to monoclonal antibodies. The enzymatic mechanisms used to generate antibody fragments are shown in Figure 1.3 on page 9.

While MAbs still represent the fastest growing class of biopharmaceuticals, smaller recombinant antibody fragments such as classic monovalent antibody fragments (Fab, scFv, etc.) are now emerging as credible alternatives (see reference 2). Moreover, recombinant antibody fragments known as diabodies, triabodies, and minibodies, as well as single-domain antibodies are under evaluation as biopharmaceuticals. These fragments possess the targeting specificity of whole MAbs, but can be produced more economically while having a range of diagnostic and therapeutic applications. For a review of recombinant antibody fragments for therapeutic use, see reference 2.

Recombinant antibodies

For research, diagnostic, and therapeutic applications the potential uses for antibody fusion proteins are vast. Combining a fusion partner with all or part of an antibody can enable the antibody or fragment to access specific areas of the host (e.g., crossing the blood-brain barrier), carry an enzyme to a specific site (e.g., for therapy or to create a drug at site) or carry a toxin to a specific target for therapy.

Antibody fusion proteins are divided into two groups:

1. Fab and F(ab')$_2$ fusions, in which the single or double antigen binding site(s) is/are retained and a fusion partner either replaces or is linked to the Fc domain.
2. Fc fusions, also known as immuno-adhesions, in which the antigen recognition site is replaced by the fusion partner, but the Fc region is retained. Depending upon the type of immunoglobulin involved, an Fc fusion will retain effector functions and can confer a longer half-life to the fusion protein.

Tagged recombinant antibodies and fragments

Amplification of a protein containing a tag of known size and biological function greatly simplifies subsequent isolation, purification, and detection. For example, (histidine)$_6$ or GST tags are now in common use to enable simple affinity purification at any scale. In some cases, protein yield can also increased. Adding tags of this type is also extremely useful if the target molecule has no Fc region (the presence an Fc region enables purification with Protein A Sepharose™ or Protein G Sepharose affinity media).

Epitope tags (short peptide sequences to which strongly binding, highly specific antibodies have already been produced) are used for detection and purification in many immunological methods. Table 1.2 reviews some of the practical advantages and disadvantages of using tagged antibodies.

Table 1.2. Advantages and disadvantages of tagged recombinant antibodies

Advantages	Disadvantages
Solubility and stability can be improved	Tag may interfere with protein structure and affect folding and biological activity
Targeting information can be incorporated into a tag	
Tags provide a marker for expression	If tag needs to be removed, 100% cleavage may not always be achieved and amino acids may sometimes remain on the antibody
Simple purification is possible using affinity chromatography under denaturing or nondenaturing conditions. Generic two-step purification protocols can often be set up for lab-scale protein production platforms	Recombinant proteins accumulated in inclusion bodies, which are insoluble aggregates of misfolded protein lacking biological activity. The isolation of proteins from inclusion bodies often leads to difficulties in refolding and full recovery of biological activity
Detection of the tag instead of the target protein moiety allows for a generic detection method in, for example, protein production platforms for structural biology	Tag cleavage and purification difficult in practice. Intellectual property issue restrict use of tagged recombinant antibodies
Some tags allow strong binding to chromatography media in the presence of denaturants, making on-column refolding better	Tag cleavage and purification difficult in practice. Intellectual property issues restrict use of tagged recombinant antibodies
Ideal for secreted proteins as the product is easily isolated from the growth medium. Some tags allow strong binding to chromatography media in the presence of denaturants, making on-column refolding possible	Tag cleavage and purification difficult in practice. Intellectual property issues restrict use of tagged recombinant antibodies

General guidelines for the amplification and purification of recombinant proteins, as well as the handling of inclusion bodies are covered in detail in the *Recombinant Protein Purification Handbook: Principles and Methods* (code number 18-1142-75) and the *GST Gene Fusion System Handbook* (18-1157-58) from GE Healthcare.

References

1. Lonberg, N. Human antibodies from transgenic animals. *Nature Biotechnology* **23 (9)**, 1117–1125 (2005).
2. Holliger, P. and Hudson, P. J. Engineered antibody fragments and the rise of single domains. *Nature Biotechnology* **23 (9)**, 1126–1136 (2005).

Chapter 2
Sample preparation

Sources and their associated contaminants

Antibodies and antibody fragments are produced from native and recombinant sources. Table 2.1 reviews some of the most common options.

The choice of source material can affect the selection of techniques for sample preparation and purification due to the differences in specific contaminants and the required quantity of target molecule. However, in many cases, the high selectivity of an affinity purification medium for a specific molecule minimizes contamination and produces a sample of high purity in a single step.

Table 2.1. Summary of sources of contamination of native and recombinant antibodies

	Molecular types	Quantity	Significant contaminants
Source: native			
Human serum	Polyclonal IgG, IgM, IgA, IgD, IgE	IgG 8–16 mg/ml IgM 0.5–2 mg/ml IgA 1–4 mg/ml IgE 10–400 ng/ml IgD up to 0.4 mg/ml	Albumin, transferrin, α_2-macroglobulin, and other serum proteins
Hybridoma: cell culture supernatant	Monoclonal	Up to 1 mg/ml	Phenol red, albumin, transferrin, bovine IgG, α_2-macroglobulin, other serum proteins, viruses
Hybridoma: cell culture supernatant, serum-free	Monoclonal	1–4 mg/ml	Albumin, transferrin (often added as supplements)
Ascites	Monoclonal	1–15 mg/ml	Lipids, albumin, transferrin, lipoproteins, endogenous IgG, other host proteins
Egg yolk	IgY	3–4 mg/ml	Lipids, lipoproteins, vitellin
Source: recombinant			
Extracellular protein expressed into supernatant	Tagged antibodies, antibody fusion proteins, Fab, or F(ab')$_2$ fragments	Depends upon expression system	Proteins from the host, e.g., *E. coli*. General low level of contamination
Intracellular protein expression		Depends upon expression system	Proteins from the host, e.g., *E. coli*, phage

An advantage of cell culture systems is the unlimited volume and quantity of material that can be produced. For ascites, production is limited and in certain countries, significant legal restrictions are imposed on production.

Extraction of recombinant antibodies and antibody fragments

The source and location of the recombinant molecule will determine the extraction procedure. Bacterial or mammalian origin, inter- or intra-cellular expression systems giving soluble product or inclusion bodies will all have special demands.

Buffer components should be selected to provide favorable extraction conditions. Table 2.2 reviews some commonly used buffers and additives. Selection of an extraction technique depends as much on the equipment available and scale of operation as on the type of sample. Examples of common extraction processes are shown in Table 2.3.

- Use procedures that are as gentle as possible; there is a trade off between efficient extraction and risk for proteolytic degradation.
- Use additives (see Table 2.2) only if essential for stabilization of the product or to improve extraction. Select additives that are easily removed, otherwise an additional purification step may be required.
- Denaturating additives such as 8 M urea or 6 M guanidine hydrochloride may be necessary if solubilization of the protein is needed e.g., if the protein is expressed as an inclusion body.

Table 2.2. Common buffers and additives for extraction of recombinant antibodies

	Typical conditions for use	Purpose
Buffer components		
Tris	20 mM, pH 7.4	Stabilize pH
NaCl	100 mM	Maintain ionic strength of medium
EDTA	10 mM	Reduce oxidation damage, chelate metal ions
Sucrose or glucose	25 mM	Stabilize lysosomal membranes, reduce protease release
Ionic or nonionic detergent		Solubilize poorly soluble proteins For details on handling inclusion bodies, please see Recombinant Protein Purification Handbook
DNAse and RNAse	1 µg/ml	Degradation of nucleic acids in order to reduce viscosity of sample solution
Protease inhibitors[1]		**Inhibits**
PMSF	0.5–1 mM	Serine proteases
APMSF	0.4–4 mM	Serine proteases
Benzamidine-HCl	0.2 mM	Serine proteases
Pepstatin	1 µM	Aspartic proteases
Leupeptin	10–100 µM	Cysteine and serine proteases
Chymostatin	10–100 µM	Chymotrypsin, papain, cysteine proteases
Antipain-HCl	1–100 µM	Papain, cysteine and serine proteases
EDTA	2–10 mM	Metal-dependent proteases (zinc and iron)
EGTA	2–10 mM	Metal-dependent proteases (calcium)

Table continues on next page.

Table 2.2 cont.

	Typical conditions for use	Purpose
Reducing agents		
1, 4 dithiothreitol, DTT	1–10 mM	Keep cysteine residues reduced
1, 4 dithioerythritol, DTE	1–10 mM	Keep cysteine residues reduced
Tris(2-carboxyethyl)phosphine hydrochloride, TCEP	0.5–5 mM	Keep cysteine residues reduced
Others		
Glycerol	5% to 10%	For stabilization, up to 50% can be used if required

PMSF – Phenylmethylsulfonyl fluoride
APMSF – 4-Aminophenyl-methylsulfonyl fluoride
[1] Protease inhibitors are available in premade mixes from several suppliers
Details from: Scopes, R. K., Protein Purification, Principles and Practice*, Springer, (1994), Janson, J. C. and Rydén, L.,* Protein Purification, Principles, High Resolution Methods and Applications*, 2nd ed. John Wiley and Sons, Inc., New York, (1998)*

PMSF is a hazardous chemical with a halflife time in aqueous solution of 35 min. PMSF is usually stored as 10 mM or 100 mM stock solution (1.74 or 17.4 mg/ml) in isopropanol at -20°C.

Table 2.3. Common sample extraction processes for recombinant antibodies and antibody fragments

Extraction process	Typical conditions	Comment
Gentle		
Cell lysis (osmotic shock)	Two volumes water to one volume packed prewashed cells	Reduced protease release, but lower product yield
Enzymatic digestion	Lysozyme 0.2 mg/ml, 37°C, 15 min	Laboratory scale only, often combined with mechanical disruption
Moderate		
Grinding with abrasive, e.g., glass beads	Add glass beads to prewashed cells, vortex, centrifuge, repeat up to five times, pooling supernatants	Physical method. Chemical conditions are less important for cell lysis but may be important for subsequent removal of cell debris and purification steps
Freeze/thaw	Freeze cells, thaw, resuspend pellet by pipetting or gentle vortexing in room temperature lysis buffer. Incubate, centrifuge, retain supernatant	Several cycles
Vigorous		
Ultrasonication or bead milling	Follow equipment instructions	Small scale; release of nucleic acids may cause viscosity problems (DNase bead milling to decrease viscosity); inclusion bodies must be resolubilized
Manton-Gaulin homogenizer	Follow equipment instructions	Large scale
French press	Follow equipment instructions	Laboratory scale
Fractional precipitation	See page 20	Precipitates must be resolubilized

Extraction should be performed quickly at subambient temperatures in the presence of a suitable buffer (see Table 2.2) in order to maintain pH and ionic strength and to stabilize the sample. If lysates are too viscous to handle due to a high concentration of host nucleic acid, continue to sonicate on ice for a longer period, or follow one of the following procedures (A–C):

A. Add DNase I to a final concentration of 10 µg/ml or
B. Add RNase A to a final concentration of 10 µg/ml and DNase I to 5 µg/ml, and incubate on ice for 10–15 min **or**
C. Draw the lysate through a syringe needle several times to avoid adding enzymes.

Clarification of serum, ascites, cell culture supernatant, or cell lysate

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

Centrifuge and filter any sample immediately before chromatographic purification.

Lipids and lipoproteins can clog chromatography columns and should be removed prior to purification. Ascites have a particularly high lipoprotein content. For removal of lipoproteins, see page 19.

Phenol red is often added to cell culture supernatants as a pH indicator. Since phenol red may bind to certain chromatography media, it is advisable to remove it prior to purification, see page 20.

Centrifugation and filtration

Centrifugation removes most particulate matter, such as cell debris. 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 2.4 as a second step.

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

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).

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.

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

Filtration removes particulate matter. Membrane filters that give the least amount of nonspecific binding of proteins are composed of cellulose acetate or polyvinylidene fluoride (PVDF). For sample preparation before chromatography, select a filter pore size in relation to the bead size of the chromatographic medium as shown in Table 2.4.

Table 2.4. Selecting filter pore sizes

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

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

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

Sample preparation before purification

The main tasks of the sample preparation stage prior to purification are:

- Removal of specific impurities such as lipoproteins or phenol red from the source material.
- Removal of gross impurities such as bulk protein from the source material.
- Buffer exchange and desalting to transfer sample to the correct buffer conditions (pH and salt concentration) and to remove unwanted small molecules.

Removal of specific impurities before purification

Lipoproteins

Lipoproteins and other lipid material can clog chromatography columns. It is advisable to remove them before beginning purification. Ascites often have a high content of lipid material.

The alternatives described here are suitable for treatment of serum, ascites, and cell culture supernatant.

Centrifuge samples to avoid the risk of nonspecific binding of the target molecule to a filter. Samples such as serum can be filtered through glass wool to remove remaining lipids.

Alternative 1:
Dextran sulfate precipitates lipoproteins in the presence of divalent cations, such as Ca^{2+}. The precipitate can be removed by centrifugation.

1. Add 0.04 ml 10% dextran sulfate solution and 1 ml 1 M $CaCl_2$/ml of sample.
2. Mix for 15 min.
3. Centrifuge 10 000 × g for 10 min.
4. Discard the precipitate.
5. Transfer the sample into a buffer suitable for purification using a desalting column (see page 24).

Alternative 2:
Polyvinylpyrrolidine (PVP) produces a pH-dependent precipitation effect. Note that 8% PVP precipitates β-lipoproteins and euglobulins at pH 7.0, but lipoproteins do not precipitate below pH 4.0.

1. Add solid PVP to the sample solution to a final concentration of 3% (w/v).
2. Stir for 4 h at 4°C.
3. Centrifuge at 17 000 × g.
4. Discard the precipitate.
5. Transfer the sample into a buffer suitable for purification using a desalting column (see page 24).

Phenol red

Phenol red is used as a pH indicator in laboratory-scale cell culture. Although not directly interfering with purification, phenol red may bind to certain purification media and should be removed as early as possible. Phenol red is known to bind to anion exchange media at pH > 7.

Use a desalting column to simultaneously remove the low molecular weight phenol red and transfer sample to the correct buffer conditions for further purification (see page 24).

Removal of gross impurities by precipitation

Low molecular weight contaminants

If samples contain a high level of low molecular weight contaminants, use a desalting column as described further on p 24 to prepare the sample for the first chromatography step.

Fractional precipitation

Increased salt concentration can enhance hydrophobic interaction between proteins. Differences in hydrophobicity result in a selective precipitation. Fractional precipitation is occasionally used at laboratory scale and in small-scale commercial production to remove gross impurities from small sample volumes.

When using a HiTrap™ affinity purification column at laboratory scale, it is unlikely that fractional precipitation will be required.

Fractional precipitation can be applied to remove gross impurities in three different ways, as shown in Figure 2.1.

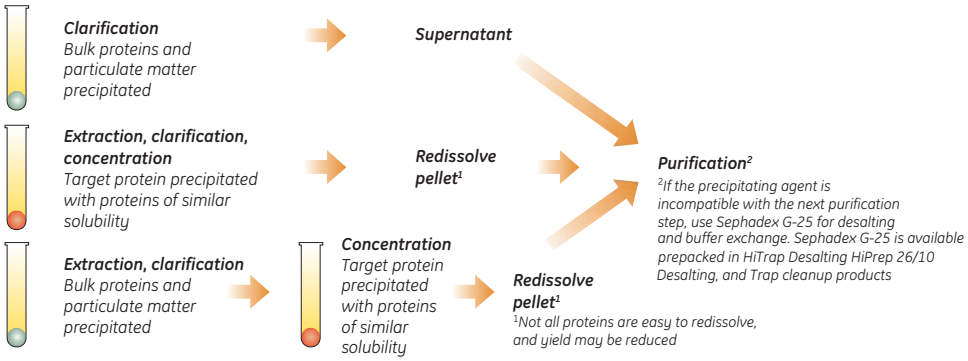


Fig 2.1. Three ways to use precipitation.

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

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

Examples of precipitation agents are reviewed in Table 2.5. The most common precipitation method using ammonium sulfate is described in more detail below.

Table 2.5. Examples of precipitation techniques

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

Details from: Scopes, R. K., Protein Purification, Principles and Practice, *Springer, (1994), Janson, J. C. and Rydén, L.,* Protein Purification, Principles, High Resolution Methods and Applications, *2nd ed. John Wiley and Sons, Inc., New York, (1998)*

Ammonium sulfate precipitation

Ammonium sulfate precipitation is frequently used for initial sample concentration and cleanup. This method is particularly effective for removing the bulk of albumin, transferrin, and other highly soluble contaminants.

Salt precipitation of monoclonal antibodies is, however, not recommended as salt-fractionated monoclonals are invariably contaminated with polyclonal host antibody.

The principle of the method is to increase the concentration of ammonium sulfate to a level where antibodies will begin to "salt out." Different antibodies salt out at different

concentrations, a process that can be used to advantage to remove contaminating proteins from the crude extract. The salt concentration needs to be optimized to remove contaminants and not the desired antibody. An additional step with increased salt concentration should then precipitate the antibody. If the antibody cannot be safely precipitated and redissolved, only the first step should be employed.

HIC is often an excellent follow-up, as the sample already contains a high salt concentration and can be applied directly to the HIC column with little or no additional preparation. The elevated salt level enhances the interaction between the hydrophobic components of the sample and the chromatography medium.

Solutions needed for precipitation:

Saturated ammonium sulfate solution: add 100 g ammonium sulfate to 100 ml distilled water, stir to dissolve

1 M Tris-HCl, pH 8.0

Buffer for the first purification step

1. Filter (0.45 µm) or centrifuge the sample (10 000 × g at 4°C).
2. Add 1 part 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently. Add ammonium sulfate solution, drop by drop, up to 50% saturation*. Stir for 1 h.
4. Centrifuge 20 min at 10 000 × g.

Discard any lipoproteins that form a layer after centrifugation. Samples can be filtered through glass wool to remove any remaining lipids.

5. Remove supernatant. Wash the pellet twice by resuspension in an equal volume of ammonium sulfate solution of the same concentration (i.e., a solution that will not redissolve the precipitated protein or cause further precipitation). Centrifuge again.
6. Dissolve pellet in a small volume of the buffer to be used for the next step.
7. Ammonium sulfate is removed during clarification/buffer exchange steps with Sephadex G-25, using desalting columns.

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

Some antibodies may be damaged by direct application of solid ammonium sulfate to the sample. The precipitating ammonium sulfate should be added as an aqueous concentrate and care taken to minimize introduction of air during resuspension.

It may be practical to use HIC as second step after an initial ammonium sulfate precipitation.

For routine, reproducible purification, precipitation with ammonium sulfate should be avoided in favor of chromatography using protein G or protein A Sepharose media.

In general, salt precipitation is rarely effective for protein concentrations below 1 mg/ml.

Adding an equal volume of saturated (or even 35% to 40% saturated) solution reduces contamination by transferrin and albumin.

The quantity of ammonium sulfate required to reach a given degree of saturation varies according to temperature. Table 2.6 shows the quantities required at 20°C.

Table 2.6. Quantities of ammonium sulfate required to reach given degrees of saturation at 20°C

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

Caprylic acid precipitation

Caprylic (octanoic) acid is as effective as ammonium sulfate and can be used to precipitate the bulk of proteins from sera and ascites. Caprylic acid is one of several fatty acids that have been evaluated for antibody precipitation and the only fatty acid used for the precipitation of monoclonal antibodies.

Using caprylic acid can help to avoid the formation of protein aggregates.

Unlike ammonium sulfate, caprylic acid does not concentrate the immunoglobulins as these are left in solution.

This technique is not recommended for cell culture supernatants because of low yields and sample dilution.

Poorly soluble antibodies may precipitate with the contaminants. Check recovery.

A protocol for caprylic acid precipitation of a monoclonal antibody from ascites is provided on the next page as a starting point from which other specific protocols can be developed.

Solutions needed for precipitation:

Caprylic (octanoic) acid
2 M HCl
2 M NaOH
Buffer for the first purification step

1. Mix volume of ascites with twice the volume of 50 mM acetate buffer, pH 4.0.
2. Adjust to pH 4.5 with 2 M HCl or NaOH.
3. Slowly add caprylic acid (1:15 w/w), stirring constantly.
4. Continue stirring for 30 min.
5. Centrifuge at 1 000 × g for 10 min.
6. Remove supernatant and adjust to pH 6.0 with 2 M NaOH.
7. Remove the caprylic acid and prepare the sample for further purification using a desalting column.

Resolubilization of antibody precipitates

Many antibodies are easily resolubilized in a small amount of the buffer to be used in the next chromatographic step. However, a denaturing agent may be required for less soluble antibodies. Specific conditions will depend upon the specific antibody. Denaturing agents must always be removed to allow complete refolding of the antibody and to maximize recovery of mass and activity. A chromatographic step often removes a denaturant during purification. Table 2.7 gives examples of common denaturing agents.

Table 2.7. Denaturing agents used for solubilization of less soluble proteins and their removal from solution

Denaturing agent	Typical conditions for use	Removal/comment
Urea	2–8 M	Remove using Sephadex G-25
Guanidine hydrochloride	3–6 M	Remove using Sephadex G-25 or IEX
Triton™ X-100	2%	Remove using Sephadex G-25 or IEX
Sarcosyl	1.5%	Remove using Sephadex G-25 or IEX
N-octyl glucoside	2%	Remove using Sephadex G-25 or IEX
Sodium dodecyl sulfate	0.1% to 0.5%	Exchange for nonionic detergent during first chromatographic step; avoid anion exchange chromatography
Alkaline pH	> pH 9, NaOH	May need to adjust pH during chromatography to maintain solubility

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

Desalting and buffer exchange

General considerations

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

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 2.8). These products contain Sephadex G-25, a gel filtration medium that allows effective removal of low molecular weight substances from antibodies with a molecular weight > 5000.

Use desalting/buffer exchange when needed, before and/or between purification steps. Remember that each extra step can reduce yield and that desalting often dilutes the sample (centrifugation protocols do not dilute samples).

Remove salts and other low molecular compounds from proteins with molecular weight > 5000.

Table 2.8. Selection guide for desalting/buffer exchange columns

Columns and 96-well plates	Medium	Loaded volume (ml)	Eluted volume (ml)	Dilution factor	Operation
HiTrap Desalting	Sephadex G-25 Superfine	0.25	1.0	4	Syringe/pump/ chromatography system
		0.5	1.5	3	Syringe/pump/ chromatography system
		1.0	2.0	2	Syringe/pump/ chromatography system
		1.5 (max.)	2.0	1.3	Syringe/pump/ chromatography system
2× HiTrap Desalting	Sephadex G-25 Superfine	3.0 (max.)	4.0–5.0	1.3–1.7	Syringe/pump/ chromatography system
3× HiTrap Desalting	Sephadex G-25 Superfine	4.5 (max.)	6.0–7.0	1.3–1.7	Syringe/pump/ chromatography system
HiPrep 26/10 Desalting	Sephadex G-25 Fine	10	10–15	1.0–1.5	Pump/chromato-graphy system
		15 (max.)	15–20	1.0–1.3	Pump/chromato-graphy system
2× HiPrep 26/10 Desalting	Sephadex G-25 Fine	30 (max.)	30–40	1.0–1.3	Pump/chromato-graphy system
3× HiPrep 26/10 Desalting	Sephadex G-25 Fine	45 (max.)	45–55	1.0–1.2	Pump/chromato-graphy system
4× HiPrep 26/10 Desalting	Sephadex G-25 Fine	60 (max.)	60–70	1.0–1.2	Pump/chromato-graphy system
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 or vacuum
PD MiniTrap G-25	Sephadex G-25 Medium	0.2–0.5	0.1–0.5	No dilution	Centrifuge
	Sephadex G-25 Medium	0.1-0.5	1.0	2	Gravity flow
PD MidiTrap G-25	Sephadex G-25 Medium	0.5–1.0	0.5–1.0	No dilution	Centrifuge
	Sephadex G-25 Medicum	0.1-0.5	1.5	1.5	Gravity flow
PD-10 Desalting Columns	Sephadex G-25 Medium	1.0–2.5	1.0–2.5	No dilution	Centrifuge
	Sephadex G-25 Medium	0.1-0.5	3.5	1-1.5	Gravity flow

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 antibodies does not exceed approximately 70 mg/ml when using normal aqueous buffers, and provided that the antibody is stable and soluble at the concentration used.

When desalting is the first chromatography step, the sample should first be clarified; centrifugation and/or filtration are recommended.

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

Desalting provides several advantages over dialysis, which is generally a slow technique requiring large volumes of buffer and carries the risk of losing material during handling.

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

Buffer exchange can sometimes be avoided by dilution to reduce ion strength, addition of ammonium sulfate before hydrophobic interaction chromatography (HIC) or titration to adjust pH.

Small-scale desalting of samples

For sample volumes ranging from 0.2 ml to 2.5 ml, it is possible to run multiple samples in parallel with PD-10 Desalting Columns, PD MidiTrap™ G-25, and PD MiniTrap™ G-25 gravity columns. Two different protocols are available for these gravity columns: one for manual use on the laboratory bench; and one for use together with a standard centrifuge in combination with a Spin Adapter.

For smaller sample volumes in the range of 70 to 130 µl, multiple samples can be run on PD SpinTrap™ G-25 spin columns together with a microcentrifuge or PD MultiTrap™ G-25 96-well plate using centrifugation or vacuum for extraction (Fig 2 A-D).

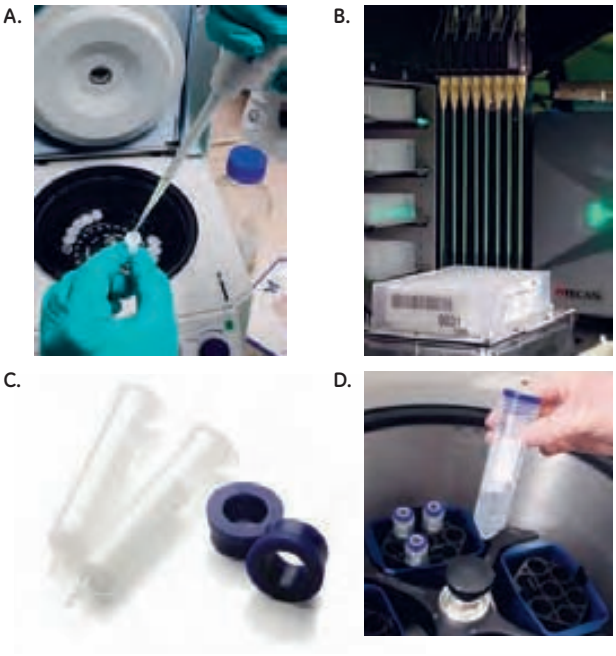


Fig 2.2. (**A**) PD SpinTrap G-25 sample preparation. (**B**) PD MultiTrap G-25 sample automated preparation in a robotic system. (**C** and **D**) Spin Adapters are used together with PD-10 Desalting Columns, PD MidiTrap G-25, and PD MiniTrap G-25 to enable use in a standard centrifuge.

Desalting larger sample volumes using HiTrap and HiPrep columns

Connect up to three HiTrap Desalting columns in series to increase the sample volume capacity, for example, two columns allow a sample volume of 3 ml; three columns allow a sample volume of 4.5 ml (Table 2.8, page 25).

Connect up to four HiPrep™ 26/10 Desalting columns in series to increase the sample volume capacity, for example, two columns allow a sample volume of 30 ml; four columns allow a sample volume of 60 ml. Even with four columns in series, the sample can be processed in 20 to 30 min (Table 2.8).

Buffer preparation

For substances carrying charged groups, an eluent containing a buffer salt is recommended. A salt concentration of at least 150 mM is recommended to prevent possible ionic interactions with the medium. Sodium chloride is often used for this purpose. Often a buffer with 25 to 50 mM concentration of the buffering substance is sufficient.

At salt concentrations above 1 M, hydrophobic substances may be retarded or bind to the medium. At even higher salt concentrations (> 1.5 M ammonium sulfate), the column packing shrinks.

Sample preparation

Sample concentration does not influence the separation as long as the viscosity does not differ by more than a factor of 1.5 from that of the buffer used. This corresponds to a maximum concentration of 70 mg/ml for proteins, when normal, aqueous buffers are used.

The sample should be fully solubilized. Centrifuge or filter (0.45 µm filter) immediately before loading to remove particulate material if necessary.

Protein solubility often depends on pH and/or ionic strength (salt concentration), and the exchange of buffer may therefore result in precipitation of the protein. Also, protein activity can be lost if the change of pH takes it outside of the range where the protein is active.

The protocols in the following sections describe desalting and buffer exchange using different formats of prepacked columns.

Manual desalting with HiTrap columns

Fig 2.3. HiTrap Desalting column allows efficient, easy-to-perform group separations with a syringe, pump, or chromatography system.

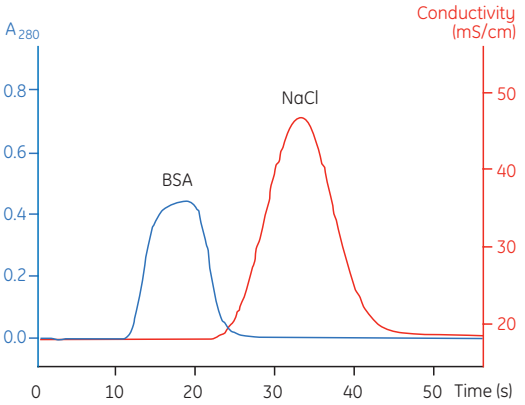
HiTrap Desalting is a 5 ml column (Fig 2.3) packed with the tried-and-tested gel filtration medium, Sephadex™ G-25 Superfine. The medium is based on cross-linked dextran beads that allow excellent resolution and high flow rates. The fractionation range for globular proteins is between M_r 1 000 and 5 000, with an exclusion limit of approximately M_r 5 000.

This ensures group separations of proteins/peptides larger than M_r 5 000 from molecules with a molecular weight less than M_r 1 000.

HiTrap Desalting can be used with aqueous solutions in the pH range 2 to 13. The prepacked medium is stable in all commonly used buffers, solutions of urea (8 M), guanidine hydrochloride (6 M), and all nonionic and ionic detergents. Lower alcohols (methanol, ethanol, propanol) can be used in the buffer or the sample, but we recommend that the concentration be kept below 25% v/v. Prolonged exposure (hours) to pH below 2 or above 13, or to oxidizing agents, should be avoided.

The recommended range of sample volumes is 0.1 to 1.5 ml when complete removal of low molecular weight components is desired. The separation is not affected by the flow rate, in the range of 1 to 10 ml/min. The maximum recommended flow rate is 15 ml/min. Separations are easily performed with a syringe, pump, or chromatography system. Up to three columns can be connected in series, allowing larger sample volumes to be handled.

Figure 2.4 shows a typical desalting and buffer exchange separation achieved using HiTrap Desalting and monitored by following changes in UV absorption and conductivity.



Column: HiTrap Desalting
Sample: 2 mg/ml BSA in 50 mM sodium phosphate, 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
Detection: UV (280 nm, 5 mm cell) and conductivity

Fig 2.4. Highly efficient desalting in 30 s using HiTrap Desalting.

To avoid cross-contamination, only use the column with the same type of sample.

Column equilibration

1. Fill the syringe or pump tubing with buffer. Remove the stopper. To avoid introducing air into the column, connect the column "drop to drop" to either the syringe (*via* the connector) or to the pump tubing.
2. Remove the snap-off end at the column outlet.
3. Wash the column with 25 ml of buffer at 5 ml/min to completely remove the storage buffer, which contains 20% ethanol*. If air is trapped in the column, wash with degassed buffer until the air disappears. Air introduced into the column by accident during sample application does not influence the separation.

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

Manual desalting using a syringe

1. To operate the column with a syringe, connect the syringe to the column using the supplied connector.
2. Equilibrate the column, see previous page Column equilibration.
3. Apply the sample using a 2 to 5 ml syringe at a flow rate between 1 and 10 ml/min. Discard the liquid eluted from the column. If the sample volume is less than 1.5 ml, change to buffer and proceed with the injection until a total of 1.5 ml has been eluted. Discard the eluted liquid.
4. Elute the protein with the appropriate volume selected from Table 2.8, see page 25. Collect the desalted protein.

The maximum recommended sample volume when using one HiTrap Desalting 5 ml column is 1.5 ml. See Table 2.8 on page 25 for information on application of smaller sample volumes.

Table 2.9. Recommended sample and elution volumes using HiTrap Desalting with a syringe, with examples of typical yields and remaining salt in the desalted sample

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

The void volume of the column is 1.5 ml. High molecular weight components elute between 1.5 and 4.5 ml, depending on the sample volume. Low molecular weight components start to elute after 3.5 ml.

Certain types of molecules, such as small heterocyclic or homocyclic aromatic compounds (purines, pyrimidines, dyes) can interact with Sephadex and are therefore eluted later than expected. Larger sample volumes can be used in these cases, but the separation has to be optimized for each type of contaminating compound.

Desalting using a pump

1. Equilibrate the column: see Column equilibration on the previous page.
2. Apply up to 1.5 ml of sample. Monitor the effluent from the column with a UV monitor and/or a conductivity monitor. Keep the flow rate in range 1 to 10 ml/min. Collect fractions.
3. Elute the column with approximately 10 ml of buffer before applying the next sample. Collect fractions.

Automated desalting with HiTrap Desalting columns on ÄKTAprime plus

ÄKTAprime™ plus contains preprogrammed templates for individual HiTrap Desalting and HiPrep Desalting 26/10 columns. The procedure below uses a HiTrap Desalting 5 ml column.

Buffer preparation

Equilibration buffer (port A1): 20 mM sodium phosphate, 150 mM NaCl, pH 7.0
Prepare at least 500 ml of the required buffer

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

Sample preparation

Pass the sample through a 0.45 µm filter.

The maximum recommended sample volume is 1.5 ml.

Preparing ÄKTAprime plus

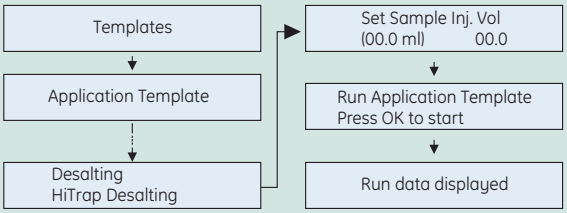
1. Place the inlet tubing from port A (port valve) and port B (2-port valve) in to the buffer.
2. Place the three brown waste tubings in the waste flask.
3. Connect the column between port 1 on the injection valve (7-port valve) and the UV flow cell.
4. Fill the fraction collector rack with 18 mm tubes (minimum 20) and position the white plate on the fractionation arm against the first tube.
5. Connect a sample loop large enough for your sample between ports 2 and 6 on the injection valve. Use a syringe to manually fill the loop.

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

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

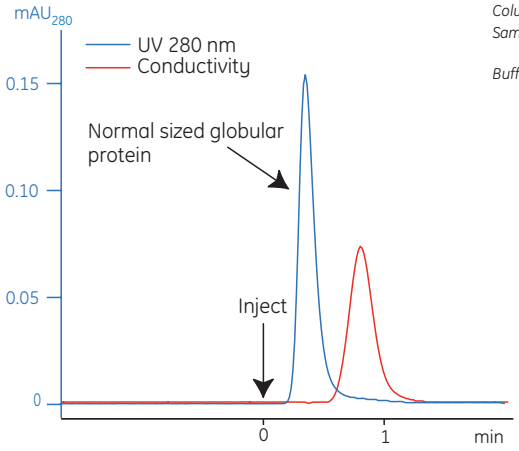
Selecting Application Template and starting the method

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



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

Figure 2.5 shows a typical result for desalting of a normal sized globular protein using HiTrap Desalting column and ÄKTAprime plus chromatography system. The result shown in this Figure would also be expected in buffer exchange of antibodies. The UV and conductivity traces enable the appropriate desalted fractions to be pooled.



Column: HiTrap Desalting
Sample: Normal sized globular protein in 20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4
Buffer (A1): 20 mM sodium phosphate, 150 mM NaCl, pH 7.0

Fig 2.5. Typical desalting of a normal sized globular protein using a chromatography system.

Scaling up desalting from HiTrap to HiPrep

For separation of sample volumes larger than 1.5 ml, or to increase the resolution between high and low molecular weight components, up to three HiTrap Desalting columns can easily be connected in series (see Table 2.8 on page 25). For syringe operations, the volumes suggested in Table 2.8 should be increased proportionally and the recommended flow rate maintained. The dilution of the sample is dependent on the sample volume and the number of columns used in series. Lower dilution factors than those proposed in Table 2.8 can be obtained, but the elution volumes have to be optimized for each combination of sample volume and number of columns in series. The backpressure for each column is approximately 0.25 bar at 10 ml/min.

HiPrep 26/10 Desalting is packed with Sephadex G-25 Fine. It provides group separation of high (M_r > 5 000) from low molecular weight substances (M_r < 1 000), allowing reliable and reproducible desalting and buffer exchange with sample sizes of 15 ml per column. Two to four columns can be used in series (Table 2.8) for sample volumes of of 30 to 60 ml (Fig 2.6).

Fig 2.6. A 60 ml sample volume can be run on four HiPrep 26/10 Desalting columns connected in series.

Automated buffer exchange on HiPrep 26/10 Desalting with ÄKTAprime plus

Buffer preparation

Equilibration buffer (port A1): 20 mM sodium phosphate, 150 mM NaCl, pH 7.0.
Prepare at least 500 ml of eluent

Sample preparation

- Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.45 µm filter before use.
- Pass the sample through a 0.45 µm filter.
- The maximum recommended sample volume is 15 ml.

Preparing ÄKTAprime plus

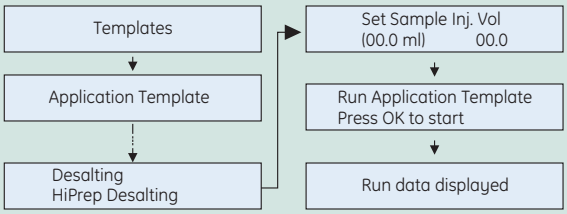
1. Place the inlet tubing from port A (8-port valve) and port B (2-port valve) in the buffer.
2. Place the three brown waste tubings in the waste flask.
3. Connect the column between port 1 on the injection valve (7-port valve) and the UV flow cell.
4. Fill the fraction collector rack with 18 mm tubes (minimum 25) and position the white plate on the fractionation arm against the first tube.
5. Connect a sample loop large enough for your sample between port 2 and 6 on the injection valve. Use a syringe to manually fill the loop.

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

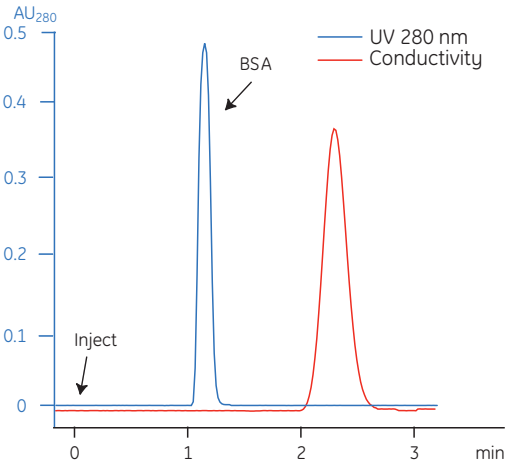
Once the system is prepared, the remaining steps (under Selecting Application Template and starting the method) will be performed automatically.

Selecting Application Template and starting the method

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



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



Column: HiPrep 26/10 Desalting
Sample: BSA and sodium chloride
Buffer (port A1): 20 mM phosphate, 150 mM NaCl, pH 7.0

Fig 2.7. A typical desalting of BSA using a chromatography system.

Small-scale desalting and buffer exchange with PD desalting columns

PD-10 Desalting Columns, PD MidiTrap G-25, PD MiniTrap G-25, PD SpinTrap G-25, and PD MultiTrap G-25 columns and 96-well filter plates are prepacked with Sephadex G-25 Medium for group separation of high (M_r > 5 000) from low molecular weight substances (M_r < 1 000) by desalting and buffer exchange.

These products are members of the Trap platform, which addresses the need for flexible, small-scale preparation of protein sample or other biomolecules prior to downstream analytical techniques such as gel electrophoresis, liquid chromatography, LC-MS, and MS. This collection of columns and plates covers the sample volume range from 70 µl to 2.5 ml and supports processing multiple samples in parallel. PD-10 Desalting Columns, PD MidiTrap G-25, and PD MiniTrap G-25, are also optimized to enable centrifugation, which results in no dilution of the eluted sample.

PD SpinTrap G-25

Fig 2.8. PD SpinTrap G-25 columns are single-use columns for rapid desalting and buffer exchange of biomolecules with a molecular weight > 5000.

PD SpinTrap G-25 is a single-use spin column that is designed for rapid, highly reproducible desalting and buffer exchange of 70 to 130 µl sample using a standard microcentrifuge (Fig 2.2 A and 2.8). The columns provide highly reproducible, parallel desalting/buffer exchange and cleanup of protein samples without sample dilution. The spin columns are prepacked with Sephadex G-25 Medium, a gel filtration medium that allows effective removal of low molecular weight substances from biomolecules with a molecular weight > 5000.

Each pack of PD SpinTrap G-25 contains prepacked columns and collection tubes for 50 preparations.

Buffer

Equilibration buffer: Ultrapure water

Desalting procedure

1. Suspend the medium by vortexing. Loosen screw cap lid and remove bottom closure using the plastic bottom cap removal tool.
2. Place the column in an appropriately sized collection tube and remove the storage solution by centrifugation for 1 min at 800 × g.
3. Equilibrate by adding 300 µl equilibration buffer and centrifuge for 1 min at 800 × g. Discard the flowthrough and replace the collection tube. Repeat this procedure four times.

To ensure optimal results, it is critical to equilibrate the spin column with 1.5 ml of equilibration buffer in total to completely remove the storage solution.

4. Replace the used collection tube with a new clean collection tube for sample collection.
5. Apply 70 to 130 µl sample slowly to the middle of the prepacked column.
6. Elute by centrifugation at 800 × g for 2 min.

For desalting larger sample volumes, use larger scale Trap PD cleanup products or HiTrap and HiPrep columns, see Table 2.8 on page 25. For desalting of multiple samples, use PD MultiTrap G-25.

Recovery is dependent on type of protein or other biomolecule. Typically, recovery is in the range of 70% to 90%. Concentration of the sample can improve recovery. Recovery can be improved for sample volumes less than 100 µl by adding 30 µl equilibration buffer after the sample has fully absorbed into the column bed.

PD MultiTrap G-25

Fig 2.9. PD MultiTrap G-25 96-well plates offer rapid, highly reproducible cleanup of biomolecules with a molecular weight > 5000.

PD MultiTrap G-25 96-well plates are designed for high-throughput desalting, buffer exchange, and cleanup of proteins, with high reproducibility well-to-well and plate-to-plate (Fig 2.9). Using the 96-well plates, multiple samples can be run conveniently and reproducibly in parallel (Fig 2.10). PD MultiTrap G-25 can be operated manually or in automated mode using a robotic system to desalt or buffer exchange sample volumes ranging from 70 to 130 µl. Elution can be performed by either centrifugation or by gravity flow.

The wells are prepacked with Sephadex G-25 Medium, a gel filtration medium that allows effective removal of low molecular weight substances from biomolecules with a molecular weight > 5000.

Each pack of PD MultiTrap G-25 contains four prepacked 96-well plates, allowing desalting or buffer exchange of up to 384 samples. A protocol for elution by centrifugation is included. Convenient collection plates (five per pack) are available separately (see Ordering information).

96-well plate: PD MultiTrap G-25
Sample: 1 mg/ml bovine serum albumin (BSA) in 1 M NaCl
Sample volume: 130 µl in each well
Equilibration buffer: Ultrapure water

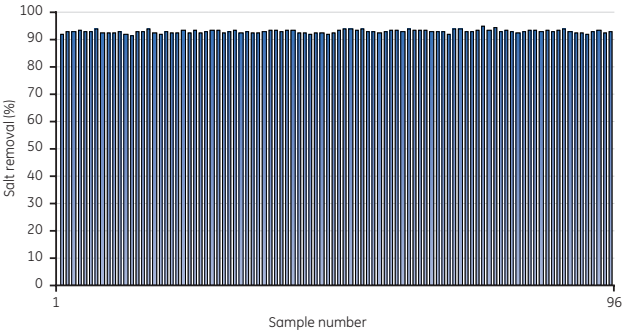


Fig 2.10. Removal of NaCl from BSA on a PD MultiTrap G-25 96-well plate showed highly reproducible results. The average desalting capacity was 93% and the well-to-well variation was 1% (relative standard deviation).

Centrifugation protocol

Buffer

Equilibration buffer: Ultrapure water

Desalting procedure

1. Suspend the medium by gently shaking the plate upside down. Remove top and bottom seals and place plate on the collection plate.
2. Remove the storage solution by centrifugation for 1 min at 800 × g.
3. Equilibrate by adding 300 µl equilibration buffer per well. Centrifuge for 1 min at 800 × g. Discard the flowthrough and replace the collection plate. Repeat this procedure four times.

To ensure optimal results, it is critical to equilibrate each well with 1.5 ml of equilibration buffer in total to completely remove the storage solution.

4. Replace the used collection plate with a new, clean collection plate for sample collection.
5. Apply 70 to 130 µl of sample to the middle of the prepacked wells.
6. Elute by centrifugation at 800 × g for 2 min.

For desalting larger sample volumes, use larger scale Trap PD cleanup products or HiTrap and HiPrep columns, see Table 2.8 on page 25.

Recovery is dependent on type of protein or other biomolecule. Typically, the recovery is in the range of 70% to 90%. Concentration of the sample can improve recovery. Recovery can be improved for sample volumes less than 100 µl by adding 30 µl equilibration buffer after the sample has fully absorbed into the column bed.

PD MiniTrap G-25

Fig 2.11. PD MiniTrap G-25 is a prepacked column for cleanup of proteins with a molecular weight > 5000 in sample volumes up to 500 µl.

PD MiniTrap G-25 is designed for convenient desalting and buffer exchange of 100 to 500 µl volume of protein sample (Fig 2.11). The columns are prepacked with Sephadex G-25 Medium, a gel filtration medium that allows effective removal of low molecular weight substances from proteins with a molecular weight > 5000. These columns provide an excellent alternative to PD SpinTrap G-25 columns on account of the increased sample volume capacity.

For increased flexibility, the product has two alternative application protocols, using either gravity or centrifugation. The gravity protocol allows simple cleanup of multiple samples in parallel without the need for a purification system. With the centrufigation protocol, samples are run in a standard centrifuge with minimal dilution of the eluted sample.

Each pack of PD MiniTrap G-25 contains 50 prepacked columns and four adapters that are required when using the centrifugation protocol.

Gravity protocol

Buffer

Equilibration buffer: Ultrapure water

Desalting procedure

1. Remove the top cap and pour off the column storage solution. Remove the bottom cap.
2. Fill the column with equilibration buffer and allow the equilibration buffer to enter the packed bed completely. Repeat twice and discard the flowthrough.

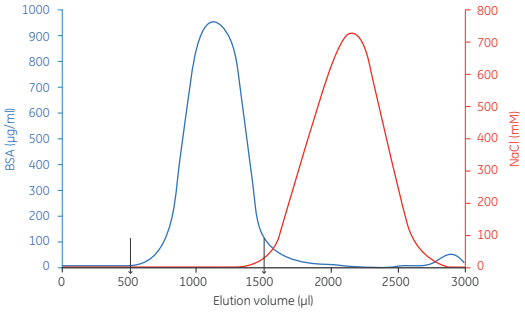
To ensure optimal results, it is critical to equilibrate the column with 8 ml of equilibration buffer in total to completely remove the storage solution.

3. Add 100 to 500 µl of sample to the column. For sample volumes lower than 500 µl, add equilibration buffer to adjust the volume up to 500 µl *after the sample has entered the packed bed completely*.
4. Allow the sample and equilibration buffer to enter the packed bed completely. Discard the flowthrough.
5. Place a test tube for sample collection under the column and elute with 1 ml buffer. Collect the desalted sample.

For desalting larger sample volumes, use HiTrap and HiPrep columns, see Table 2.8 on page 25.

Recovery is dependent on type of protein. Typically, recovery is in the range of 70% to 90%. Concentration of the sample can improve recovery. Recovery and desalting capacity are higher when using gravity flow compared with centrifugation.

A typical result for desalting of a protein is shown in Figure 2.12. Although the desalted protein shown in the Figure is BSA, a similar result would be expected in the desalting of antibodies using PD MiniTrap G-25.



Column: PD MiniTrap G-25
Sample: 1 mg/ml bovine serum albumin (BSA) in 1 M NaCl
Sample volume: 500 µl
Equilibration buffer: Water (ultrapure)

Fig 2.12. Removal of NaCl from BSA using the gravity protocol. The protein recovery was 95%.

Centrifugation protocol

Buffer

Equilibration buffer: Ultrapure water

Desalting procedure

1. Remove the top cap and pour off the column storage solution.
2. Remove the top filter using forceps. Remove the bottom cap.
3. Place the PD MiniTrap G-25 into a 15 ml collection tube and connect the supplied column adapter to the top of the tube.
4. Fill the column with equilibration buffer and allow the equilibration buffer to enter the packed bed completely. Repeat and discard the flowthrough.
5. Fill the column with equilibration buffer again and centrifuge at 1000 × g for 2 min and discard the flowthrough.

To ensure optimal results, it is critical to equilibrate the column with 8 ml of equilibration buffer in total (steps 4 and 5) to completely remove the storage solution.

6. Add 200 to 500 µl of sample slowly to the middle of the packed bed.
7. Place the PD MiniTrap G-25 into a new 15 ml collection tube.
8. Elute by centrifugation 1000 × g for 2 min and collect the eluate.

For desalting larger sample volumes, use HiTrap and HiPrep columns, see Table 2.8 on page 25.

Recovery is dependent on type of protein. Typically, recovery is in the range of 70% to 90%. Concentration of the sample can improve recovery. Recovery and desalting capacity are higher using gravity flow compared with centrifugation.

PD MidiTrap G-25

Fig 2.13. PD MidiTrap G-25 is a prepacked column for cleanup of proteins with a molecular weight > 5000 in sample volumes up to 1 ml.

PD MidiTrap G-25 is designed for convenient desalting and buffer exchange of 0.5 to 1.0 ml volume of protein sample (Fig 2.13). The columns are prepacked with Sephadex G-25 Medium, a gel filtration medium that allows effective removal of low molecular weight substances from proteins with a molecular weight > 5000. These columns provide an excellent alternative to PD MiniTrap G-25 columns on account of the increased sample volume capacity.

For increased flexibility, the product has two alternative application protocols, using either gravity or centrifugation. The gravity protocol allows simple cleanup of multiple samples in parallel without the need for a purification system. With the centrifugation protocol, samples are run in a standard centrifuge with minimal dilution of the eluted sample.

Each pack of PD MidiTrap G-25 contains 50 prepacked columns and four adapters that are required when using the centrifugation protocol.

Gravity protocol

Buffer

Equilibration buffer: Ultrapure water

Desalting procedure

1. Remove the top cap and pour off the column storage solution. Remove the bottom cap.
2. Fill the column with equilibration buffer and allow the equilibration buffer to enter the packed bed completely. Repeat twice, discarding the flowthrough each time.

To ensure optimal results, it is critical to equilibrate the column with 15 ml of equilibration buffer in total to completely remove the storage solution.

3. Add 0.5 to 1 ml of sample to the column. For sample volumes lower than 1 ml, add equilibration buffer to adjust the volume up to 1 ml *after the sample has entered the packed bed completely.*
4. Allow the sample and equilibration buffer to enter the packed bed completely. Discard the flowthrough.
5. Place a test tube for sample collection under the column and elute with 1.5 ml buffer. Collect the desalted sample.

For desalting larger sample volumes, use HiTrap and HiPrep columns, see Table 2.8 on page 25.

Recovery is dependent on type of protein. Typically, recovery is in the range of 70% to 90%. Concentration of the sample can improve recovery. Recovery and desalting capacity are higher using gravity flow compared with centrifugation.

Centrifugation protocol

Buffer

Equilibration buffer: Ultrapure water

Desalting procedure

1. Remove the top cap and pour off the column storage solution.
2. Remove the top filter using forceps. Remove the bottom cap.
3. Place the PD MidiTrap G-25 into a 50 ml collection tube and connect the supplied column adapter to the top of the tube.
4. Fill the column with equilibration buffer and allow the equilibration buffer to enter the packed bed completely. Repeat and discard the flowthrough.
5. Fill the column with equilibration buffer again and centrifuge at 1000 × g for 2 min and discard the flowthrough.

To ensure optimal results, it is critical to equilibrate the column with 15 ml of equilibration buffer in total (steps 4 and 5) to completely remove the storage solution.

6. Add 0.75 to 1.0 ml of sample slowly to the middle of the packed bed.
7. Place the PD MidiTrap G-25 into a new 50 ml collection tube.
8. Elute by centrifugation 1000 × g for 2 min and collect the eluate.

For desalting larger sample volumes, use HiTrap and HiPrep columns, see Table 2.8 on page 25.

Recovery is dependent on type of protein. Typically, recovery is in the range of 70% to 90%. Concentration of the sample can improve recovery. Recovery and desalting capacity are higher using gravity flow compared with centrifugation.

Disposable PD-10 Desalting Columns

PD-10 Desalting Columns are designed for convenient desalting and buffer exchange of 1.0 to 2.5 ml volume of protein sample. The columns are prepacked with Sephadex G-25 Medium, a gel filtration medium that allows effective removal of low molecular weight substances from proteins with a molecular weight > 5000. These columns provide an excellent alternative to PD MidiTrap G-25 columns on account of the increased sample volume capacity.

For increased flexibility, the product has two alternative application protocols, using either gravity or centrifugation. The gravity protocol allows simple cleanup of multiple samples in parallel without the need for a purification system. Using the centrifugation protocol, samples are run in a standard centrifuge with minimal dilution of the eluted sample.

Each pack of PD-10 Desalting Columns contains 30 prepacked columns. To simplify the use of PD-10 Desalting Columns with the gravity protocol, LabMate™ PD-10 Buffer Reservoir may be used (see Ordering information). Using the buffer reservoir, wash and equilibration buffers can be applied in one step.

A typical separation is shown in Figure 2.14. Although the Figure shows a typical desalting of albumin, this would be an expected result for the desalting of antibodies.

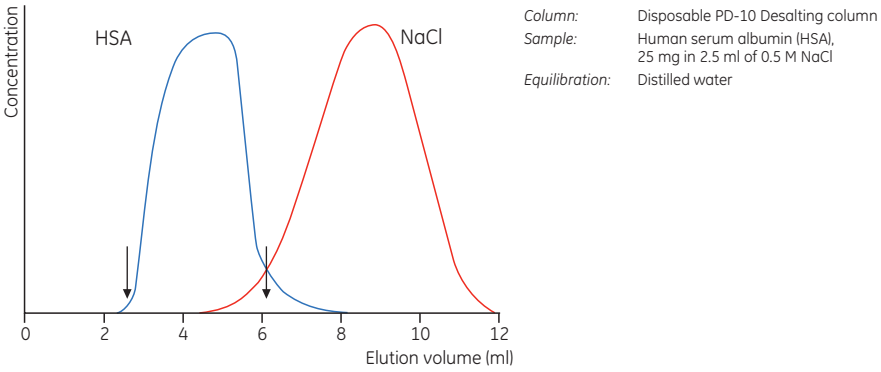


Fig 2.14. Removal of NaCl from albumin solution. A PD-10 Desalting column was equilibrated with distilled water. The sample contained human serum albumin (25 mg) dissolved in 2.5 ml of 0.5 M NaCl solution. A total of 23.8 mg albumin was recovered in 3.5 ml eluent corresponding to a yield of 95.3% (between arrows). Initial total salt content of sample before desalting was 2%.

Gravity protocol

Buffer

Equilibration buffer: Ultrapure water

Desalting procedure

1. Cut off bottom tip, remove top cap, and pour off excess liquid.
2. If available, mount the LabMate Buffer Reservoir on top of the PD-10 Desalting column and place the columns in the PD-10 Desalting Workmate.
3. Equilibrate the column with approximately 25 ml of buffer. Discard the flowthrough (use the plastic tray to collect flowthrough).

To ensure optimal results, it is critical to equilibrate the column with 25 ml of equilibration buffer in total to completely remove the storage solution.

4. Add sample of a total volume of 2.5 ml. If the sample is less than 2.5 ml, add buffer until the total volume of 2.5 ml is achieved. Discard the flowthrough.
5. Elute with 3.5 ml of buffer and collect the flowthrough.

For desalting larger sample volumes, use HiTrap and HiPrep columns, see Table 2.8 on page 25.

Recovery is dependent on type of protein. Typically, recovery is in the range of 70% to 90%. Concentration of the sample can improve recovery. Recovery and desalting capacity are higher using gravity flow compared with centrifugation.

Centrifugation protocol

Buffer

Equilibration buffer: Ultrapure water

Desalting procedure

1. Remove the top cap and pour off the column storage solution.
2. Remove the top filter using forceps. Remove the bottom cap.
3. Place the PD-10 Desalting Column into a 50 ml collection tube and connect the supplied column adapter to the top of the tube.
4. Fill the column with equilibration buffer and allow the equilibration buffer to enter the packed bed completely. Repeat three times, discarding the flowthrough each time.
5. Fill the column with equilibration buffer again and centrifuge at 1000 × g for 2 min and discard the flowthrough.

To ensure optimal results, it is critical to equilibrate the column with 25 ml of equilibration buffer in total (steps 4 and 5) to completely remove the storage solution.

6. Add 1.75 to 2.5 ml of sample slowly to the middle of the packed bed.
7. Place the PD-10 Desalting column into a new 50 ml collection tube.
8. Elute by centrifugation 1000 × g for 2 min and collect the eluate.

For desalting larger sample volumes, use HiTrap and HiPrep columns, see Table 2.8 on page 25.

Recovery is dependent on type of protein. Typically, recovery is in the range of 70% to 90%. Concentration of the sample can improve recovery. Recovery and desalting capacity are higher using gravity flow compared with centrifugation.

Chapter 3
Small-scale purification by affinity chromatography

A significant advantage for the purification of antibodies and antibody fragments, from any source, is that a great deal of information is available about the properties of the target molecule and the major contaminants (see Chapter 2, Table 2.1).

When there is an immunospecific interaction, affinity chromatography (AC) is often the first and sometimes the only step required. However, to achieve satisfactory sample homogeneity, a further polishing step, often gel filtration (GF), may be required. Affinity purification offers high selectivity, and usually, high capacity for the target protein(s). The target molecule is concentrated into a smaller volume and purity levels often above 95% are possible in one step. Purification can be performed in batch, by using gravity-flow, spin columns, 96-well plates, or prepacked HiTrap columns.

This chapter describes the affinity media and prepacked formats available from GE Healthcare for small-scale purification of antibodies using bulk media, prepacked in chromatography columns, as well as in 96-well plates and spin columns.

Recent advances in the production and purification of genetically engineered antibodies and antibody fragments have opened up many possibilities, not only to manipulate their biological properties, but also to facilitate their purification. For example, tags can be introduced into target molecules for which no affinity media were previously available to allow effective affinity purification.

For additional information on the purification of recombinant proteins, including purification of GST and $(histidine)_6$ tagged proteins, please refer to *Recombinant Protein Purification Handbook: Principles and Methods (18-1142-75),* and the *GST Gene Fusion System Handbook (18-1157-58).* Further details on the purification of protein A fusion proteins can be found in the handbook *Affinity Chromatography: Principles and Methods (18-1022-29).*

General considerations

Sample preparation

For sample preparation, follow procedures according to the source of the antibody, as recommended in Chapter 2. Use high quality water and chemicals. Filtration of buffers is recommended. Centrifuge or filter samples immediately before use. If the sample is too viscous, dilute with binding buffer. Sample binding properties can be improved by adjusting the sample to the composition of the binding buffer: perform a buffer exchange using a desalting column (see Chapter 2), or dilute in binding buffer.

Types of media and formats for one-step purification

The basis for antibody affinity purification is the high affinity and specificity of protein G and protein A for the Fc region of IgG from a variety of species. Protein G and protein A have been immobilized to several different matrixes resulting in an excellent means of isolating IgG and IgG subclasses from ascites, cell culture supernatants, and serum.

Sepharose bulk media

Protein G and protein A are bacterial proteins from Group G *Streptococci* and *Staphylococcus aureus*, respectively. When coupled to Sepharose, protein G and protein A create extremely useful, easy-to-use media for routine purification of antibodies.

GE Healthcare offers a recombinant form of protein G grown in *E. coli* from which the albumin-binding region of the native protein has been genetically deleted. This recombinant protein G ligand is coupled to both Protein G Sepharose 4 Fast Flow and Protein G Sepharose High Performance media and is available in prepacked formats including HiTrap and SpinTrap columns, as well as MultiTrap 96-well plates. Moreover, Protein G Sepharose 4 Fast Flow is available in bulk (lab packs) when larger scale purification is desired.

Native protein A (nProtein A) is also available coupled to Sepharose 4 Fast Flow and Sepharose High Performance while recombinant protein A (rProtein A) coupled to Sepharose Fast Flow is also offered. Recombinant protein A bound to Sepharose offers several potential advantages compared to native protein A. rProtein A has been engineered to favor single-point oriented immobilization *via* thioether coupling, which results in enhanced binding capacity for IgG. Furthermore, rProtein A is produced in *E. coli* and no human IgG affinity step is used during validated fermentation and purification processes, minimizing risk of human IgG contamination. nProtein A Sepharose 4 Fast Flow and rProtein A Sepharose Fast Flow can be packed in laboratory and larger scale columns and are also available in prepacked HiTrap columns for convenient, small-scale purification. For purification scale-up, Sepharose media can be packed in XK and Tricorn™ columns.

Protein G is a good choice for general purpose capture of antibodies at laboratory scale since it binds a broader range of IgG from eukaryotic species and also binds to more subclasses of IgG. Usually protein G has greater affinity for IgG than protein A and exhibits minimal binding to albumin, resulting in cleaner preparations and greater yields. The binding strength of protein G for IgG depends on the source species and subclass of the immunoglobulin. The dynamic binding capacity depends on the binding strength and also on several other factors, such as flow rate during sample application.

MabSelect media have been designed for capturing monoclonal antibodies from large volumes of feed. The recombinant protein A ligand of MabSelect is engineered to favor an oriented coupling that delivers enhanced binding capacity. MabSelect SuRe uses an alkali-tolerant recombinant protein A ligand that is resistant to harsh cleaning agents (e.g., 0.1 to 0.5 M NaOH). MabSelect Xtra uses the same recombinant protein A ligand as MabSelect, but the medium has a smaller particle size and greater porosity for increased dynamic binding capacity at higher flow rates. MabSelect, MabSelect SuRe, and MabSelect Xtra are available in bulk (lab packs), as well as in prepacked HiTrap column format for small-scale purification and use in process development.

Prepacked formats

Different sizes and types of prepacked columns, as well as prepacked 96-well plates from GE Healthcare provide a fast and convenient alternative to empty columns that require packing (Fig 3.1). Antibodies can be purified by simply passing the sample through a column packed with an appropriate medium. These various formats are prepacked with media that are also available in bulk for packing of larger scale purification columns such as XK and Tricorn columns. All prepacked columns and 96-well plates are supplied with a detailed protocol that outlines the buffers and steps required for optimal results.

When purification is performed at small scale, as is the case in antibody screening experiments, prepacked MultiTrap 96-well plates are available. Each well has the capacity to bind up to about 0.5 mg of antibody. Samples are pipetted into the prepacked wells and washing and elution can be performed using centrifugation or vacuum. Using these plates, high-throughput processing of samples can be performed. When many plates are used simultaneously, a robotic system can be used for plate handling.

Prepacked SpinTrap columns are designed for use in a microcentrifuge and can offer an alternative to screening in 96-well plate format when fewer samples are to be screened. Ab SpinTrap, for example, is a column designed for rapid purification and screening of antibodies and each column has the capacity to bind approximately 1 mg of antibody.

Prepacked HiTrap columns provide flexibility and convenience in antibody purification as they can be connected in series for purification scale-up and can be operated using syringe, pump, or chromatography system.

Fig 3.1. MultiTrap 96-well plates, SpinTrap columns, and HiTrap columns are designed for fast and convenient screening and small-scale purification of monoclonal and polyclonal IgG from serum, cell culture supernatants, and ascites.

Most media are available for scaling up and packing larger columns. Custom-designed affinity media can be manufactured.

Reuse of affinity media depends on the nature of the sample and should only be considered when processing identical samples to avoid cross-contamination.

Adding a polishing step after intitial purification

One-step affinity purification generally achieves satisfactory purity of the target antibody. To achieve adequate homogeneity of the purified antibody, however, an additional polishing step using gel filtration (GF) is recommended. Table 3.1 presents product recommendations for laboratory-scale purification of antibodies in terms of scale and gel filtration polishing step.

Table 3.1. Suitable products for purification of antibodies. Products for capture, an additional polishing step (gel filtration), and desalting are shown

	Screening	Laboratory scale	Scaling up
Capture	Protein G HP MultiTrap Protein A HP MultiTrap Protein G HP SpinTrap Protein A HP SpinTrap Ab Spin Trap	HiTrap Protein G HP MabTrap Kit HiTrap Protein A HP HiTrap rProtein A FF HiTrap MabSelect HiTrap MabSelect SuRe HiTrap MabSelect Xtra	Protein G Sepharose 4 Fast Flow nProtein A Sepharose 4 Fast Flow rProtein A Sepharose Fast Flow MabSelect MabSelect SuRe MabSelect Xtra
Polishing	Superdex 200 5/150 GL Superdex 75 5/150 GL	Superdex 200 10/300 GL Superdex 75 10/300 GL	HiLoad 16/60 Superdex 200 pg HiLoad 26/60 Superdex 200 pg HiLoad 16/60 Superdex 75 pg HiLoad 26/60 Superdex 75 pg
Buffer exchange/ desalting/sample cleanup	PD SpinTrap G-25 PD MultiTrap G-25 PD MiniTrap G-25 PD MidiTrap G-25	HiTrap Desalting PD-10 Desalting columns HiPrep 26/10 Desalting	HiPrep 26/10 Desalting columns connected in series

An example of a typical polishing step using high-resolution gel filtration is shown in Figure 3.2. Superdex™ media in prepacked columns can be used to separate any contaminant molecules on the basis of differences in size, and also to transfer the sample into storage buffer, and remove excess salt and other small molecules.

In the case of antibodies, a polishing step is often used to separate dimeric and monomeric forms (Fig 3.2), as well as remove large aggregate forms of antibody.

Multistep purification strategies are described in Chapter 6.

Column:	Superdex 200 10/300 GL
Column volume:	24 ml
Sample:	Monoclonal antibody
Sample volume:	100 µl
Elution buffer:	20 mM Tris-HCl, 150 mM NaCl, pH 7.5
Flow rate:	0.25 ml/min
System:	ÄKTAexplorer 100

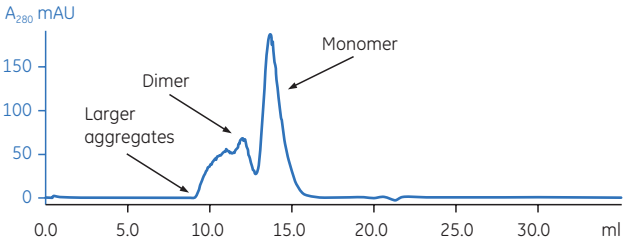


Fig 3.2. Gel filtration is a technique often used as a polishing step in antibody purification, as well as a method to remove dimeric, monomeric, and large aggregate forms of antibody. The chromatogram shows a separation of monomer, dimer, and larger aggregates of a monoclonal antibody using a prepacked Superdex 200 10/300 GL column.

Protein G and protein A bind to different IgG

The high affinity of protein G and protein A for the Fc region of polyclonal and monoclonal IgG-type antibodies forms the basis for purification of IgG, IgG fragments containing the Fc region, and subclasses.

Protein G and protein A are bacterial proteins from Group G *Streptococci* and *Staphylococcus aureus* respectively. When coupled to Sepharose, protein G and protein A create extremely useful, easy-to-use media for routine purification of antibodies. Examples include the purification of monoclonal IgG-type antibodies, purification of polyclonal IgG and its subclasses, adsorption and purification of immune complexes involving IgG, and fusion proteins. IgG subclasses can be isolated from cell culture supernatants, serum, and ascites.

Table 3.2 shows a comparison of the relative binding strengths of protein G and protein A to different immunoglobulins. The information has been compiled from various publications. Binding strengths are tested with free protein G or protein A and can be used as guidelines to predict the binding behavior to a protein G or protein A purification medium. However, when coupled to an affinity matrix, the interaction may be altered. For example, rat IgG_1 binds to Protein G Sepharose, but not to Protein A Sepharose.

Table 3.2. Relative binding strengths of antibodies from various species to protein G and protein A as measured in a competitive ELISA test. The amount of IgG required to give a 50% inhibition of binding of rabbit IgG conjugated with alkaline phosphatase was determined

Species	Subclass	Protein G binding	Protein A binding
Human	IgA	—	variable
	IgD	—	—
	IgE	—	—
	IgG_1	++++	++++
	IgG_2	++++	++++
	IgG_3	++++	—
	IgG_4	++++	++++
	IgM*	—	variable
Avian egg yolk	IgY†	—	—
Cow		++++	++
Dog		+	++
Goat		++	—
Guinea pig	IgG_1	++	++++
Hamster		++	+
Horse		++++	++
Koala		+	—
Llama		+	—
Monkey (rhesus)		++++	++++
Mouse	IgG_1	++++	+
	IgG_{2a}	++++	++++
	IgG_2	+++	+++
	IgG_3	+++	++
	IgM*	—	variable
Pig		+++	+++
Rabbit		+++	++++
Rat	IgG_1	+	—
	IgG_{2a}	++++	—
	IgG_{2b}	++	—
	IgG_3	++	+
Sheep		++	+/—

** Purified using HiTrap IgM Purification HP columns*
† Purified using HiTrap IgY Purification HP columns
++++ = strong binding
++ = medium binding
— = weak or no binding

Single-step purification based on Fc region specificity will co-purify host IgG and may even bind trace amounts of serum proteins. To avoid trace amounts of contaminating IgG, consider alternative techniques such as immunospecific affinity using anti-host IgG antibodies coupled to for example NHS-activated Sepharose, ion exchange chromatography (IEX) with, for example, Capto™ adhere, or hydrophobic interaction chromatography (HIC, see Chapter 6).

Purification using Protein G Sepharose media

Protein G, a cell-surface protein from Group G *streptococci*, is a type III Fc-receptor. Protein G binds IgG through a nonimmune mechanism. As with protein A, protein G binds specifically to the Fc region of IgG, but binds more strongly to several polyclonal IgG and human IgG_3. Under standard buffer conditions, protein G binds to all human subclasses and all mouse IgG subclasses, including mouse IgG_1. Protein G also binds rat IgG_{2a} and IgG_{2b}, which either do not bind or bind only weakly to protein A, see Table 3.2.

Protein G is a good choice for general purpose capture of antibodies at laboratory scale since it binds a broader range of IgG from eukaryotic species and binds more classes of IgG than protein A. Usually protein G has greater affinity for IgG than protein A and exhibits minimal binding to albumin, resulting in cleaner preparations and greater yields. The binding strength of protein G for IgG depends on the source species and subclass of the immunoglobulin. The dynamic binding capacity depends on the binding strength and also on several other factors, such as flow rate during sample application.

Many antibodies also interact *via* the Fab region with a low affinity site on protein G. Protein G does not appear to bind human myeloma IgM, IgA, or IgE. Human IgA and IgM have, however, been shown to bind weakly to protein A.

Leakage of ligands from an affinity medium must be considered, especially if harsh elution conditions are used. The multipoint attachment of protein G to Sepharose media results in very low protein G ligand leakage over a wide range of elution conditions. Removal of ligand contaminant can be achieved by adding a polishing step using gel filtration or ion exchange chromatography.

Table 3.3 on the next page shows the options for purification of antibodies using Protein G Sepharose media.

Table 3.3. Purification options for IgG using Protein G Sepharose 4 Fast Flow and Protein G Sepharose High Performance

Product	Format or column size	Binding capacity (mg IgG/ml medium)	Description
Protein G Sepharose 4 Fast Flow	5 ml 25 ml	> 20 (human) 23 (cow) 19 (goat) 17 (guinea pig) 10 (mouse) 7 (rat)	Supplied as suspension for packing in, e.g., XK and Tricorn columns; used for scaling up purification
Protein G HP MultiTrap	96-well plates	> 25 (human)	For small-scale purification, screening of antibody constructs, optimization of buffer conditions, protein enrichment by immunoprecipitation
Ab SpinTrap	100 µl spin columns	> 25 (human)	For small-scale purification of IgG and fragments, including human IgG_3 using a microcentrifuge. Strong affinity to mouse monoclonal IgG_1 and rat IgG
Protein G HP SpinTrap	100 µl spin columns	> 25 (human)	For small-scale purification of IgG and fragments, including human IgG_3 and protein enrichment of target antigens by immunoprecipitation using a microcentrifuge. Strong affinity to mouse monoclonal IgG_1 and rat IgG. Supplied with a protocol for antibody purification and for capture of target antigens by immunoprecipitation
HiTrap Protein G HP	1 ml, 5 ml columns	> 25 (human)	Laboratory-scale purification of IgG and fragments, including human IgG_3. Strong affinity to monoclonal mouse IgG_1 and rat IgG. Prepacked 1 ml or 5 ml columns can be connected in series to facilitate scale-up
MAbTrap Kit	1 Kit	> 25 (human)	Purification of IgG and fragments, including human IgG_3. Strong affinity to mouse monoclonal IgG_1 and rat IgG. Kit contains HiTrap Protein G HP (1 × 1 ml), accessories, premade buffers for 10 purifications, and detailed experimental protocols
Companion product Ab Buffer Kit	1 Kit		Premade buffers recommended for antibody purification using Ab SpinTrap, Protein G HP SpinTrap, HiTrap Protein G HP, and Protein G HP MultiTrap

Protein G Sepharose 4 Fast Flow

Protein G Sepharose 4 Fast Flow consists of 90 µm beads of highly cross-linked agarose, which provide a robust and stable chromatography matrix that allows high flow rates. Protein G Sepharose 4 Fast Flow binds a broader range of IgG from eukaryotic species than protein A Sepharose based media and binds more classes of IgG (Table 3.2).

The ligand of Protein G Sepharose 4 Fast Flow is a recombinant form of protein G from which the albumin-binding region of the native molecule has been genetically deleted, thereby avoiding undesirable reactions with albumin. Recombinant protein G contains two Fc binding regions, which are extremely useful for isolation of immune complexes. The medium is a good choice for general-purpose capture of antibodies and scale-up in the laboratory.

Protein G Sepharose media bind IgG over a wide pH range with a strong affinity at neutral pH. To elute the IgG, it is necessary to lower the pH to between 2.5 and 3.0 depending on the antibody. If biological activity of the antibody or antibody fragment is lost due to the low pH required for elution, try Protein A Sepharose; the elution conditions used are generally milder (see Table 3.5 on page 69)

Protein G Sepharose 4 Fast Flow is available as a bulk medium (Fig 3.3) for packing in XK and Tricorn columns (Fig 3.4) at laboratory scale. Furthermore, Protein G Sepharose 4 Fast Flow can be packed in larger columns for industrial-scale purification of monoclonal and polyclonal antibodies.

Fig 3.3. Protein G Sepharose 4 Fast Flow is available in various bulk (lab packs) sizes for laboratory- and process-scale applications.

Fig 3.4. Empty Tricorn columns for packing. For purification using a pump or chromatography system.

Column packing

Refer to Appendix 5 for general column packing guidelines.

Ideally, Sepharose 4 Fast Flow media are packed in XK or Tricorn columns in a two-step procedure: Do not exceed 0.3 bar (0.03 MPa) in the first step and 3.5 bar (0.35 MPa) in the second step. If the packing equipment does not include a pressure gauge, use a packing flow rate of 2.5 ml/min (XK 16/20 column) or 1.0 ml/min (Tricorn 10/100 column) in the first step, and 14 ml/min (XK 16/20 column) or 5.5 ml/min (Tricorn 10/100 column) in the second step.

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

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

Sample preparation

Refer to Chapter 2 for general considerations.

Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.

IgG from many species has a medium to strong affinity for protein G at approximately pH 7. The sample should have a pH around 7 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Purification

1. Prepare collection tubes by adding 60 to 200 µl of 1 M Tris-HCl, pH 9.0 per milliliter of fraction to be collected.

To preserve the activity of acid-labile IgG, we recommend adding 60 to 200 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

2. If the column contains 20% ethanol, wash it with 5 column volumes of distilled water. Use a linear flow rate of 50 to 100 cm/h*.
3. Equilibrate the column with 5 to 10 column volumes of binding buffer at a linear flow rate of 150 cm/h.
4. Apply the pretreated sample.
5. Wash with binding buffer until the absorbance reaches the baseline.

   ** See Appendix 7 for information on how to convert linear flow (cm/h) to volumetric flow rates (ml/min)*

IgG from most species and subclasses bind to protein G at near physiological pH and ionic strength. For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between antibody and ligand ligand is weak, since this may decrease yield.

6. Elute with elution buffer using a step or linear gradient. For step elution, 5 column volumes of elution buffer are usually sufficient. For linear gradient elution, a shallow gradient over 20 column volumes allows separation of proteins with similar binding strengths.
7. After elution, regenerate the column by washing it with 5 to 10 column volumes of binding buffer. The column is now ready for a new purification.

Protein G Sepharose media bind IgG over a wide pH range with a strong affinity at neutral pH. To elute the IgG, it is necessary to lower the pH to between 2.5 and 3.0 depending on the antibody. If biological activity of the antibody or antibody fragment is lost due to the low pH required for elution, try Protein A Sepharose; the elution conditions used are generally milder (see Table 3.5 on page 69).

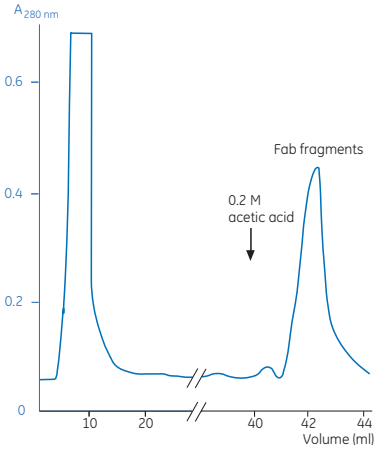
Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

Storage

Store in 20% ethanol at 4°C to 8°C.

Purifying Fab and F(ab')$_2$ fragments

Protein G also has an affinity binding site for certain Fab regions, and consequently, Protein G affinity media can in some cases be used for the purification of Fab and F(ab´)$_2$ fragments. Figure 3.5 shows the purification of recombinant mouse Fab fragments, expressed in *E. coli*, in a single affinity purification step using Protein G Sepharose 4 Fast Flow.



Medium:	Protein G Sepharose 4 Fast Flow
Sample:	15 ml recombinant mouse Fab, expressed in *E. coli.*
Binding buffer:	50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween, pH 7.4
Elution buffer:	0.2 M acetic acid pH 2.8
Flow rate:	0.8 ml/min

Fig. 3.5. Purification of recombinant mouse Fab fragments, expressed in *E. coli* using Protein G Sepharose 4 Fast Flow.

Multiwell plates and columns prepacked with Protein G Sepharose High Performance

Protein G HP MultiTrap, Ab SpinTrap, Protein G HP SpinTrap, and HiTrap Protein G HP are prepacked with Protein G Sepharose High Performance, which consists of 34 µm highly cross-linked agarose beads for high-performance purification of antibodies (Table 3.3). This medium offers the highest possible binding capacity and is compatible with additives commonly used in antibody purification. Protein G Sepharose High Performance is stable over a broad pH range. The high chemical and physical stability, as well as broad operating pH range of the medium preserves the biological activity of the antibody while ensuring a highly pure product.

Protein G Sepharose High Performance provides sharper eluted peaks and more concentrated elution of antibodies compared with Protein G Sepharose 4 Fast Flow. However, the smaller bead size of the Sepharose High Performance compared with that of the Sepharose 4 Fast Flow matrix leads to increased backpressure on the column. As a result, Sepharose 4 Fast Flow is the preferred medium for scale-up.

Protein G HP MultiTrap

Protein G HP MultiTrap are 96-well plates prepacked with Protein G Sepharose High Performance. Protein G HP MultiTrap is a versatile tool for screening of different proteins and for preparation of protein samples, enrichment of proteins of interest from clarified cell lysates and biological fluids, and small-scale purification of antibodies (Fig 3.6). Purification runs are performed in parallel, which ensures fast and reliable capture of antibodies from a large number of complex samples.

Each pack of Protein G HP MultiTrap contains four prepacked multiwell plates and protocols for protein enrichment of target antibody-antigen complexes by immunoprecipitation and for small-scale antibody purification. Collection plates (see Ordering information for code no.) for collecting eluted, purified antibody are available separately.

The procedure for small-scale screening and purification of antibodies is described below. For a description of the immunoprecipitation procedure, download Instructions 28-9067-73 at www.gelifesciences.com/protein-purification.

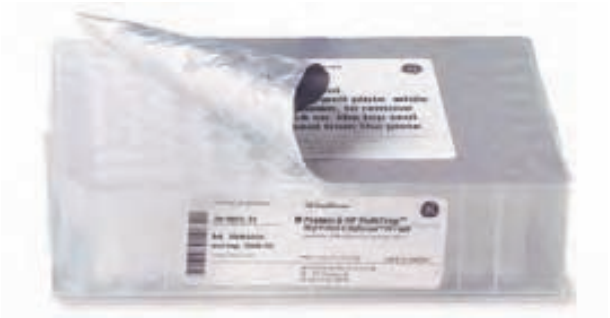


Fig 3.6. Protein G HP MultiTrap 96-well plates are used for rapid, parallel screening and small-scale purification of monoclonal and polyclonal antibodies at small scale.

Sample preparation

Refer to Chapter 2 for general considerations.

IgG from many species has a medium to strong affinity for protein G at approximately pH 7. The sample should have a pH around 7 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28-9030-59).

Purification

1. Prepare two collection plates with 15 µl neutralizing buffer per well.

To preserve the activity of acid-labile IgG, we recommend adding 15 µl of 1 M Tris-HCl pH 9.0 to collection wells in the collection plate, which ensures that the final pH of the sample will be approximately neutral.

2. Invert and gently shake the MultiTrap plate to resuspend the medium. Remove top and bottom seals and place the MultiTrap plate on a collection plate. Centrifuge for 1 min at 70 to 100 × g to remove the storage solution.
3. Equilibrate by adding 300 µl binding buffer. Centrifuge for 30 s at 70 to 100 × g.
4. Bind antibody by adding maximum 300 µl of the antibody sample. Incubate for 4 min while gently mixing. Centrifuge for 30 s at 70 to 100 × g.
5. Wash by adding 300 µl binding buffer and centrifuge for 30 s at 70 to 100 × g. Repeat this step.

IgG from most species and subclasses bind to protein G at near physiological pH and ionic strength. For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between antibody and ligand is weak, since this may decrease yield.

6. Replace the collection plate with a fresh collection plate prepared in step 1.
7. Add 200 µl of elution buffer to elute the antibody and centrifuge for 30 s at 70 × g. Collect the eluate. Repeat this step*.

***** *Most of the bound antibody is eluted after two elution steps*

Protein G Sepharose media bind IgG over a wide pH range with a strong affinity at neutral pH. To elute the IgG, it is necessary to lower the pH to between 2.5 and 3.0 depending on the antibody. If biological activity of the antibody or antibody fragment is lost due to the low pH required for elution, try Protein A Sepharose; the elution conditions used are generally milder (see Table 3.5 on page 69).

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

Storage

Store in 20% ethanol at 4°C to 8°C.

Ab SpinTrap columns

Ab SpinTrap are prepacked, single-use spin columns for minipreps of monoclonal and polyclonal antibodies from unclarified serum and cell culture supernatants (Fig 3.7). The columns are designed for small-scale purification of multiple samples in parallel and are suitable for use in antibody screening experiments. Ab SpinTrap contains Protein G Sepharose High Performance, which has a high protein binding capacity, and is compatible with all buffers commonly used in antibody purification.

The 50 columns supplied in each package can be used with a standard microcentrifuge and one purification run takes less than 20 min. Purification from unclarified samples minimizes loss of target protein caused by manual operations such as sample centrifugation, transfer of sample to centrifuge tubes, and collecting supernatant.

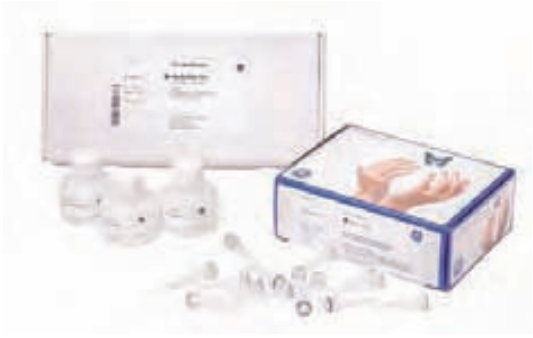
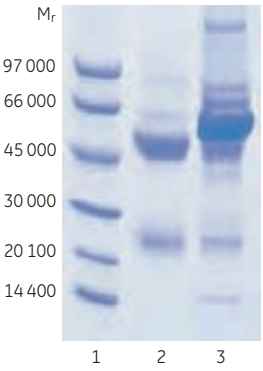


Fig 3.7. Ab SpinTrap columns and Ab Buffer Kit combine to allow rapid, small-scale purification of antibodies using a microcentrifuge.

Purification of antibodies from serum without sample clarification, dilution, or filtration is possible with Ab SpinTrap column. Figure 3.8 shows purification of anti-HSA (human serum albumin, lane 2 of SDS gel) from the undiluted serum of an immunized rabbit.



Column: Ab SpinTrap
Sample: 600 µl of undiluted serum from rabbit containing anti-HSA
Equilibration: 600 µl of 20 mM sodium phosphate, pH 7.0
Incubation: 4 min
Wash: 2 × 600 µl of 20 mM sodium phosphate, pH 7.0
Elution: 2 × 400 µl of 0.1 M glycine-HCl, pH 2.7

Lane

1. LMW markers
2. Eluted pool (diluted 1:5)
3. Start material (diluted 1:50)

Fig 3.8. SDS-PAGE (reducing conditions; ExcelGel™ SDS Gradient 8–18; Coomassie™ Blue staining) of eluted pool of purified anti-HSA in undiluted serum from an immunized rabbit.

Sample preparation

Refer to Chapter 2 for general considerations.

IgG from many species has a medium to strong affinity for protein G at approximately pH 7. The sample should have a pH around 7 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28-9030-59).

Purification

1. Prepare two collection tubes per sample for eluted fractions, each containing 30 µl neutralizing buffer.

To preserve the activity of acid-labile IgG, we recommend adding 30 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures the final pH of the sample will be approximately neutral.

2. Invert and shake the column repeatedly to resuspend the medium. Remove the bottom cap from the column using the plastic bottom cap removal tool. Save the bottom cap. Centrifuge for 30 s at 70 to 100 × g to remove the storage solution.
3. Equilibrate by adding 600 µl binding buffer, centrifuge for 30 s at 70 to 100 × g.
4. Bind antibody by adding max. 600 µl of antibody sample. Secure the top cap tightly and incubate for 4 min while gently mixing. Centrifuge for 30 s at 70 to 100 × g.
5. Wash by adding 600 µl binding buffer, centrifuge for 30 s at 70 to 100 × g.

Protein G Sepharose media bind IgG over a wide pH range with a strong affinity at neutral pH. To elute the IgG, it is necessary to lower the pH to between 2.5 and 3.0 depending on the antibody. If biological activity of the antibody or antibody fragment is lost due to the low pH required for elution, try Protein A Sepharose; the elution conditions used are generally milder (see Table 3.5 on page 69).

IgG from most species and subclasses bind to protein G at near physiological pH and ionic strength. For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between the antibody and ligand is weak, since this may decrease yield.

6. Add 400 µl of elution buffer and mix by inversion. Place the column in a 2 ml microcentrifuge tube containing 30 µl neutralizing buffer (see step 1). Elute by centrifugation for 30 s at 70 × g and collect the eluate.
7. Place the column in a new 2 ml microcentrifuge tube containing 30 µl neutralizing buffer (see step 1). Centrifuge for 30 s at 70 × g and collect the second eluate.

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

Storage

Store in 20% ethanol at 4°C to 8°C.

Protein G HP SpinTrap columns

Protein G HP SpinTrap are prepacked, single-use spin columns for protein enrichment of target antigens from antibody-antigen complexes of monoclonal and polyclonal antibodies from unclarified serum and cell culture supernatants (Fig 3.9). In addition, the columns are designed for small-scale purification of multiple samples in parallel and are suitable for use in antibody screening experiments. Protein G HP SpinTrap contains Protein G Sepharose High Performance, which has a high protein binding capacity, and is compatible with all buffers commonly used in antibody purification.

The 16 columns supplied in each package can be used with a standard microcentrifuge. Each package of Protein G HP SpinTrap contains a protocol for protein enrichment of target antibody-antigen complexes by immunoprecipitation and a protocol for antibody purification. The procedure for small-scale screening and purification of antibodies is described below. For a description of the immunoprecipitation procedure, download Instructions 28-9067-72 at www.gelifesciences.com/protein-purification.

Fig 3.9. Protein G HP SpinTrap columns are used for protein enrichment of target proteins using antibodies bound to the protein G ligand and are also used for small-scale purification of antibodies.

Sample preparation

Refer to Chapter 2 for general considerations.

IgG from many species has a medium to strong affinity for protein G at approximately pH 7. The sample should have a pH around 7 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28-9030-59).

Purification

1. Prepare two collection tubes per sample for eluted fractions, each containing 30 µl neutralizing buffer.

To preserve the activity of acid-labile IgG, we recommend adding 30 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures the final pH of the sample will be approximately neutral.

2. Invert and shake the column repeatedly to resuspend the medium. Remove the bottom cap from the column using the plastic bottom cap removal tool. Save the bottom cap. Centrifuge for 30 s at 70 to 100 × g to remove the storage solution.
3. Equilibrate by adding 600 µl binding buffer, centrifuge for 30 s at 70 to 100 × g.
4. Bind antibody by adding max. 600 µl of antibody sample. Secure the top cap tightly and incubate for 4 min while gently mixing. Centrifuge for 30 s at 70 to 100 × g.
5. Wash by adding 600 µl binding buffer, centrifuge for 30 s at 70 to 100 × g.

Protein G Sepharose media bind IgG over a wide pH range with a strong affinity at neutral pH. To elute the IgG, it is necessary to lower the pH to between 2.5 and 3.0 depending on the antibody. If biological activity of the antibody or antibody fragment is lost due to the low pH required for elution, try Protein A Sepharose; the elution conditions used are generally milder (see Table 3.5 on page 69).

IgG from most species and subclasses bind to protein G at near physiological pH and ionic strength. For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between the antibody and ligand is weak, since this may decrease yield.

6. Add 400 µl of elution buffer and mix by inversion. Place the column in a 2 ml microcentrifuge tube containing 30 µl neutralizing buffer (see step 1). Elute by centrifugation for 30 s at 70 × g and collect the eluate.
7. Place the column in a new 2 ml microcentrifuge tube containing 30 µl neutralizing buffer (see step 1). Centrifuge for 30 s at 70 × g and collect the second eluate.

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

Storage

Store in 20% ethanol at 4°C to 8°C.

HiTrap Protein G HP columns

HiTrap Protein G HP (Fig 3.10) is a convenient, ready-to-use column prepacked with Protein G Sepharose High Performance, which provides improved purification of polyclonal IgG from, for example, cow, sheep, and horse compared with protein A-based media (see Table 3.2 on page 48). Furthermore, protein G-based media allow binding of rat IgG, human IgG_3, and mouse IgG_1.

The columns are available in 1 ml and 5 ml sizes. In common with all HiTrap columns, HiTrap Protein G HP can be used for rapid antibody purification with a syringe, pump, or chromatography system such as ÄKTAdesign™. Furthermore, purification capacity can be greatly increased by connecting columns in series.

Fig 3.10. HiTrap Protein G HP columns are designed for antibody purification using a syringe, pump, or chromatography system.

Figure 3.11 shows the purification of mouse monoclonal IgG_1 on HiTrap Protein G HP. The monoclonal antibody was purified from a hybridoma cell culture supernatant.

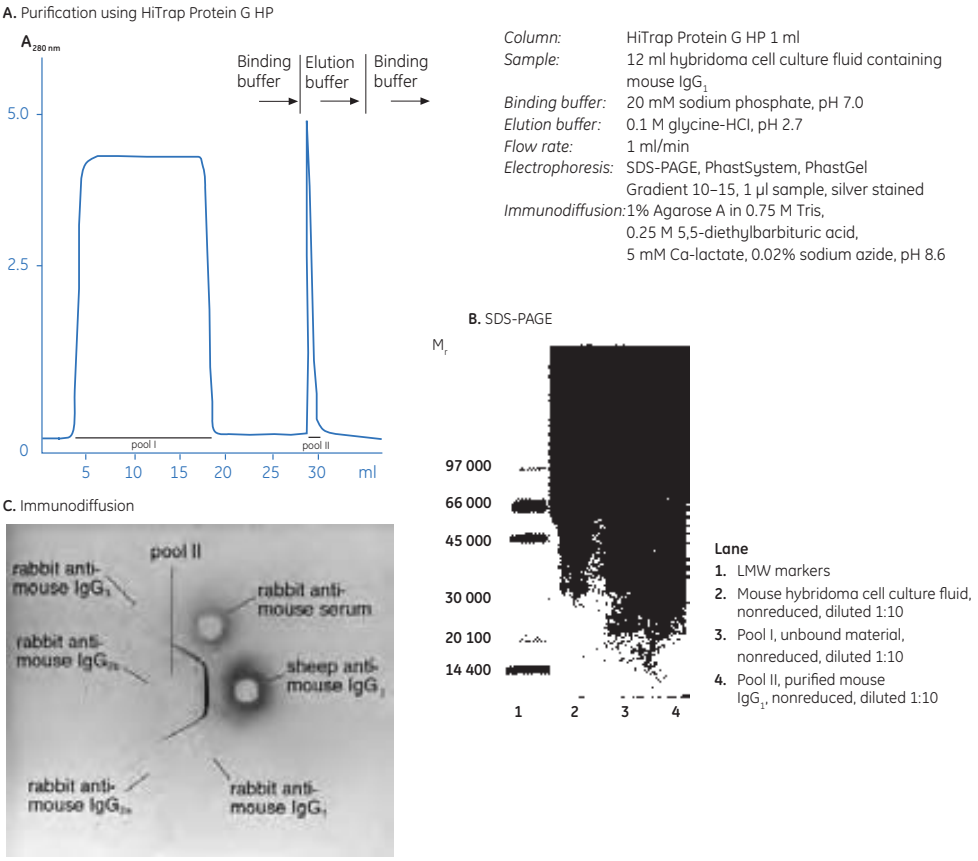


Fig 3.11. (A) Purification of mouse monoclonal IgG_1 from cell culture supernatant on HiTrap Protein G HP 1 ml column. Purity of mouse IgG_1 was confirmed by **(B)** nonreducing SDS-PAGE on PhastSystem™ using PhastGel™ 10-15 (silver stained) and **(C)** agarose-gel immunodiffusion.

Sample preparation

Refer to Chapter 2 for general considerations.

Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.

IgG from many species has a medium to strong affinity for protein G at approximately pH 7. The sample should have a pH around 7 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (Chapter 2) or dilution and pH adjustment.

If the sample is serum or ascites, dilute 1:1 with binding buffer.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

For purification using a syringe on HiTrap Protein G HP 1 ml or 5 ml columns, buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28-9030-59).

Purification

See Appendix 4 for general instructions for purification using HiTrap columns.

1. Prepare collection tubes by adding 60 to 200 µl of 1 M Tris-HCl, pH 9.0 per milliliter of fraction to be collected.

To preserve the activity of acid-labile IgG, we recommend adding 60 to 200 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

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

   **1 ml/min corresponds to approximately 30 drops/min when using a syringe with a 1 ml HiTrap column; 5 ml/min corresponds to approximately 120 drops/min when using a 5 ml HiTrap column*

IgG from most species and subclasses bind to protein G at near physiological pH and ionic strength. For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between the antibody and ligand is weak, since this may decrease yield.

8. Elute with elution buffer using a one-step or linear gradient. For step elution, 5 column volumes are usually sufficient. For linear gradient elution, 10 to 20 column volumes are usually sufficient. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for elution.

Protein G Sepharose media bind IgG over a wide pH range with a strong affinity at neutral pH. To elute the IgG, it is necessary to lower the pH to between 2.5 and 3.0 depending on the antibody. If biological activity of the antibody or antibody fragment is lost due to the low pH required for elution, try Protein A Sepharose; the elution conditions used are generally milder (see Table 3.5 on page 69).

9. After elution, regenerate the column by washing it with 3 to 5 column volumes of binding buffer. The column is now ready for a new purification of the same antibody.

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

To increase capacity, connect several HiTrap Protein G HP columns (1 ml or 5 ml) in series. HiTrap columns can be used with a syringe, a peristaltic pump or connected to a liquid chromatography system (see Chapter 5 for details of ÄKTAdesign chromatography systems).

Reuse of HiTrap Protein G Sepharose HP columns depends on the nature of the sample and should only be considered when processing identical samples to avoid cross-contamination.

Storage

Store in 20% ethanol at 4°C to 8°C.

MAbTrap Kit

MAbTrap™ Kit contains one HiTrap Protein G HP 1 ml column, binding, elution, and neutralization buffers, a syringe with fittings, and an optimized purification protocol (Fig 3.12). The kit contains sufficient material for up to 20 purifications of monoclonal or polyclonal IgG from serum, cell culture supernatant, or ascites, when using a syringe. The column can also be connected to a peristaltic pump if preferred.

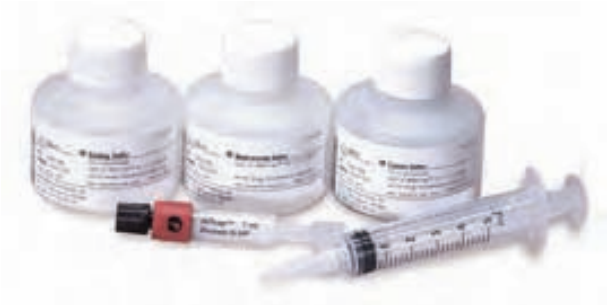
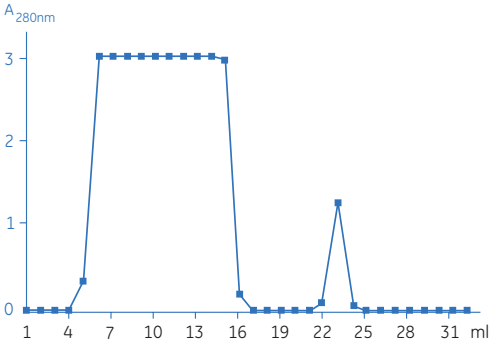


Fig 3.12. MabTrap Kit contains both a 1 ml HiTrap Protein G HP column and premade buffers for antibody purification.

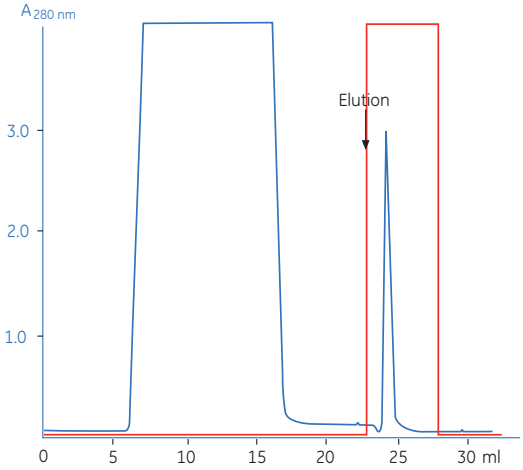
Figure 3.13 shows the purification of mouse monoclonal IgG_1 from cell culture supernatant using a syringe operation and a similar purification with pump operation. Operating HiTrap Protein G HP 1 ml with a syringe resulted in an IgG_1 pool of 3 ml with an absorbance of 0.44 (A_{280}) and a corresponding yield of 0.9 mg pure mouse monoclonal IgG_1. A similar experiment in which the column was operated with a P-1 pump resulted in an IgG_1 pool of 2 ml with an absorbance of 0.60 (A_{280}), corresponding also to a total yield of 0.9 mg pure mouse monoclonal IgG_1.

A. Purification using a syringe

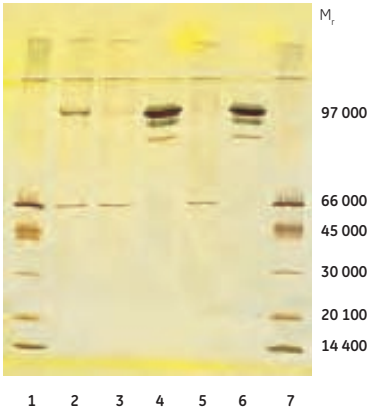


Column:	HiTrap Protein G HP 1 ml
Sample:	10 ml cell supernatant containing mouse monoclonal IgG_1, anti-transferrin
Binding buffer:	20 mM sodium phosphate, pH 7.0
Elution buffer:	0.1 M glycine-HCl, pH 2.7
Electrophoresis:	SDS-PAGE, PhastSystem, PhastGel Gradient 10–15, 1 µl sample, silver stained

B. Purification using a pump



C. SDS-PAGE analysis



Lane

1. LMW markers
2. Cell culture supernatant, mouse monoclonal IgG_1, diluted 1:1
3. Flowthrough, using peristaltic pump, diluted 1:10
4. Eluted mouse monoclonal IgG_1, using a peristaltic pump
5. Flowthrough, using a syringe, diluted 1:10
6. Eluted mouse monoclonal IgG_1, using a syringe
7. LMW markers

Fig 3.13. Purification of mouse monoclonal IgG_1 from cell culture supernatant; **(A)** using a syringe and **(B)** a peristaltic pump. **(C)** Analysis of eluted fractions by nonreducing SDS-PAGE on PhastSystem using PhastGel 10–15, silver stained.

Sample preparation

Refer to Chapter 2 for general considerations.

Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.

IgG from many species has a medium to strong affinity for protein G at approximately pH 7.0. The sample should have a pH around 7 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (Chapter 2) or dilution and pH adjustment.

If the sample is serum or ascites, dilute 1:1 with binding buffer.

Buffer preparation

Binding buffer: Dilute binding buffer concentrate 10-fold

Elution buffer: Dilute elution buffer concentrate 10-fold

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

Dilute the 10× buffer concentrates with high quality water as follows:

1. 2.5 ml binding buffer concentrate + 22.5 ml high quality water to a total volume of 25 ml.
2. 0.5 ml elution buffer concentrate + 4.5 ml high quality water to a total volume of 5 ml.

See Appendix 4 for general instructions for purification using HiTrap columns.

Purification

1. Prepare collection tubes by adding 60 to 200 µl of 1 M Tris-HCl, pH 9.0 per milliliter of fraction to be collected.

To preserve the activity of acid-labile IgG, we recommend adding 60 to 200 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

2. Fill the syringe with distilled water. Remove the stopper and connect the column to the syringe (use the connector supplied).
3. Remove the snap-off end at the column outlet.
4. Wash out the ethanol with 5 ml of distilled water.
5. Equilibrate the column with at least 3 ml of binding buffer. The recommended flow rate is 1 ml/min*.
6. Apply the sample using a syringe fitted to the Luer connector or by pumping it onto the column. For best results, use a flow rate of 0.2 to 1 ml/min during sample application.
7. Wash with binding buffer (generally at least 5 to 10 column volumes) until the absorbance reaches a steady baseline or no material remains in the effluent. Maintain a flow rate of 1 to 2 ml/min for washing.

* *1 ml/min corresponds to approximately 30 drops/min when using a syringe with a 1 ml HiTrap column.*

IgG from most species and subclasses bind to protein G at near physiological pH and ionic strength. For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between the antibody and ligand is weak, since this may decrease yield.

8. Elute with 3 to 5 ml of elution buffer using a one-step or linear gradient though larger volumes are sometimes required to break the interaction.

Protein G Sepharose media bind IgG over a wide pH range with a strong affinity at neutral pH. To elute the IgG, it is necessary to lower the pH to between 2.5 and 3.0 depending on the antibody. If biological activity of the antibody or antibody fragment is lost due to the low pH required for elution, try Protein A Sepharose; the elution conditions used are generally milder (see Table 3.5 on page 69).

9. After elution, regenerate the column by washing it with 3 to 5 ml of binding buffer. The column is now ready for a new purification.

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

Reuse of HiTrap Protein G HP columns depends on the nature of the sample and should only be considered when processing identical samples to avoid cross-contamination.

Storage

Store in 20% ethanol at 4°C to 8°C.

Purification using protein A-based media

Protein A is derived from a strain of *Staphylococcus aureus* and contains five regions that bind to the Fc region of IgG. As an affinity ligand, protein A is coupled to Sepharose so that these regions are free to bind. One molecule of coupled protein A can bind at least two molecules of IgG.

Both native protein A (nProtein A) and recombinant protein A (rProtein A) ligands are available from GE Healthcare. These molecules share similar specificity for the Fc region of IgG, but the recombinant protein A has been engineered to include a C-terminal cysteine that enables a single-point coupling when the protein is coupled to Sepharose, which ensures higher binding capacity. Besides the well-known affinity for the Fc region of IgG, protein A also has affinity for certain variants of the Fab region, and consequently, protein A affinity media can in some cases be used for the purification of Fab and $F(ab')_2$ fragments. Protein A Sepharose media from GE Healthcare also possess a considerably higher binding capacity than Protein G Sepharose media and therefore the preferred choice for capture of monoclonal antibodies in industrial-scale processes (see Chapter 7).

nProtein A Sepharose 4 Fast Flow is manufactured without using any animal-derived components. rProtein A is produced in *E. coli* and no human IgG affinity step is used during validated fermentation and purification processes, minimizing risk of human IgG contamination.

Native and recombinant Protein A Sepharose media are available in bulk media and in prepacked HiTrap columns for small-scale purification of antibodies. Native Protein A Sepharose is also available prepacked in spin column and 96-well plate format, for small-scale screening and purification.

Protein A Sepharose High Performance media provide sharper eluted peaks and more concentrated elution of antibodies compared with Protein A Sepharose 4 Fast Flow. However, the smaller bead size of the Sepharose High Performance compared with that of the Sepharose 4 Fast Flow matrix leads to increased backpressure on the column. The larger bead size of Sepharose 4 Fast Flow allows higher flow rate, which is essential when scaling up a purification.

MabSelect media are GE Healthcare's primary protein A media for the capture of MAbs in industrial purification processes. MabSelect media are composed of an agarose matrix with high-flow characteristics to facilitate the purification of large volumes of cell cultures. The recombinant protein A ligand of MabSelect is engineered to favor an oriented coupling that delivers enhanced binding capacity. MabSelect SuRe uses an alkali-tolerant recombinant protein A that is resistant to harsh cleaning agents such as sodium hydroxide. MabSelect Xtra uses the same recombinant protein A ligand as MabSelect, but the medium has a smaller particle size and greater porosity for increased dynamic binding capacity.

MabSelect media are all available in bulk and prepacked in HiTrap column format for purification screening, small-scale purification, and process development.

Protein A media are often a better choice than protein G media for isolating certain subclasses of IgG or for removing, for example, cross-species IgG contaminants from horse or fetal calf serum. Although IgG is the major human immunoglobulin, some other types have also been demonstrated to bind with protein A (see IgA and IgM page 95).

The binding strength of protein A to IgG depends upon the source species of the immunoglobulin. The dynamic binding capacity depends upon the binding strength and factors such as flow rate during sample application.

Leakage of ligands from an affinity medium must be considered, especially if harsh elution conditions are used. The multipoint attachment of protein A to Sepharose media results in very low ligand leakage over a wide range of elution conditions. Removal of ligand contaminant can be achieved by polishing using gel filtration or ion exchange chromatography.

The various purification options for Protein A Sepharose media are summarized in Table 3.4. Table 3.5 describes typical binding and elution conditions for Protein A Sepharose media.

Table 3.4. Purification options for IgG using protein A Sepharose media

Product	Format or column size	Binding capacity (mg IgG/ml medium)	Description
nProtein A Sepharose 4 Fast Flow	5 ml 25 ml	Approx. 30 (human)	Supplied as suspension for packing in, e.g., XK and Tricorn columns; used for scaling up purification.
Protein A HP MultiTrap	96-well plates	Approx. 20 (human)	For small-scale purification of IgG, screening of different protein constructs, optimization of buffer conditions, protein enrichment by immunoprecipitation.
Protein A HP SpinTrap	100 µl spin columns	Approx. 20 (human)	For small-scale purification of IgG and fragments, protein enrichment by immunoprecipitation.
HiTrap Protein A HP	1 ml, 5 ml columns	Approx. 20 (human)	Laboratory-scale purification of IgG and fragments. Prepacked 1 ml or 5 ml columns can be connected in series to facilitate scale-up.
rProtein A Sepharose Fast Flow	5 ml 25 ml	Approx. 50 (human)	Supplied as suspension for packing in, e.g., XK and Tricorn columns; used for scaling up purification. Higher binding capacity than nProtein A Sepharose 4 Fast Flow.
HiTrap rProtein A FF	1 ml, 5 ml columns	Approx. 50 (human)	Laboratory-scale purification of IgG, fragments, and subclasses. Prepacked 1 ml or 5 ml columns can be connected in series to scale up. Enhanced binding capacity.
MabSelect	25 ml	Approx. 30 (human)	Supplied as a suspension for column packing in all column scales. Retains high binding capacity at high flow rates. Suitable for scale-up applications and large-scale purification of antibodies.
HiTrap MabSelect	1 ml, 5 ml columns	Approx. 30 (human)	Laboratory-scale purification of IgG and fragments. Prepacked 1 ml or 5 ml columns can be connected in series to facilitate scale-up. Used in process development, screening, and small-scale purification.
MabSelect SuRe	25 ml	Approx. 30 (human)	Supplied as a suspension for column packing in all column scales. Retains high binding capacity at high flow rates. Tolerant of harsh cleaning conditions with e.g., NaOH. Retains high binding capacity at high flow rates.
HiTrap MabSelect SuRe	1 ml, 5 ml columns	Approx. 30 (human)	Laboratory-scale purification of IgG and fragments. Prepacked 1 ml or 5 ml columns can be connected in series to facilitate scale-up. Tolerant of harsh cleaning conditions with e.g., NaOH. Used in process development, screening, and small-scale purification.

Table continues on next page.

Product	Format or column size	Binding capacity (mg IgG/ml medium)	Description
MabSelect Xtra	25 ml	Approx. 40 (human)	Supplied as a suspension for column packing in all column scales. Suitable for scale-up and large-scale purification. Retains high binding capacity at high flow rates.
HiTrap MabSelect Xtra	1 ml, 5 ml columns	Approx. 40 (human)	Laboratory-scale purification of IgG and fragments. Prepacked 1 ml or 5 ml columns can be connected in series to scale up. High binding capacity. Used in process development, screening, and small-scale purification.
Companion product			
Ab Buffer Kit	1 Kit		Premade buffers recommended for antibody purification using Protein A HP MultiTrap, Protein A HP SpinTrap, HiTrap Protein A HP, and HiTrap rProtein A FF

Table 3.5. Binding and elution conditions commonly used with Protein A Sepharose media for purification of IgG from different species

Species	Subclass	Protein A binding pH	Protein A elution pH
Human	IgG_1	6.0–7.0	3.5–4.5
	IgG_2	6.0–7.0	3.5–4.5
	IgG_3	8.0–9.0	≤ 7.0
	IgG_4	7.0–8.0	3.0–6.0
Cow	IgG_2	n.a.	2.0
Goat	IgG_2	n.a	5.8
Guinea pig	IgG_1	n.a.	4.8
	IgG_2	n.a	4.3
Mouse	IgG_1	8.0–9.0	4.5–6.0
	IgG_{2a}	7.0–8.0	3.5–5.5
	IgG_{2b}	Approx. 7.0	3.0–4.0
	IgG_3	Approx. 7.0	3.5–5.5
Rat	IgG_1	≥ 9.0	7.0–8.0
	IgG_{2a}	≥ 9.0	≤ 8.0
	IgG_{2b}	≥ 9.0	≤ 8.0
	IgG_3	8.0–9.0	3.0–4.0 (using 3 M potassium isothiocyanate)

Binding strengths are tested with free protein A and can be used as guidelines to predict the binding behavior to a protein A purification medium. However, when coupled to an affinity matrix, the interaction may be altered. For example, rat IgG_1 does not bind to protein A, but does bind to Protein A Sepharose.

With some antibodies, for example mouse IgG_1, a high concentration of NaCl in the binding buffer may be necessary to achieve efficient binding. Recommended binding buffers are 1.5 M glycine, 3 M NaCl, pH 8.9 or 20 mM sodium phosphate, 3 M NaCl, pH 7.0.

Most antibodies and subclasses bind protein A close to physiological pH and ionic strength. Avoid excessive washing if the interaction between the protein of interest and the ligand is weak since this may decrease yield.

Use a mild elution method when labile antibodies are isolated. Reverse the flow of the wash buffer and elute with 0.1 M glycyltyrosine in 2 M NaCl, pH 7.0 at room temperature, applied in pulses. (Note: glycyltyrosine absorbs strongly at wavelengths used for detecting proteins). The specific elution is so mild that the purified IgG is unlikely to be denatured. Alternative elution buffers include: 1 M acetic acid pH 3.0, 0.1 M glycine-HCl pH 3.0, or 3 M potassium isothiocyanate. Note: potassium isothiocyanate can severely affect structure and immunological activity.

Desalt and/or transfer purified IgG fractions into a suitable buffer using a desalting column (see Chapter 2).

To increase capacity, connect several HiTrap columns (1 ml or 5 ml) in series. Alternatively pack a larger column with nProtein A Sepharose 4 Fast Flow or rProtein A Sepharose 4 Fast Flow (see Appendix 5). When working with large scale fermentation, consider using any of the MabSelect media. MabSelect is designed to retain a high binding capacity at the higher flow rates required to process large sample volumes as rapidly as possible.

Reuse of Protein A Sepharose media depends on the nature of the sample and should only be considered when processing identical samples to avoid cross-contamination.

nProtein A Sepharose 4 Fast Flow

Fig 3.14. nProtein A Sepharose 4 Fast Flow is an affinity medium for capture of monoclonal and polyclonal antibodies from cell culture supernatants, serum, and ascites.

nProtein A Sepharose 4 Fast Flow (Fig 3.14) is native protein A coupled to Sepharose 4 Fast Flow. The medium is designed for recovery and purification of monoclonal antibodies from cell culture supernatants, serum, and ascites at both laboratory and process scale.

Leakage of ligand from the Sepharose Fast Flow matrix is low. Moreover, nProtein A Sepharose 4 Fast Flow has high mechanical and chemical stability, withstanding high concentrations of hydrogen bond disrupting agents such as urea, guanidine hydrochloride, and sodium thiocyanate.

nProtein A Sepharose 4 Fast Flow is available as a bulk medium for packing in XK and Tricorn columns at laboratory scale. The low ligand leakage property and high flow rate characteristic of the Sepharose Fast Flow medium allow the use of nProtein A Sepharose 4 Fast Flow for scale-up of monoclonal and polyclonal antibody purification.

Column packing

Refer to Appendix 5 for general guidelines column packing.

Ideally, Sepharose 4 Fast Flow media are packed in XK or Tricorn columns in a two-step procedure: Do not exceed 0.3 bar (0.03 MPa) in the first step and 3.5 bar (0.35 MPa) in the second step. If the packing equipment does not include a pressure gauge, use a packing flow rate of 2.5 ml/min (XK 16/20 column) or 1.0 ml/min (Tricorn 10/100 column) in the first step, and 14 ml/min (XK 16/20 column) or 5.5 ml/min (Tricorn 10/100 column) in the second step.

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

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

Sample preparation

Refer to Chapter 2 for general considerations.

Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.

IgG from many species has a medium to strong affinity for protein A at physiological pH. Sample pH should be between 6 and 9 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0

Elution buffer: 0.1 M citric acid, pH 3.0

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Purification

1. Prepare collection tubes by adding 60 to 200 µl of 1 M Tris-HCl, pH 9.0 per milliliter of fraction to be collected.

To preserve the activity of acid-labile IgG, we recommend adding 60 to 200 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

2. If the column contains 20% ethanol, wash it with 5 column volumes of distilled water. Use a linear flow rate of 50 to 100 cm/h*.
3. Equilibrate the column with 5 to 10 column volumes of binding buffer at a linear flow rate of 150 cm/h.
4. Apply the pretreated sample.
5. Wash with binding buffer until the absorbance reaches the baseline.

   ** See Appendix 7 for information on how to convert linear flow (cm/h) to volumetric flow rates (ml/min)*

IgG from most species and subclasses bind to protein A at near physiological pH and ionic strength (see Table 3.5 on page 69). For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between the antibody and ligand is weak, since this may decrease yield.

6. Elute with elution buffer using a step or linear gradient. For step elution, 5 column volumes of elution buffer are usually sufficient. For linear gradient elution, a shallow gradient, over 20 column volumes, may separate proteins with similar binding strengths.
7. After elution, regenerate the column by washing it with 5 to 10 column volumes of binding buffer. The column is now ready for a new purification.

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

Storage

Store in 20% ethanol at 4°C to 8°C.

Protein A HP MultiTrap

Protein A HP MultiTrap is a 96-well plate prepacked with Protein A Sepharose High Performance for the preparation of protein samples, optimization of buffer conditions, enrichment of proteins of interest from clarified cell lysates and biological fluids, and purification of antibodies (Fig 3.15). Purification runs are performed in parallel, which ensures fast and reliable capture of antibodies from a large number of complex samples.

Each package of Protein A HP MultiTrap contains four prepacked multiwell plates and alternative protocols for protein enrichment of target antibody-antigen complexes by immunoprecipitation and antibody purification. Collection plates (see Ordering information) for collecting eluted, purified antibody are available separately.

The procedure described below addresses screening and purification of antibodies. For a description of immunoprecipitation using this product, download Instructions 28-9067-71 at www.gelifesciences.com/protein-purification.

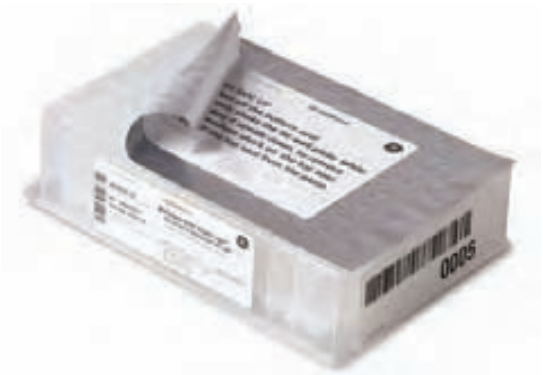


Fig 3.15. Protein A HP MultiTrap 96-well plates are used for rapid, parallel screening and small-scale purification of monoclonal and polyclonal antibodies.

Sample preparation

Refer to Chapter 2 for general considerations.

Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.

IgG from many species has a medium to strong affinity for protein A at physiological pH. Sample pH should be between 6 and 9 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28-9030-59).

Purification

1. Prepare two collection plates with 15 µl neutralizing buffer per well.

To preserve the activity of acid-labile IgG, we recommend adding 15 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

2. Invert and gently shake the MultiTrap plate to resuspend the medium. Remove top and bottom seals and place the MultiTrap plate on a collection plate. Centrifuge for 1 min at 70 to 100 × g to remove the storage solution.
3. Equilibrate by adding 300 µl binding buffer. Centrifuge for 30 s at 70 to 100 × g.
4. Bind antibody by adding maximum 300 µl of the antibody sample. Incubate for 4 min while gently mixing. Centrifuge for 30 s at 70 to 100 × g.
5. Wash by adding 300 µl binding buffer and centrifuge for 30 s at 70 to 100 × g. Repeat this step.

IgG from most species and subclasses bind to protein A at near physiological pH and ionic strength (see Table 3.5 on page 69). For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between the antibody and ligand is weak, since this may decrease yield.

6. Replace the collection plate with a fresh collection plate prepared in step 1.
7. Add 200 µl of elution buffer to elute the antibody and centrifuge for 30 s at 70 × g. Collect the eluate. Repeat this step*.

   ***** *Most of the bound antibody is eluted after two elution steps*

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

Storage

Store in 20% ethanol at 4°C to 8°C.

Protein A HP SpinTrap columns

Protein A HP SpinTrap (Fig 3.16) is a single-use spin column for protein enrichment of target antigens from antibody-antigen complexes and antibody purification from unclarified serum and cell culture supernatants. The columns are designed for small-scale purification of multiple samples in parallel and are suitable for use in antibody screening experiments. Protein A HP SpinTrap contains Protein A Sepharose High Performance, which has high protein binding capacity, and is compatible with all buffers commonly used in antibody purification.

The 16 columns supplied in each package can be used with a standard microcentrifuge. Each package of Protein A HP SpinTrap contains alternative protocols for protein enrichment of target antibody-antigen complexes by immunoprecipitation and for antibody purification. The procedure described below addresses screening and purification of antibodies. For a description of immunoprecipitation using this product, download Instructions 28-9067-70 at www.gelifesciences.com/protein-purification.

Fig 3.16. Protein A HP SpinTrap columns are used for protein enrichment of target proteins using antibodies bound to the protein A ligand or for small-scale purification of antibodies.

Sample preparation

Refer to Chapter 2 for general considerations.

Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.

IgG from many species has a medium to strong affinity for protein A at physiological pH. Sample pH should be between 6 and 9 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

Buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28-9030-59).

Purification

1. Prepare two collection tubes per sample for eluted fractions, each containing 30 µl neutralizing buffer.

To preserve the activity of acid-labile IgG, we recommend adding 30 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

2. Invert and shake the column repeatedly to resuspend the medium. Remove the bottom cap from the column using the plastic bottom cap removal tool. Save the bottom cap. Centrifuge for 30 s at 70 to 100 × g to remove the storage solution.
3. Equilibrate by adding 600 µl binding buffer, centrifuge for 30 s at 70 to 100 × g.
4. Bind antibody by adding max. 600 µl of the antibody sample. Secure the top cap tightly and incubate for 4 min while gently mixing. Centrifuge for 30 s at 70 to 100 × g.
5. Wash by adding 600 µl binding buffer, centrifuge for 30 s at 70 to 100 × g.

IgG from most species and subclasses bind to protein A at near physiological pH and ionic strength (see Table 3.5 on page 69). For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between the antibody and the ligand is weak, since this may decrease yield.

6. Add 400 µl of elution buffer and mix by inversion. Place the column in a 2 ml microcentrifuge tube containing 30 µl neutralizing buffer (see step 1). Elute by centrifugation for 30 s at 70 × g and collect the eluate.
7. Place the column in a new 2 ml microcentrifuge tube containing 30 µl neutralizing buffer (see step 1). Centrifuge for 30 s at 70 × g and collect the second eluate.

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

Storage

Store in 20% ethanol at 4°C to 8°C.

HiTrap Protein A HP columns

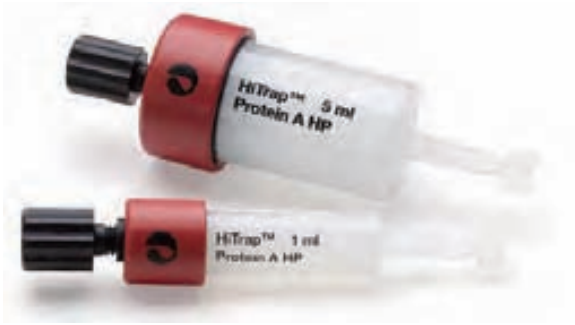
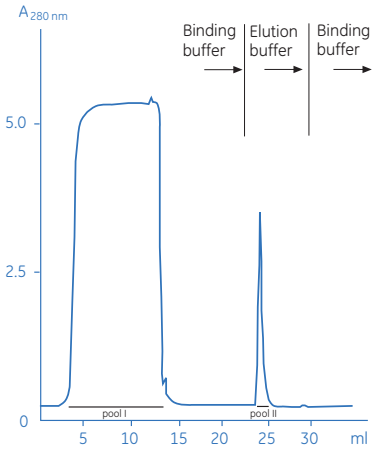


Fig 3.17 HiTrap Protein A HP columns are designed for rapid purification of antibodies using a syringe, pump, or chromatography system.

HiTrap Protein A HP are 1 ml and 5 ml ready-to-use columns prepacked with Protein A Sepharose High Performance (Fig 3.17). The columns are used for convenient purification of antibodies from cell culture supernatants, serum, and ascites. Purification can be performed using a syringe, pump, or chromatography system. Furthermore, purification capacity can be greatly increased by connecting columns in series.

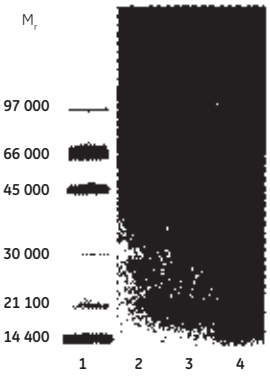
Figure 3.18 shows the purification of mouse monoclonal IgG_{2b} from a hybridoma cell culture supernatant using a 1 ml HiTrap Protein A HP column.

A. Purification using HiTrap Protein A HP



Column:	HiTrap Protein A HP 1 ml
Sample:	10 ml hybridoma cell culture containing mouse IgG_{2b}
Binding buffer:	20 mM sodium phosphate, pH 7.0
Elution buffer:	0.1 M citric acid-NaOH, pH 3.0
Flow rate:	1 ml/min
Electrophoresis:	SDS-PAGE, PhastSystem, PhastGel Gradient 10–15, 1 µl sample, silver stained
Immunodiffusion:	1% Agarose A in 0.75 M Tris, 0.25 M 5,5-diethylbarbituric acid, 5 mM Ca-lactate, 0.02% sodium azide, pH 8.6

B. SDS-PAGE analysis



Lane

1. LMW markers
2. Mouse cell culture, nonreduced, diluted 1:10
3. Pool I, unbound material, nonreduced, diluted 1:10
4. Pool II, purified mouse IgG_1, nonreduced, diluted 1:10

C. Immunodiffusion

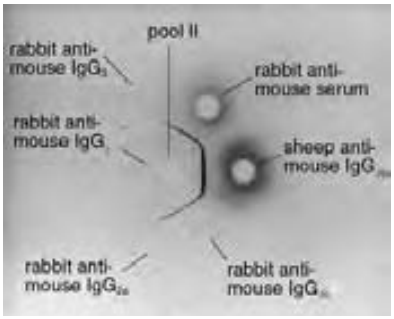


Fig 3.18. (A) Purification of mouse monoclonal IgG_{2b} from cell culture supernatant on HiTrap Protein A HP, 1 ml column. Purity of mouse IgG_{2b} was confirmed by **(B)** nonreducing SDS-PAGE on PhastSystem using PhastGel 10-15 (silver stained) and **(C)** agarose-gel immunodiffusion.

Sample preparation

Refer to Chapter 2 for general considerations.

Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.

IgG from many species has a medium to strong affinity for protein A at physiological pH. Sample pH should be between 6 and 9 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (Chapter 2) or by dilution and pH adjustment.

Buffer preparation

Binding buffer:	20 mM sodium phosphate, pH 7.0
Elution buffer:	0.1 M citric acid, pH 3–6
Neutralizing buffer:	1 M Tris-HCl, pH 9.0

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

Using a syringe on HiTrap Protein A HP 1 ml or 5 ml columns, buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28-9030-59).

Purification

See Appendix 4 for general instructions for purification using HiTrap columns.

1. Prepare collection tubes by adding 60 to 200 µl of 1 M Tris-HCl, pH 9.0 per milliliter of fraction to be collected.

To preserve the activity of acid-labile IgG, we recommend adding 60 to 200 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

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

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

IgG from most species and subclasses bind to protein A at near physiological pH and ionic strength (see Table 3.5 on page 69). For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between the antibody and ligand is weak, since this may decrease yield.

8. Elute with elution buffer using a one-step or linear gradient. For step elution, 5 column volumes is usually sufficient. For linear gradient elution, 10 to 20 column volumes are usually sufficient. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for elution.
9. After elution, regenerate the column by washing it with 3 to 5 column volumes of binding buffer. The column is now ready for a new purification.

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

To increase capacity, connect several HiTrap Protein A HP columns (1 ml or 5 ml) in series. HiTrap columns can be used with a syringe, peristaltic pump, or can be connected to a liquid chromatography system (see Chapter 5 for details of ÄKTAdesign chromatography systems).

Reuse of HiTrap Protein A HP columns depends on the nature of the sample and should only be considered when processing identical samples to avoid cross-contamination.

Storage

Store in 20% ethanol at 4°C to 8°C.

rProtein A Sepharose Fast Flow

Fig 3.19. rProtein A Sepharose Fast Flow is an affinity medium with high binding capacity for antibodies, enabling capture of up to 50 mg antibody/ml medium.

rProtein A Sepharose Fast Flow (Fig 3.19) is an affinity medium with high binding capacity for monoclonal and polyclonal antibodies. The binding capacity of rProtein A Sepharose Fast Flow is considerably higher than for nProtein A Sepharose 4 Fast Flow. The recombinant protein A ligand of rProtein A Sepharose Fast Flow has been specially engineered to favor an oriented coupling giving a matrix with enhanced binding capacity. Ligand leakage is low.

rProtein A Sepharose Fast Flow is produced in *E. coli* and no human IgG affinity step is used during validated fermentation and purification processes, minimizing risk of human IgG contamination.

rProtein A is available as a bulk medium for packing in XK and Tricorn columns at laboratory scale. The low ligand leakage property and high flow rate characteristic of the Sepharose Fast Flow medium allow the use of rProtein A Sepharose Fast Flow for scaling up purification of monoclonal and polyclonal antibodies. The medium is also available in prepacked 1 ml and 5 ml HiTrap rProtein A FF columns, which allow convenient, one-step purification. Furthermore, purification capacity can be greatly increased by connecting columns in series.

Column packing

Refer to Appendix 5 for general column packing guidelines.

Ideally, Sepharose 4 Fast Flow media are packed in XK or Tricorn columns in a two-step procedure: Do not exceed 0.3 bar (0.03 MPa) in the first step and 3.5 bar (0.35 MPa) in the second step. If the packing equipment does not include a pressure gauge, use a packing flow rate of 2.5 ml/min (XK 16/20 column) or 1.0 ml/min (Tricorn 10/100 column) in the first step, and 14 ml/min (XK 16/20 column) or 5.5 ml/min (Tricorn 10/100 column) in the second step.

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

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

Sample preparation

Refer to Chapter 2 for general considerations.

Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.

IgG from many species has a medium to strong affinity for protein A at physiological pH. Sample pH should be between 6 and 9 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0

Elution buffer: 0.1 M sodium citrate, pH 3–6

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Purification

1. Prepare collection tubes by adding 60 to 200 µl of 1 M Tris-HCl, pH 9.0 per milliliter of fraction to be collected.

To preserve the activity of acid-labile IgG, we recommend adding 60 to 200 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

2. If the column contains 20% ethanol, wash it with 5 column volumes of distilled water. Use a linear flow rate of 50 to 100 cm/h*.
3. Equilibrate the column with 5 to 10 column volumes of binding buffer at a linear flow rate of 150 cm/h.
4. Apply the pretreated sample.
5. Wash with binding buffer until the absorbance reaches the baseline.

* *See Appendix 7 for information on how to convert linear flow (cm/h) to volumetric flow rates (ml/min)*

IgG from most species and subclasses bind to protein A at near physiological pH and ionic strength (see Table 3.5 on page 69). For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between the antibody and ligand is weak, since this may decrease yield.

6. Elute with elution buffer using a step or linear gradient. For step elution, 5 column volumes of elution buffer are usually sufficient. For linear gradient elution, a shallow gradient, over 20 column volumes, may separate proteins with similar binding strengths.
7. After elution, regenerate the column by washing it with 5 to 10 column volumes of binding buffer. The column is now ready for a new purification.

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

Storage

Store in 20% ethanol at 4°C to 8°C.

HiTrap rProtein A FF columns

Fig 3.20. HiTrap rProtein A FF are columns prepacked with rProtein A Sepharose Fast Flow, which has very high binding capacity for monoclonal antibodies.

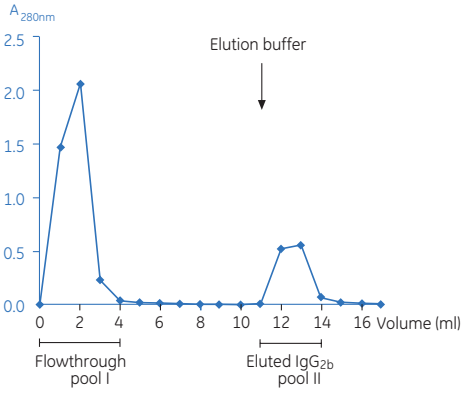
HiTrap rProtein A FF is a ready-to-use column prepacked with rProtein A Sepharose Fast Flow (Fig 3.20) for the convenient purification of monoclonal antibodies from cell culture supernatants, serum, and ascites. The column is suitable for small-scale purification of monoclonal antibodies from multiple species, screening, and process development.

The columns are available in 1 ml and 5 ml sizes. In common with all HiTrap columns, HiTrap rProtein A FF can be used for rapid purification using a syringe, pump, or chromatography system. Furthermore, purification capacity can be greatly increased by connecting columns in series.

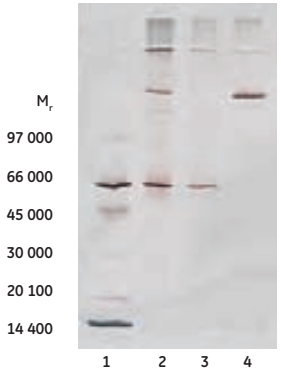
Figure 3.21 shows the purification of mouse IgG_{2b} from ascites on a HiTrap rProtein A FF 1 ml column using a syringe. The eluted pool contained 1 mg of IgG_{2b} and the silver stained SDS-PAGE gel confirmed a purity level of over 95%.

A. Purification using HiTrap rProtein A FF

Column:	HiTrap rProtein A FF, 1 ml
Sample:	1 ml mouse ascites containing IgG_{2b} filtered through a 0.45 µm filter. The sample was a kind gift from Dr. N. Linde, EC Diagnostics, Sweden
Binding buffer:	20 mM sodium phosphate, pH 7.0
Elution buffer:	0.1 M sodium citrate, pH 3.0
Flow rate:	Approx. 1 ml/min
Instrumentation:	Syringe



B. SDS-PAGE analysis



Lane

1. LMW markers
2. Mouse cell culture, nonreduced, diluted 1:10
3. Pool I, unbound material, nonreduced, diluted 1:10
4. Pool II, purified mouse IgG_{2b}, nonreduced, diluted 1:10

Fig 3.21. (A) Purification of mouse IgG_{2b} from ascites on a 1 ml HiTrap rProtein A FF column using a syringe. **(B)** Analysis of eluted IgG_{2b} by SDS-PAGE on PhastSystem and a PhastGel Gradient 10-15 precast gel revealed a purity of 95% (silver staining).

Sample preparation

Refer to Chapter 2 for general considerations.

Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.

IgG from many species has a medium to strong affinity for protein A at physiological pH. Sample pH should be between 6 and 9 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0

Elution buffer: 0.1 M sodium citrate, pH 3-6

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Using a syringe on HiTrap rProtein A FF 1 ml or 5 ml columns, buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28-9030-59).

Purification

See Appendix 4 for general instructions for purification using HiTrap columns.

1. Prepare collection tubes by adding 60 to 200 µl of 1 M Tris-HCl, pH 9.0 per milliliter of fraction to be collected.

To preserve the activity of acid-labile IgG, we recommend adding 60 to 200 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

2. Fill the syringe or pump tubing with distilled water. Remove the stopper and connect the column to the syringe (use the connector supplied), laboratory pump, or chromatography system "drop to drop" to avoid introducing air into the system.
3. Remove the snap-off end at the column outlet.
4. Wash out the ethanol with 3 to 5 column volumes of distilled water.
5. Equilibrate the column with at least 5 column volumes of binding buffer. Recommended flow rates are 1 ml/min (1 ml column) and 5 ml/min (5 ml column)*.
6. Apply the pretreated sample using a syringe fitted to the Luer connector or by pumping it onto the column. For best results, use a flow rate of 0.2 to 1 ml/min (1 ml column) and 0.5 to 5 ml/min (5 ml column) during sample application.

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

7. Wash with binding buffer (generally at least 5 to 10 column volumes) until the absorbance reaches a steady baseline or no material remains in the effluent. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for washing.

IgG from most species and subclasses bind to protein A at near physiological pH and ionic strength (see Table 3.5 on page 69). For the optimum binding conditions for IgG from a particular species, consult the most recent literature. Avoid excessive washing if the interaction between the antibody and ligand is weak, since this may decrease yield.

8. Elute with elution buffer using a one-step or linear gradient. For step elution, 5 column volumes is usually sufficient. For linear gradient elution, 10 to 20 column volumes are usually sufficient. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for elution.
9. After elution, regenerate the column by washing it with 3 to 5 column volumes of binding buffer. The column is now ready for a new purification.

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

To increase capacity, connect several HiTrap rProtein A FF columns (1 ml or 5 ml) in series. HiTrap columns can be used with a syringe, a peristaltic pump or connected to a liquid chromatography system (see Chapter 5 for details of ÄKTAdesign chromatography systems).

Reuse of HiTrap rProtein A FF columns depends on the nature of the sample and should only be considered when processing identical samples to avoid cross-contamination.

Storage

Store in 20% ethanol at 4°C to 8°C.

MabSelect media and prepacked columns

The MabSelect family of media consists of MabSelect, MabSelect SuRe, and MabSelect Xtra bulk media, as well as prepacked HiTrap columns for purification of MAbs in the laboratory and for process development. MabSelect media are BioProcess™ affinity media for capture of monoclonal antibodies from large volumes of feed by packed bed chromatography. The recombinant protein A ligand is engineered for oriented coupling to the highly cross-linked agarose base matrix to give a robust affinity medium with enhanced binding capacity for IgG. The low ligand leakage of the ligand combined with the stability of the novel base matrix make MabSelect media suitable for purification of MAbs at process scale.

MabSelect media are available prepacked in 1 ml and 5 ml HiTrap columns (Fig 3.22) for fast purification of MAbs in the laboratory, scale-up, and process development. HiTrap columns can be connected in series to allow scale-up. The prepacked MabSelect medium withstands the high flow rates and high pressure used in purification scale-up and retains the high binding capacity of the bulk medium (Fig 3.23).

Fig 3.22. HiTrap MabSelect, HiTrap MabSelect SuRe, and HiTrap MabSelect Xtra columns for laboratory scale purification of monoclonal antibodies with optimized binding capacity at high flow rates.

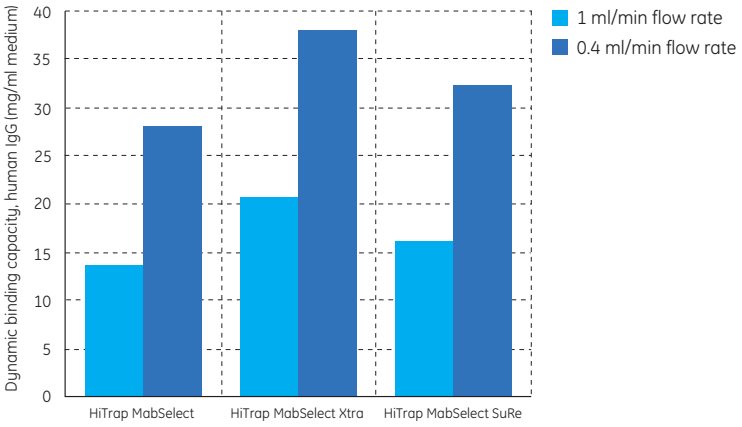


Fig 3.23. MabSelect media feature a highly cross-linked agarose, produced using a manufacturing process that gives a very rigid matrix that is optimal for high-throughput affinity chromatography of IgG.

MabSelect SuRe has been developed from the same highly cross-linked agarose matrix used for MabSelect, which enables high flow rates at low backpressure. In contrast to the recombinant protein A ligand of MabSelect, however, the alkali-tolerant recombinant protein A ligand of MabSelect SuRe is resistant to harsh cleaning agents (NaOH), resulting in significant cost savings. The high alkaline tolerance of the medium also provides the possibility to extend the number of cycles in regular large-scale production.

HiTrap MabSelect SuRe columns are prepacked with MabSelect SuRe. These columns can be used to develop an effective cleaning-in-place (CIP) protocol for purification of MAbs at industrial scale.

MabSelect Xtra has been developed to meet the demands of ever-increasing levels of expression in monoclonal antibody feedstocks. MabSelect Xtra uses the same recombinant protein A ligand as MabSelect, but has a smaller particle size and greater porosity, which ensures increased dynamic binding capacity at high flow rates. The medium provides a lower overall production cost due to the possibility of processing concentrated feedstocks in fewer batches. Process development, screening of purification conditions and small-scale purification of MAbs can be performed using HiTrap MabSelect Xtra columns.

Packing Tricorn 10/100 columns with MabSelect or MabSelect SuRe

Refer to Appendix 5 for general column packing guidelines.

Preparing the suspension

Suspension solution: 50 mM phosphate buffer, 0.15 M NaCl, pH 7.0

Packing solution: 0.15 M NaCl

10 ml MabSelect or MabSelect SuRe (corresponds to 14 ml MabSelect or MabSelect SuRe/20% ethanol suspension)

Sintered glass filter funnel (medium grade, G3 type)

Filtering flask

1. Equilibrate all materials to room temperature.
2. Mount the glass filter funnel onto the filtering flask.
3. Pour 14 ml of MabSelect or MabSelect SuRe/20% ethanol suspension into the funnel and wash with 2 × 20 ml distilled water followed by 2 × 20 ml packing solution.
4. Transfer the sedimented medium from the funnel into a beaker and add 9.5 ml packing solution. This will give a 51% medium suspension.

Assembling and packing the column

Equipment needed:

Tricorn 10/100 column,

Tricorn 10 Coarse Filter Kit

Tricorn Packing Connector 10-10

Tricorn Glass Tube 10/100

Tricorn 10 bottom unit with a 10 mm filter mounted

Pump P-901 or similar

20 ml pipettes

See Ordering information for packing accessory code numbers

1. Details of column parts and packing equipment can be found in the instructions supplied with the column. Before packing, ensure that all parts are clean and intact.
2. Wet a bottom coarse filter from the Tricorn 10 Coarse Filter Kit in 70% ethanol. Insert into the column.
3. Insert the filter holder into the column tube. Ensure that the keyed part of the filter holder fits into the slot on the threaded section on the column tube. Push the filter holder into place.
4. Screw the end cap onto the column tube. Insert a stop plug into the bottom unit.
5. Screw Tricorn Packing Connector 10-10 onto the top of the column tube. The packing connector must be fitted with suitable O-rings (included with Tricorn Packing Connector 10-10).
6. Mount the column and packing unit vertically.
7. Screw Tricorn Glass Tube 10/100 into the upper fitting of Tricorn Packing Connector 10-10.
8. Transfer the resuspended 51% suspension of MabSelect or MabSelect SuRe into the glass tube in a continuous motion using a 20 ml pipette.

Pipetting the suspension down the column wall minimizes formation of air bubbles.

9. Attach the Tricorn 10 Bottom Unit mounted with a 10 mm filter to the top of the glass tube. The filter will distribute an even flow during packing. Place a beaker beneath the column tube and connect a pump to the top of the packing unit. Remove the stop plug from the bottom of the column tube. Packing is performed in two steps: For MabSelect, pack at 2.5 ml/min (191 cm/h) for 20 min, followed by 10 ml/min (764 cm/h) for 2 min. For MabSelect SuRe, pack at 0.8 ml/min (61 cm/h) for 20 min, followed by 10 ml/min (764 cm/h) for 2 min.

Ensure that backpressure does not exceed the pressure limits (< 5 MPa) of the column during packing.

10. Switch off and disconnect the pump, re-fit the stop plug into Tricorn 10 Bottom Unit. Take the column from the stand and remove Tricorn 10/100 Glass Tube and packing connector over a sink. Remount the column vertically.
11. If necessary, remove excess medium by resuspending the top of the packed bed and remove with a Pasteur pipette or spatula. For a 10 cm bed height, the surface of the packed bed should be leveled with the lower end of the glass tube threads. Top-off the column with packing solution.
12. Wet a top coarse filter from the Tricorn 10 Coarse Filter Kit, the bottom of the adapter, and O-ring in 70% ethanol. Place the top coarse filter at the center of the adapter with the glossy side towards the adapter. Mount the adapter unit onto the column tube. Connect the pump.
13. Remove the stop plug and continue to pack at 10 ml/min (764 cm/h) for 2 min.
14. Mark the position of the bed surface on the column. Stop the pump, re-fit the stop plug into the bottom of the column. Reposition the adapter to approximately 1 mm below the marked position.
15. Wash the column with 15 ml of distilled water at 5 ml/min (382 cm/h) before checking packing quality.

Packing Tricorn 10/100 columns with MabSelect Xtra

Refer to Appendix 5 for general column packing guidelines.

Preparing the suspension

Suspension solution: 50 mM phosphate buffer, 0.15 M NaCl, pH 7.0

Packing solution: 0.15 M NaCl

4 M NaCl

10 ml MabSelect Xtra (corresponds to 14 ml MabSelect Xtra/20% ethanol suspension)

Sintered glass filter funnel (medium grade, G3 type)

Filtering flask

1. Equilibrate all materials to room temperature.
2. Mount the glass filter funnel onto the filtering flask.
3. Pour 14 ml of MabSelect Xtra/20% ethanol suspension into the funnel and wash with 2 × 20 ml distilled water followed by 2 × 20 ml packing solution.
4. Transfer the sedimented medium from the funnel into a beaker and add 15 ml packing solution. This will give a 40% medium suspension.

Assembling and packing the column

Equipment needed:

Tricorn 10/100 column

Tricorn 10 Coarse Filter Kit

Tricorn Packing Connector 10-10

Tricorn Glass Tube 10/300

Tricorn 10 bottom unit with a 10 mm filter mounted

Pump P-901 or similar

25 ml pipettes

See Ordering information for packing accessory code numbers

1. Details of the column parts and packing equipment can be found in the instructions supplied with the column. Before packing, ensure that all parts are clean and intact.
2. Wet a bottom coarse filter from the Tricorn 10 Coarse Filter Kit in 70% ethanol. Insert into the column.
3. Insert the filter holder into the column tube. Ensure that the keyed part of the filter holder fits into the slot on the threaded section on the column tube. Push the filter holder into place.
4. Screw the end cap onto the column tube. Insert a stop plug into the bottom unit.
5. Screw Tricorn Packing Connector 10-10 onto the top of the column tube. The packing connector must be fitted with suitable O-rings (included with Tricorn Packing Connector 10-10).
6. Mount the column and packing unit vertically.
7. Screw Tricorn Glass Tube 10/300 into the upper fitting of Tricorn Packing Connector 10-10.
8. Fill the Tricorn column with 14 ml of 4 M NaCl using a pipette.
9. Transfer the resuspended 40% suspension of MabSelect Xtra into the glass tube in a continuous motion using a 25 ml pipette.

Pipetting the suspension down the column wall minimizes formation of air bubbles.

The suspension should form a layer over the 4 M NaCl solution.

10. Attach the Tricorn 10 Bottom Unit mounted with a 10 mm filter to the top of the glass tube. The filter will distribute an even flow during packing. Place a beaker beneath the column tube and connect a pump to the top of the packing unit. Remove the stop plug from the bottom of the column tube. Packing is performed in two steps: Pack at 0.8 ml/min for (61 cm/h) 20 min, followed by 10 ml/min (764 cm/h) for 2 min.

Ensure that backpressure does not exceed the pressure limits (< 5 MPa) of the column during packing.

11. Switch off and disconnect the pump, re-fit the stop plug into Tricorn 10 Bottom Unit. Take the column from the stand and remove Tricorn 10/300 Glass Tube and packing connector over a sink. Remount the column vertically.
12. If necessary, remove excess medium by resuspending the top of the packed bed and remove with a Pasteur pipette or spatula. For a 10 cm bed height, the surface of the packed bed should be leveled with the lower end of the glass tube threads. Top-off the column with packing solution.

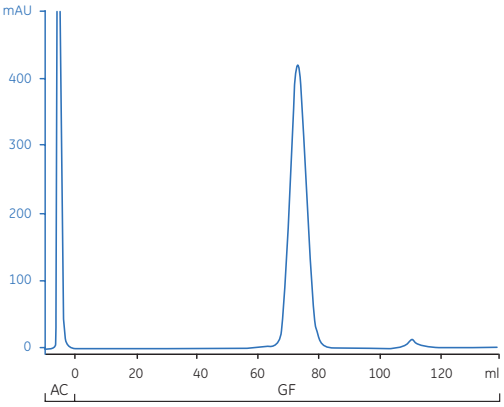
13. Wet a top coarse filter from the Tricorn 10 Coarse Filter Kit, the bottom of the adapter, and O-ring in 70% ethanol. Place the top coarse filter at the center of the adapter with the glossy side towards the adapter. Mount the adapter unit onto the column tube. Connect the pump.
14. Remove the stop plug and continue to pack at 10 ml/min (764 cm/h) for 1 min.
15. Mark the position of the bed surface on the column. Stop the pump, re-fit the stop plug into the bottom of the column. Reposition the adapter to approximately 1 mm below the marked position.
16. Wash the column with 15 ml of distilled water at 5 ml/min (382 cm/h) before checking packing quality.

HiTrap MabSelect and HiTrap MabSelect Xtra

This section describes a general procedure for purification of MAbs using HiTrap MabSelect and HiTrap MabSelect Xtra prepacked columns.

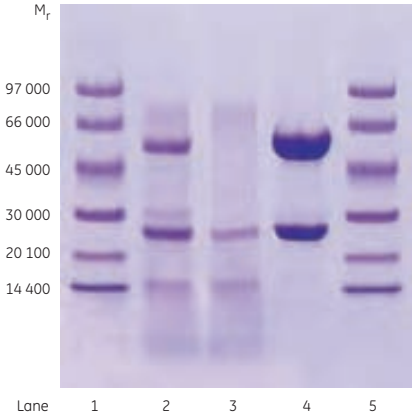
Figure 3.24 shows capture of mouse monoclonal IgG_{2a} by affinity chromatography (AC) using HiTrap MabSelect followed by a gel fitration (GF) polishing step. The purified MAb is seen in lane 4 of the SDS-PAGE gel.

A. Purification using HiTrap MabSelect on ÄKTAxpress MAb



Affinity column:	HiTrap MabSelect 1 ml
Gel filtration column:	HiLoad 16/60 Superdex 200 pg
Sample:	Filtered mouse myeloma cell culture, 165 mg/l IgG_{2a}
Sample volume:	75 ml
Binding buffer (affinity):	20 mM phosphate, 0.15 M NaCl, pH 7.4
Elution buffer (affinity):	0.1 M sodium citrate, pH 3.0
Buffer (gel filtration):	0.15 M NaCl
Flow rate:	
affinity:	1 ml/min
gel filtration:	1.5 ml/min
System:	ÄKTAxpress MAb

B. SDS-PAGE analysis



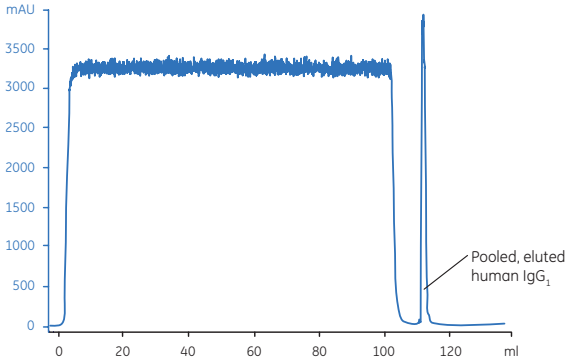
Lane

1. LMW markers
2. Start material
3. Flowthrough
4. Pool eluted purified mouse IgG_{2a}
5. LMW markers

Fig 3.24. (A) Purification of IgG_{2a} using HiTrap MabSelect 1 ml in an automated, two-step purification on ÄKTAxpress™ MAb **(B)** SDS-PAGE analysis on ExcelGel SDS Gradient 8-18, reducing conditions.

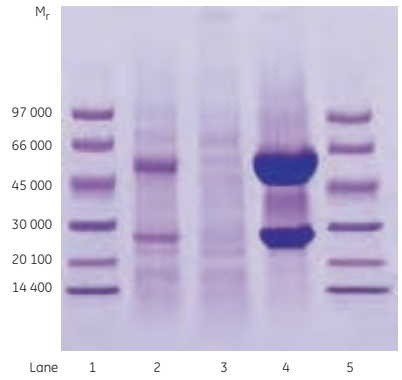
Successful purification of human monoclonal IgG_1 in one step using HiTrap MabSelect Xtra is shown in Figure 3.25. Highly pure MAb is seen in lane 4 of the SDS-PAGE gel.

A. Purification using HiTrap MabSelect Xtra on ÄKTAexplorer 10



Column: HiTrap MabSelect Xtra 1 ml
Sample: Clarified CHO cell culture, 0.11 mg/ml human IgG_1
Sample volume: 100 ml
Binding buffer: 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4
Elution buffer: 0.1 M sodium citrate, pH 3.0
Flow rate: 1 ml/min
System: ÄKTAexplorer 10

B. SDS-PAGE analysis



Lane
1. LMW markers
2. Start material
3. Flowthrough
4. Pool of eluted, purified human IgG_1
5. LMW markers

Fig 3.25. (A) Purification of human IgG_1 in one step on HiTrap MabSelect Xtra 1 ml on ÄKTAexplorer™ 10. **(B)** SDS-PAGE analysis on ExcelGel SDS Gradient 8-18, reducing conditions.

Sample preparation

Refer to Chapter 2 for general considerations.

Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.

IgG from many species has a medium to strong affinity for protein A at physiological pH. Sample pH should be between 6 and 9 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 0.15 M NaCl, pH 7.2

Elution buffer: 0.1 M sodium citrate, pH 3–3.6

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Purification

See Appendix 4 for general instructions for purification using HiTrap columns.

1. Prepare collection tubes by adding 60 to 200 µl of 1 M Tris-HCl, pH 9.0 per milliliter of fraction to be collected.

To preserve the activity of acid-labile IgG, we recommend adding 60 to 200 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

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

   ** 1 ml/min corresponds to approximately 30 drops/min when using a syringe with a 1 ml HiTrap column; 5 ml/min corresponds to approximately 120 drops/min when using a 5 ml HiTrap column*

Stepwise elution allows the target antibody to be eluted in a more concentrated form, thus decreasing buffer consumption and shortening cycle times. Decreasing the flow rate may be necessary due to the high concentrations of protein in the eluted pool.

When optimizing elution conditions, determine the highest pH that allows efficient elution of antibody from the column. This will prevent denaturing sensitive antibodies due to low pH.

9. After elution, regenerate the column by washing it with 3 to 5 column volumes of binding buffer. The column is now ready for a new purification.
10. If required, perform cleaning-in-place (see Cleaning-in-place on the next page).

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

Reuse of HiTrap columns depends on the nature of the sample and should only be considered when processing identical samples to avoid cross-contamination.

Cleaning-in-place (CIP)

For cleaning-in-place protocols for the removal of unwanted precipitated or denatured contaminants and hydrophobically bound substances, see Appendix 1 and the Instructions for HiTrap MabSelect/HiTrap MabSelect Xtra (28-4084-14), which are available for download at www.gelifesciences.com/protein-purification.

Storage

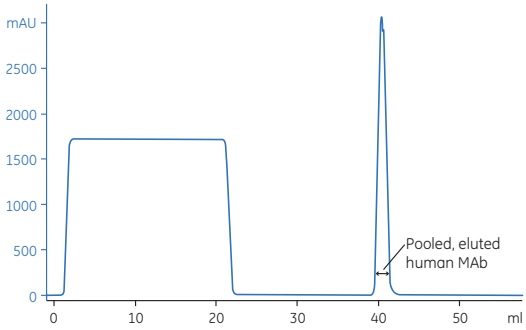
Store in 20% ethanol at 4°C to 8°C.

HiTrap MabSelect SuRe

This section describes purification of monoclonal antibodies with HiTrap MabSelect SuRe. The protocol includes a CIP procedure to minimize cross-contamination from different antibodies between purification runs, as well as for general column cleaning after a purification run.

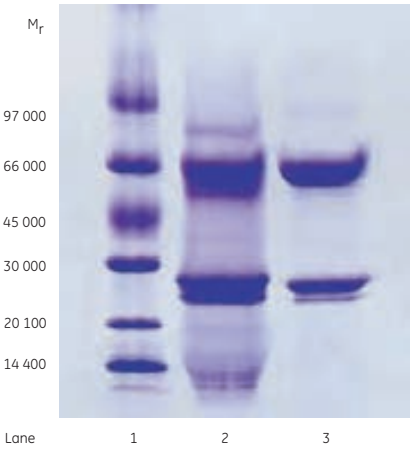
Figure 3.26 shows capture of a human monoclonal antibody by affinity chromatography using HiTrap MabSelect SuRe. SDS-PAGE of pooled, eluted fractions shows the high purity of the human MAb obtained in a single-step purification (SDS gel, lane 3).

A. Purification using HiTrap MabSelect SuRe on ÄKTAexplorer 100



Column:	HiTrap MabSelect SuRe 1 ml
Sample:	20 ml clarified cell supernatant containing a human monoclonal antibody
Binding buffer:	20 mM sodium phosphate, 0.15 M NaCl, pH 7.4
Elution buffer:	0.1 M glycine-HCl, pH 3.5
Flow rate:	
Sample loading:	0.4 ml/min
Wash and elution:	1 ml/min
System:	ÄKTAexplorer 100

B. SDS-PAGE analysis



Lane
1. LMW markers
2. Start material
3. Pool of eluted, purified human MAb

Fig 3.26. (A) One-step purification of a human monoclonal antibody on HiTrap MabSelect SuRe 1 ml on ÄKTAexplorer 100. **(B)** SDS-PAGE analysis on ExcelGel SDS Gradient 8-18, reducing conditions.

Sample preparation

Refer to Chapter 2 for general considerations.

Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.

IgG from many species has a medium to strong affinity for protein A at physiological pH. Sample pH should be between 6 and 9 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 0.15 M NaCl, pH 7.2

Elution buffer: 0.1 M sodium citrate, pH 3–3.6

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Purification

See Appendix 4 for general instructions for purification using HiTrap columns.

1. Prepare collection tubes by adding 60 to 200 µl of 1 M Tris-HCl, pH 9.0 per milliliter of fraction to be collected.

To preserve the activity of acid-labile IgG, we recommend adding 60 to 200 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

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

   **1 ml/min corresponds to approximately 30 drops/min when using a syringe with a 1 ml HiTrap column; 5 ml/min corresponds to approximately 120 drops/min when using a 5 ml HiTrap column*

When optimizing elution conditions, determine the highest pH that allows efficient elution of antibody from the column. This will prevent denaturing sensitive antibodies due to exposure to low pH.

Stepwise elution allows the target antibody to be eluted in a more concentrated form, thus decreasing buffer consumption and shortening cycle times. It might be necessary to decrease the flow rate due to the high concentrations of protein in the eluted pool.

9. If no cleaning-in-place is planned after elution, regenerate the column with 5 column volumes of elution buffer or and wash with 3 column volumes of binding buffer. If required, perform cleaning-in-place is directly after elution with at least 2 column volumes of 0.1 to 0.5 M NaOH ensuring a contact time of 10 to 15 min. Wash with 5 column volumes of binding buffer.
10. Re-equilibrate the column with 5 to 10 column volumes binding buffer (or until the column has reached the same pH as the binding buffer).

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).

Reuse of HiTrap columns depends on the nature of the sample and should only be considered when processing identical samples to avoid cross-contamination.

Cleaning-in-place (CIP)

CIP can be performed to remove very tightly bound, precipitated, or denatured substances from the medium. If such contaminants are allowed to accumulate, they may affect the chromatographic properties of the column, reducing capacity and potentially contaminating subsequent purification runs. If the fouling is severe, it may block the column, increase backpressure, and reduce flow rate.

Regular CIP prevents the build-up of contaminants and helps to maintain the capacity, flow properties, and general performance of HiTrap MabSelect SuRe. When an increase in backpressure is seen, the column should be cleaned. We recommend performing a blank run, including CIP, before the first purification is started to wash out possible trace amounts of leached protein A.

For more information on CIP for HiTrap MabSelect SuRe, see Appendix 1 and Instructions 11-0034-89 at www.gelifesciences.com/protein-purification.

Storage

Store in 20% ethanol at 4°C to 8°C.

Purification of other classes of antibodies

IgA

Protein A can interact with human colostral IgA as well as human myeloma IgA_2 but not IgA_1. Polyclonal IgA from pig, dog, and cat and monoclonal canine IgA have also exhibited binding affinity for protein A.

For routine purification, it may be worth developing an immunospecific purification with an anti-IgA monoclonal antibody coupled to a pre-activated affinity matrix to provide a high resolution, and highly selective affinity purification medium (see page 100). Alternatively, a multistep purification strategy could be employed (see Chapter 6).

IgD

Protein G and protein A do not bind to IgD.

For routine purification, it may be worth developing an immunospecific purification with an anti-IgD monoclonal antibody coupled to a pre-activated affinity matrix to provide a high resolution and highly selective affinity purification medium (see page 100). Alternatively, a multistep purification strategy could be employed (see Chapter 6).

IgE

IgE is present at very low concentrations in both human and mouse serum and can make a simple purification more difficult to develop and perform. Protein G and protein A do not bind to IgE.

For routine purification it may be worth developing an immunospecific purification with an anti-IgE monoclonal antibody coupled to a pre-activated affinity matrix to provide a high resolution and highly selective affinity purification medium (see page 100).

IgM

IgM present in human and mouse serum binds weakly to protein A. Capture of IgM on thiophilic adsorption ligands is, however, a tried-and-tested method for purification of this subspecies.

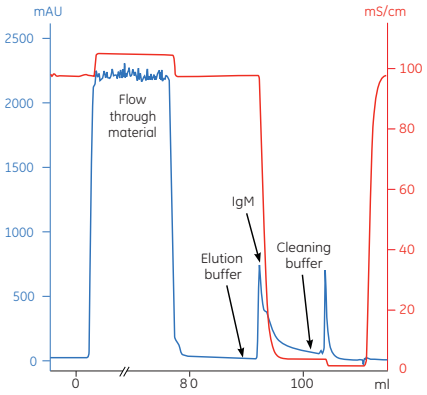
The technique using HiTrap IgM Purification HP described below is optimized for purification of monoclonal IgM from hybridoma cell culture, but it can be used as a starting point to determine the binding and elution conditions required for IgM from other species.

Purifying IgM using HiTrap IgM Purification HP

HiTrap IgM Purification HP 1 ml columns are prepacked with a thiophilic adsorption medium (2-mercaptopyridine coupled to Sepharose High Performance). The binding capacity of HiTrap IgM Purification HP is 5 mg/ml of medium. The column can be used for purification of native human and human monoclonal IgM. The interaction between the protein and the ligand has been suggested to result from the combined electron donating- and accepting-action of the ligand in a mixed-mode hydrophilic-hydrophobic interaction.

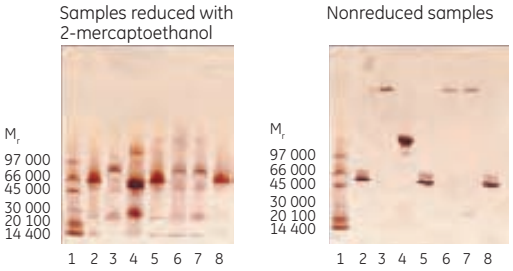
Protein A Sepharose media may offer an alternative solution to HiTrap IgM Purification HP since some human monoclonal IgM, some IgM from normal and macroglobulinemic sera, and some monoclonal canine IgM and polyclonal IgA from pig, dog, and cat can bind to protein A.

Figure 3.28 shows the results from the purification of monoclonal α-Shigella IgM from hybridoma cell culture supernatant. Analysis by SDS-PAGE (Fig 3.29) demonstrated a purity level of over 80%. Results from an ELISA (not shown) indicated high activity in the purified fraction.



Column: HiTrap IgM Purification HP
Sample: 75 ml of cell culture supernatant containing α-Shigella IgM, filtered through a 0.45 µm filter
Binding buffer: 20 mM sodium phosphate buffer, 0.5 M potassium sulfate, pH 7.5
Elution buffer: 20 mM sodium phosphate buffer, pH 7.5
Cleaning buffer: 20 mM sodium phosphate buffer, pH 7.5, 30% isopropanol
Flow rate: 1 ml/min

Fig 3.28. Purification of α Shigella IgM on HiTrap IgM Purification HP.



Lane
1. LMW markers
2. Cell culture supernatant, starting material, diluted 20-fold
3. IgM, human
4. IgG
5. Flowthrough pool, diluted 20-fold
6. Eluted IgM, fraction 8, diluted 8-fold
7. Eluted IgM, fraction 9, diluted 8-fold
8. Washing out unbound material pool, diluted 3-fold

Fig 3.29. SDS-PAGE on PhastSystem using PhastGel 4–15, silver staining.

Sample preparation

Refer to Chapter 2 for general considerations.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 0.8 M ammonium sulfate, pH 7.5

Elution buffer: 20 mM sodium phosphate, pH 7.5

Wash buffer: 20 mM sodium phosphate, pH 7.5 with 30% isopropanol

The sample must have the same concentration of ammonium sulfate as in the binding buffer (0.8 M). Slowly add small amounts of solid ammonium sulfate to the sample from the hybridoma cell culture until the final concentration is 0.8 M. Stir slowly and continuously. Pass the sample through a 0.45 µm filter immediately before applying it to the column. Some monoclonal IgM may not bind to the column at a concentration of 0.8 M ammonium sulfate. Binding can be improved by increasing the ammonium sulfate concentration to 1 M.

To avoid precipitation of IgM, it is important to add the ammonium sulfate slowly. An increased concentration of ammonium sulfate will cause more IgG to bind, which might be a problem if serum has been added to the cell culture medium. If there is IgG contamination of the purified IgM, the IgG can be removed by using HiTrap Protein A HP, HiTrap rProtein A FF, or HiTrap Protein G HP.

Ammonium sulfate can be replaced by 0.5 M potassium sulfate. Most monoclonal IgM binds to the column in the presence of 0.5 M potassium sulfate and the purity of IgM is comparable to the purity achieved with 0.8 M ammonium sulfate.

Purification

See Appendix 4 for general instructions for purification using HiTrap columns.

1. Fill the syringe or pump tubing with distilled water. Remove the stopper and connect the column to the syringe (use the connector supplied).
2. Remove the snap-off end at the column outlet.
3. Wash out the ethanol with 5 ml of distilled water.
4. Equilibrate the column with 5 ml of binding buffer. The recommended flow rate is 1 ml/min*.
5. Apply the sample using a syringe fitted to the Luer connector or by pumping it onto the column. For best results, use a flow rate of 0.2 to 1 ml/min during sample application.
6. Wash with 15 ml of binding buffer or until the absorbance reaches a steady baseline or no material remains in the effluent. Maintain a flow rate of 1 to 2 ml/min for washing.
7. Elute with 12 ml of elution buffer using a one-step or linear gradient though larger volumes are sometimes required to break the interaction.
8. After elution, regenerate the column by washing it with 7 ml of wash buffer and re-equilibrate the column with 5 ml of binding buffer. The column is now ready for a new purification of the same antibody.

   * *1 ml/min corresponds to approximately 30 drops/min when using a syringe with a 1 ml HiTrap column*

Some monoclonal IgM may bind too strongly to the column matrix for complete elution. The remaining IgM will be eluted during cleaning, but the high concentration of isopropanol will cause precipitation of IgM. Perform an immediate buffer exchange (see Chapter 2) or dilute the sample to preserve the IgM. Lower concentrations of isopropanol may elute the IgM and decrease the risk of precipitation.

Reuse of HiTrap IgM Purification HP depends on the nature of the sample and should only be considered when processing identical samples to avoid cross-contamination.

To increase capacity, connect several HiTrap IgM Purification HP columns in series. HiTrap columns can be used with a syringe, a peristaltic pump, or connected to a liquid chromatography system.

Storage

Store in 20% ethanol at 4°C to 8°C.

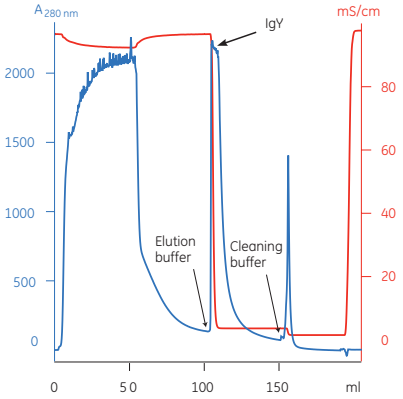
IgY

IgY is an avian antibody that cannot be purified using protein G or protein A. IgY is, however, easily purified from avian egg yolk using HiTrap IgY Purification HP to yield a product with greater than 70% purity.

Purifying IgY using HiTrap IgY Purification HP

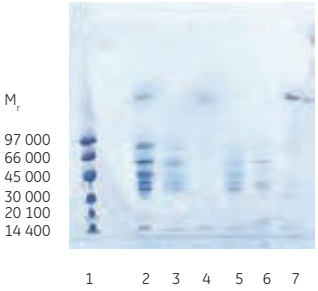
HiTrap IgY Purification HP 5 ml columns are prepacked with a thiophilic adsorption medium (2-mercaptopyridine coupled to Sepharose High Performance). The interaction between the protein and the ligand has been suggested to result from the combined electron donating- and accepting-action of the ligand in a mixed-mode hydrophobic-hydrophilic interaction.

Figure 3.30 shows the purification of α–Hb IgY from 45 ml of egg yolk extract (corresponding to one quarter of a yolk) and the SDS-PAGE analysis (Fig 3.31) indicating a purity of over 70%.



Column:	HiTrap IgY Purification HP
Sample:	45 ml of egg yolk extract (corresponding to 1/4 of an egg yolk) containing α-Hb IgY, filtered through a 0.45 µm filter
Binding buffer:	20 mM sodium phosphate buffer, 0.5 M potassium sulfate, pH 7.5
Elution buffer:	20 mM sodium phosphate buffer, pH 7.5
Cleaning buffer:	20 mM sodium phosphate buffer, pH 7.5, 30% isopropanol
Flow rate:	5 ml/min

Fig 3.30. Purification of IgY on HiTrap IgY Purification HP.



Lane

1. LMW markers
2. Egg yolk extract
3. Flowthrough pool
4. Eluted IgY
5. Egg yolk extract, diluted four-fold
6. Flowthrough pool, diluted four-fold
7. Eluted IgY, diluted four-fold

Fig 3.31. SDS-PAGE of nonreduced samples on PhastSystem using PhastGel 4–15, Coomassie staining.

Sample preparation

Refer to Chapter 2 for general considerations.

As much as possible of the egg yolk lipid must be removed before purification. Water or polyethylene glycol can be used to precipitate the lipids. Precipitation with water is described below.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 0.5 M potassium sulfate, pH 7.5

Elution buffer: 20 mM sodium phosphate, pH 7.5

Wash buffer: 20 mM sodium phosphate, pH 7.5 with 30% isopropanol

To improve recovery of total IgY or a specific IgY antibody, replace 0.5 M potassium sulfate with 0.6 to 0.8 M sodium sulfate in the binding buffer. The sample should have the same concentration of sodium sulfate as the binding buffer. Using more than the recommended salt concentration in the binding buffer will reduce the purity of the eluted IgY.

1. Separate the egg yolk from the egg white.
2. Add nine parts of distilled water to one part egg yolk.
3. Mix and stir slowly for 6 h at 4°C.
4. Centrifuge at 10 000 × g, at 4°C for 25 min to precipitate the lipids.
5. Collect the supernatant containing the IgY.
6. Slowly add potassium sulfate to the sample, stirring constantly, to a final concentration of 0.5 M.
7. Adjust pH to 7.5.
8. Pass the sample through a 0.45 µm filter immediately before applying it to the column.

Purification

See Appendix 4 for general instructions for purification using HiTrap columns.

1. Fill the syringe or pump tubing with distilled water. Remove the stopper and connect the column to the syringe (use the connector supplied).
2. Snap off the tab on the column outlet.
3. Wash out the ethanol with 25 ml of distilled water.
4. Equilibrate the column with 25 ml of binding buffer. The recommended flow rate is 5 ml/min*.
5. Apply the sample using a syringe fitted to the Luer connector or by pumping it onto the column. For best results, use a flow rate of 0.5 to 5 ml/min during sample application
6. Wash with at least 50 ml of binding buffer or until the absorbance reaches a steady baseline or no material remains in the effluent. Maintain a flow rate of 5 to 10 ml/min for washing.
7. Elute with 50 ml of elution buffer using a one-step or linear gradient though larger volumes are sometimes required to break the interaction.
8. After elution, regenerate the column by washing it with 35 ml of wash buffer and re-equilibrate the column with 25 ml of binding buffer. The column is now ready for a new purification.

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

The purity of the eluted IgY may be improved by using gradient elution with, for example, a linear gradient 0% to 100% elution buffer over 10 column volumes, followed by 100% elution buffer for several column volumes.

To increase binding capacity, connect several HiTrap IgY Purification HP columns in series. A HiTrap column can be used with a syringe, a peristaltic pump, or connected to a liquid chromatography system.

Reuse of HiTrap IgY Purification HP depends on the nature of the sample. To prevent cross-contamination, it should only be reused when processing identical samples.

Storage

Store in 20% ethanol at 4°C to 8°C.

Making immunospecific purification media with custom ligands

If an affinity medium is not available, a ligand (such as a pure antigen or an antibody) can be covalently coupled to a suitable matrix to create an immunospecific affinity medium for purification. Although this process requires careful development and optimization, it is often worthwhile, for example when a specific protein needs to be prepared on a regular basis. Immunospecific purification is particularly useful if the target molecules bind weakly or not at all to protein A or protein G and can also be used to remove key contaminants.

This section describes the simplest coupling method, that is, when a ligand is coupled *via* its primary amine group to a pre-activated medium. GE Healthcare offers two pre-activated media for coupling of antigen or antibody ligands: NHS-activated Sepharose 4 Fast Flow, which is available in bulk packs for packing columns, and NHS-activated Sepharose High Performance, which is available in convenient, prepacked HiTrap NHS-activated HP columns.

The excellent hydrophilic properties of the base matrixes of NHS-activated Sepharose media minimize nonspecific adsorption of proteins that can reduce the binding capacity of the target protein. The pH range for coupling is well suited to the stability characteristics of many immunoglobulins. Furthermore, the media are stable at high pH to allow stringent washing procedures (subject to the pH stability of the coupled ligand).

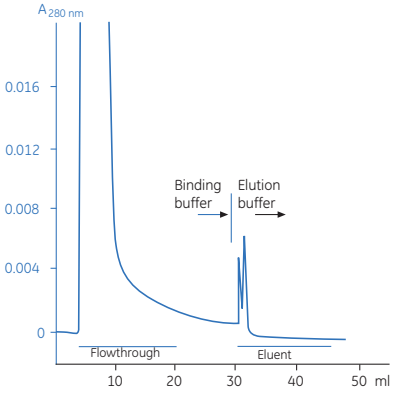
NHS-activated Sepharose media are used for the initial capture step; a gel filtration step is commonly used after immunocapture to ensure a highly pure and homogenous target protein, (removal of monomers, dimers, and any leached ligand).

If no primary amine group on the ligand to be coupled is available, ligand attachment *via* carboxyl, thiol, or hydroxyl groups can be considered. These procedures are described in the handbook *Affinity Chromatography: Principles and Methods* (18-1022-29).

A *pure* ligand is required that has a proven *reversible* high affinity for the target molecule. Using an antigen or an anti-antibody as a ligand will give a high degree of purification. If possible, test the affinity of the interaction.

Immunospecific interactions often require harsh elution conditions. Collect fractions into a neutralizing buffer, such as 60 to 200 µl 1 M Tris-HCl, pH 9.0 per milliliter fraction.

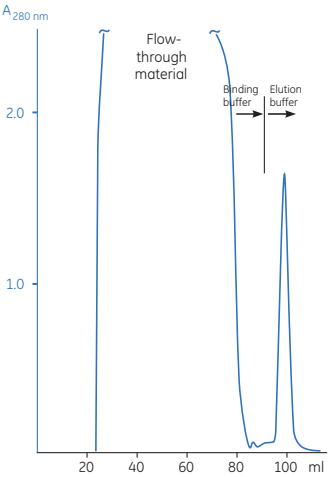
Figure 3.32 shows the partial purification of an IgE-stimulating factor from a human T-cell line, using IgE as the specific affinity ligand coupled to HiTrap NHS-activated HP 1 ml column. Figure 3.33 shows purification of anti-mouse Fc-IgG from sheep serum using mouse IgG_1 coupled to HiTrap NHS-activated HP 1 ml column.

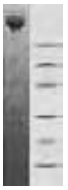


Column: IgE coupled to HiTrap NHS-activated HP 1 ml
Sample: 2 ml of a 65-fold concentrated serum-free cell culture supernatant of the human T-cell line MO
Binding buffer: 20 mM sodium phosphate, 150 mM NaCl, pH 7.4
Elution buffer: 100 mM glycine, 500 mM NaCl, pH 3.0
Flow rate: 0.25 ml/min

Fig 3.32. Purification of an IgE-stimulating factor from a human T-cell line.

Column: HiTrap NHS-activated HP 1 ml. Mouse IgG, (10 mg, 3.2 ml) was coupled in 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3, room temp., recycled with a peristaltic pump for 1 h. Yield was 95% (9.5 mg)
Sample: 50 ml sheep anti-mouse Fc serum filtered 0.45 µm
Binding buffer: 75 mM Tris-HCl, pH 8.0
Elution buffer: 100 mM glycine-HCl, 0.5 M NaCl, pH 2.7
Flow rate: 1.0 ml/min
Electrophoresis: SDS-PAGE, PhastSystem PhastGel Gradient 8–25, 1 µl sample, Coomassie stained





Lane
1. Eluted material, nonreduced
2. LMW markers, reduced

Lane
1. Eluted material, reduced
2. LMW markers, reduced

Fig 3.33. Purification of anti-mouse Fc-IgG from sheep antiserum.

Coupling ligands to HiTrap NHS-activated HP columns

The protocol below describes the preparation of a prepacked HiTrap NHS-activated HP column and a recommendation for a preliminary purification protocol. Many of these details are generally applicable to NHS-activated Sepharose media. Coupling can take place within the pH range of 6.5 to 9 with a maximum yield achieved at around pH 8.

A general column packing procedure is described in Appendix 5.

Buffer preparation

Acidification solution: 1 mM HCl (kept on ice)

Coupling buffer: 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3

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

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

Ligand and HiTrap column preparation

1. Dissolve the desired ligand in the coupling buffer to a final concentration of 0.5 to 10 mg/ml (for protein ligands) or perform a buffer exchange using a desalting column (see Chapter 2). The optimal concentration depends on the ligand. Dissolve the ligand in one column volume of coupling buffer.
2. Remove the top cap and apply a drop of acidification solution to the top of the column to avoid air bubbles.
3. Connect the Luer adapter (or tubing if using a pump or system) to the top of the column.
4. Remove the snap-off end at the column outlet.

Ligand coupling

1. Wash out the isopropanol with acidification solution. Use 3 × 2 ml for HiTrap 1 ml and 3 × 10 ml for HiTrap 5 ml.

Do not exceed flow rates of 1 ml/min for HiTrap 1 ml columns and 5 ml/min for HiTrap 5 ml columns at this stage to avoid irreversible compression of the prepacked medium.

2. Immediately inject 1 ml (HiTrap 1 ml) or 5 ml (HiTrap 5 ml) of the ligand solution onto the column.
3. Seal the column and leave for 15 to 30 min at 25°C or 4 h at 4°C*.

   * *Coupling efficiency can be measured after this step. Procedures are supplied with each HiTrap NHS-activated HP column*

If larger volumes of ligand solution are used, recirculate the solution by connecting a second syringe to the outlet of the column and gently pump the solution back and forth for 15 to 30 min. Recirculation can also be performed by connecting a peristaltic pump, for example, Pump P-1.

Washing and deactivation

Deactivate any excess active groups that have not coupled to the ligand, and wash out the nonspecifically bound ligands, by following the procedure below:

Buffers required:

Buffer A: 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3

Buffer B: 0.1 M acetate, 0.5 M NaCl, pH 4

1. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer A.
2. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer B.
3. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer A.
4. Leave the column for 15 to 30 min at room temperature or approximately 4 h at 4 °C.
5. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer B.
6. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer A.
7. Inject 3 × 2 ml (HiTrap 1 ml) or 3 × 10 ml (HiTrap 5 ml) of Buffer B.
8. Finally, inject 2 ml (HiTrap 1 ml) or 10 ml (HiTrap 5 ml) of a buffer with neutral pH to adjust the pH.

Storage

Store the column in a solution that maintains the stability of the ligand and contains a bacteriostatic agent, for example phosphate-buffered saline (PBS), 0.05% sodium azide, pH 7.2.

pH stability of the media when coupled to the selected ligand depends on the stability of the ligand. Sodium azide can interfere with many coupling methods and some biological assays. It can be removed using a desalting column.

Sodium azide is carcinogenic, handle with care.

Performing a purification on a coupled HiTrap NHS-activated column

Use high quality water and chemicals. Filtration through 0.45 µm filters is recommended. Optimal binding and elution conditions for purification of the target protein must be determined separately for each ligand (see below for suggested elution buffers). The general protocol given here can be used for preliminary purification.

For the first run, perform a blank run to ensure that any loosely bound ligand is removed. Samples should be centrifuged immediately before use and/or filtered through a 0.45 µm filter. If the sample is too viscous, dilute with binding buffer.

Sample binding properties can be improved by adjusting the sample to the composition of the binding buffer. Perform a buffer exchange using a desalting column (see Chapter 2) or dilute the sample in binding buffer.

1. Prepare the column by washing with:

   a. 3 ml (HiTrap 1 ml) or 15 ml (HiTrap 5 ml) binding buffer.

   b. 3 ml (HiTrap 1 ml) or 15 ml (HiTrap 5 ml) elution buffer (see below for advice on elution buffers).
2. Equilibrate the column with 10 column volumes of binding buffer.
3. Sample preparation. The sample should be adjusted to the composition of the binding buffer. This can be done by either diluting the sample with binding buffer or by buffer exchange or desalting (see Chapter 2). The sample should be filtered through a 0.45 µm filter or centrifuged immediately before it is applied to the column.
4. Apply the sample, using a syringe fitted to the Luer adapter or by pumping it onto the column. Recommended flow rates: 0.2 to 1 ml/min (HiTrap 1 ml) or 1 to 5 ml/min (HiTrap 5 ml)*. The optimal flow rate is dependent on the binding constant of the ligand.
5. Wash with binding buffer, 5 to 10 column volumes or until no material appears in the effluent. Excessive washing should be avoided if the interaction between the protein of interest and the ligand is weak, since this may decrease the yield.
6. Elute with elution buffer; 1 to 3 column volumes is usually sufficient but larger volumes may be necessary.
7. The purified fractions can be desalted (see Chapter 2).
8. Re-equilibrate the column by washing with 5 to 10 column volumes of binding buffer. The columns is now ready for a new purification of the same kind of sample.

   ** 1 ml/min corresponds to approximately 30 drops/min when using a syringe with a 1 ml HiTrap column; 5 ml/min corresponds to approximately 120 drops/min when using a syringe with a 5 ml HiTrap column*

To preserve the activity of acid-labile IgG, we recommend adding 60 to 200 µl of 1 M Tris-HCl pH 9.0 to collection tubes, which ensures that the final pH of the sample will be approximately neutral.

Elution buffers

Immunospecific interactions can be very strong and sometimes difficult to reverse. The specific nature of the interaction determines the elution conditions. Always check the reversibility of the interaction before coupling a ligand to an affinity matrix. If standard elution buffers do not reverse the interaction, alternative elution buffers that may be useful are listed below:

- Low pH (below pH 2.5)
- High pH (up to pH 11)
- Substances that reduce the polarity of a buffer may facilitate elution without affecting protein activity: dioxane (up to 10%), ethylene glycol (up to 50%)

Chapter 4
Removal of specific contaminants after initial purification

For many applications at laboratory scale, contaminant molecules may not be a significant problem. Affinity chromatography (AC) will provide sufficient purity and, as long as the presence of any minor contaminants does not interfere with the intended application, the purified sample can be used directly.

However, as outlined in Table 2.1 on page 15, source materials will be associated with major contaminants which may need to be removed either before purification begins (e.g., lipid material or phenol red) or after initial purification.

Common contaminants after initial purification are albumin, transferrin, antibody aggregates, leached protein A, DNA, and host or bovine immunoglobulins that originate from ascites or cell culture serum. The problem of contaminants of animal origin in, for example, bovine cell culture systems for monoclonal antibody (MAb) production, has been largely circumvented by use of serum-free systems. The three main contaminants albumin, transferrin, and host or bovine immunoglobulins pose three different purification problems: albumin because of its abundance; transferrin because of its similarity to the charge characteristics of many antibodies; and host or bovine immunoglobulins because of the similarity to that of the target immunoglobulin.

- For some cell culture preparations, it is possible to decrease the level of serum during growth, thereby reducing or eliminating many of these impurities before purification. An alternative solution is to consider the use of a different host that does not require these supplements.
- Select chromatography techniques that utilize differences in the characteristics of the contaminant and target molecule: ion exchange chromatography (IEX) for separation by differences in charge; hydrophobic interaction chromatography (HIC) for separation by differences in hydrophobicity; and gel filtration for separation by size. See Appendix 9 for an overview of the principles of the chromatography techniques used at laboratory scale.
- If the pI value of the antibody is sufficiently different from the contaminants, a cation exchange medium (negatively charged) can be used for removal of the contaminants at a pH above the pI of the impurities and below that of the antibody. This will ensure that the antibody (positively charged) binds to the column while the impurities, including negatively charged nucleic acids, pass through.

Bovine immunoglobulins

Co-purification of host or bovine immunoglobulins is a problem associated with any affinity purification of antibodies from a native source or a source to which supplements such as calf serum or bovine serum albumin are added. This contamination problem has been largely circumvented in the large-scale manufacture of MAbs for therapeutic use through the widespread use of serum-free cell culture systems.

Difficulties have also been encountered when murine monoclonal antibodies are the target molecule. The similarities between the physical characteristics of the target antibody and

the contaminants require careful selection and optimization to find the most suitable chromatography technique for purification. Both HIC and IEX can be used.

The hydrophobicity of proteins is difficult to predict. Screen several chromatography media with different hydrophobicities (e.g., using HiTrap HIC Selection Kit) to find the medium that gives the best results.

HiTrap HIC Selection Kit includes seven HIC media with different hydrophobic properties for small-scale screening and selection of optimal binding and elution conditions. Figure 4.1 shows an example of media screening of a mouse monoclonal antibody on different HIC media prepacked in HiTrap 1 ml columns. In this case, the optimal medium for purification was HiTrap Phenyl HP.

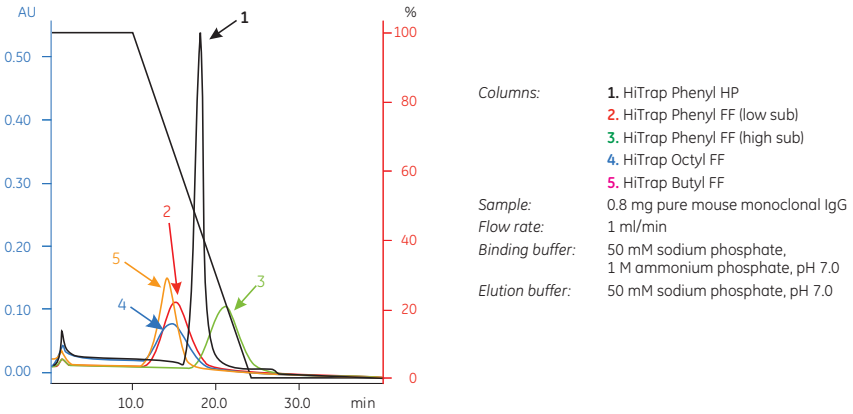


Fig 4.1. Screening of a mouse monoclonal antibody for optimal purification conditions using HiTrap HIC columns.

RESOURCE™ HIC Test Kit contains columns prepacked with SOURCE™ media with different ligands than those in the HiTrap HIC Selection Kit. Refer to *Hydrophobic Interaction and Reversed Phase Chromatography: Principles and Methods*, code number 11-0012-69 AA, for more information.

Albumin and transferrin

Ion exchange and hydrophobic interaction chromatography are two methods used for removing albumin and transferrin, separating the molecules on the basis of differences in their isoelectric points or hydrophobicities (see Appendix 9 for the principles of these techniques).

After an IEX purification, albumin and transferrin may be present if their charge properties are similar to those of the target antibody. In some cases, it may be possible to optimize pH and elution conditions in the IEX step to improve the separation between the antibody and the contaminants (Appendix 9).

Since most monoclonal antibodies are more hydrophobic than albumin and transferrin, HIC can be used to bind the antibody and allow these contaminants to wash through the column.

However, AC using Blue Sepharose 6 Fast Flow is a useful alternative to IEX and HIC for removing albumin.

Removal of albumin using Blue Sepharose media

Blue Sepharose 6 Fast Flow or prepacked HiTrap Blue HP 1 ml and 5 ml columns (Fig 4.2) can be used to remove albumin either before or after other purification steps (see Table 4.1). The albumin binds in a nonspecific manner by electrostatic and/or hydrophobic interactions with the aromatic anionic ligand, Cibacron™ Blue F3G-A, coupled to Sepharose.

Fig 4.2. Prepacked with Blue Sepharose High Performance, HiTrap Blue HP columns offer fast and simple removal of albumin by affinity chromatography.

Use HiTrap Blue HP 1 ml or 5 ml columns to remove host albumin from mammalian expression systems or when the sample is known to contain high levels of albumin that may mask the UV absorption of other protein peaks.

Do not use Blue Sepharose media if the immunoglobulin or other target molecule has a hydrophobicity similar to that of albumin.

Table 4.1. Options for the removal of albumin by affinity chromatography using Blue Sepharose media

	Capacity/ml medium[1]	Comments
HiTrap Blue HP	HSA 20 mg	Removal of albumin Prepacked 1 ml and 5 ml columns
Blue Sepharose 6 Fast Flow	HSA >18 mg	Supplied as a suspension ready for column packing

[1] *Protein binding capacity varies for different proteins*

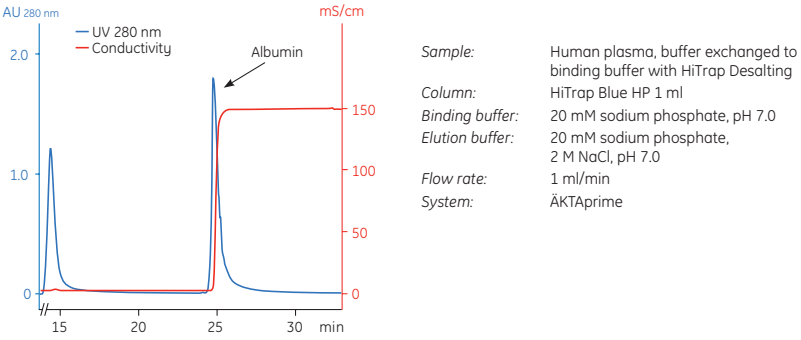


Fig 4.3. Effective removal of albumin from human plasma using a HiTrap Blue HP 1 ml column.

Figure 4.3 shows the removal of human serum albumin from plasma using HiTrap Blue HP 1 ml.

The protocol for removal of albumin using HiTrap Blue HP 1 ml and 5 ml columns is described below.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0, or 50 mM KH_2PO_4, pH 7.0

Elution buffer: 20 mM sodium phosphate, 2 M NaCl, pH 7.0, or 50 mM KH_2PO_4, 1.5 M KCl, pH 7.0

Albumin removal

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

** 1 ml/min corresponds to approximately 30 drops/min when using a syringe with a 1 ml HiTrap column; 5 ml/min corresponds to approximately 120 drops/min when using a 5 ml HiTrap column*

Storage

Store in 20% ethanol at 4°C to 8°C.

α_2-macroglobulin and haptoglobulin

α_2-macroglobulin, haptoglobulin and, other minor proteins such as ceruloplasmin may be present in preparations made from native sources or in the presence of serum.

Since α_2-macroglobulin (M_r 820 000) is closely related in size to IgM, it is easily separated from smaller molecules such as IgG by gel filtration. Similarly, haptoglobulin will separate from IgM on a suitable gel filtration (GF) column.

In general, ion exchange media and carefully selected running conditions (pH and conductivity) will ensure that these contaminants are removed. For the removal of α_2-macroglobulin, Blue Sepharose 6 Fast Flow and Chelating Sepharose Fast Flow can also be considered.

Dimers and aggregates

A frequent difficulty when purifying immunoglobulins is the appearance of dimers and other aggregates. Aggregates are often formed when working with proteins at higher concentrations. In the presence of high salt concentrations, dimers or polymers can be formed during freezing and thawing. These aggregates can lower the biological activity of the sample.

Gel filtration is one of the techniques for removing aggregates at laboratory scale and is used as the final polishing step in many purification strategies. A medium such as Superdex 200 will give the best possible separation between monomer and dimer. The principles of gel filtration are outlined in Appendix 9.

In large-scale purification of antibodies, ion exchange using, for example, Capto adhere is an effective method for removal of aggregates (see Chapter 7 for details).

Gel filtration is highly recommended as a final polishing step after any affinity purification. The sample will be transferred into a final buffer at the correct pH and the low molecular weight molecules, such as salt, will be removed.

Gel filtration is not a binding technique so sample loading is limited from 1% to 3% of the total column volume in most cases.

For purification with larger sample volumes, use HiLoad™ 16/60 Superdex 200 pg or HiLoad 26/60 Superdex 200 pg prepacked columns.

Removal of aggregates and dimers at manufacturing scale is often achieved through use of ion exchange media after initial protein A antibody capture. Multimodal ion exchangers such as Capto MMC and Capto adhere are recommended for removal of contaminants downstream of protein A capture, see Chapter 7.

Figure 4.4 shows an example of the purification of human IgG monomers and dimers on Superdex 200 10/300 GL gel filtration column.

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

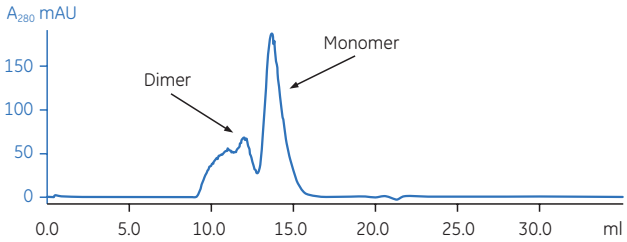


Fig 4.4. Separation of the monomer and dimer of a monoclonal antibody on Superdex 200 10/300 GL.

DNA and endotoxins

For large-scale purification, the need to assay for critical impurities is often essential as the products may be used for clinical or diagnostic applications. In practice, when a protein is purified for research purposes, it is often too time-consuming to identify and set up specific assays for harmful contaminants, such as DNA and endotoxins. A practical approach is to purify the protein to a certain level and perform SDS-PAGE after storage to check for protease degradation. Suitable control experiments should be included within bioassays to indicate if impurities are interfering with results. Information on the degree of purity and quantity of aggregates can also be obtained by analytical gel filtration using Superdex 200 gel filtration columns.

- Nucleic acids often dissociate from proteins at high salt concentrations. This makes hydrophobic interaction chromatography a suitable technique for capturing the target protein and removing nucleic acids.
- Since DNA and endotoxins are negatively charged over a wide pH interval, a cation exchange chromatography step at a pH below the isoelectric point of the antibody will bind the target protein and allow the negatively charged molecules to wash through the column. Consequently, if anion exchange is used as the initial capture step, these contaminants will be removed at an early stage in purification.
- If endotoxins or DNA need to be removed from a purified product, anion exchange chromatography at using Capto Q or Capto adhere a pH value slightly below the isoelectric point of the antibody will bind the endotoxins and DNA while the antibody will wash through the column. Alternatively, use a pH that binds both molecular species, but allows them to be clearly separated during gradient elution from the column.

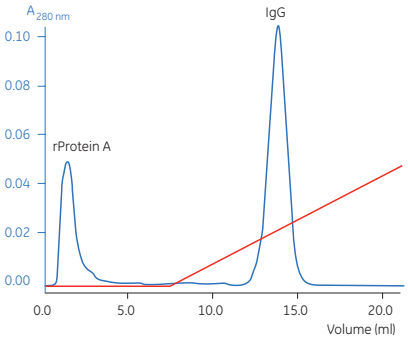
Removal of DNA and endotoxins in the large-scale purification schemes used in process manufacturing and development is discussed in Chapter 7.

Affinity ligands

With any affinity chromatography medium, ligand leakage from the matrix can occur, particularly if harsh conditions are required to elute the target molecule. In many cases, this leakage is negligible and satisfactory purity is achieved. At laboratory scale, leakage of ligand is not a significant problem.

GE Healthcare offers a range of Sepharose and high-flow agarose media with negligible ligand leakage (see Chapter 3). MabSelect SuRe, for example, is a high-flow agarose medium with a protein A ligand designed to withstand the harsh purification conditions used in biopharmaceutical production. Ligand leakage from MabSelect SuRe is negligible, which makes the medium particularly useful in the capture step employed in large-scale purification of MAbs, where trace amounts of ligand in the final product are not acceptable.

Figure 4.5 shows an example of the removal of leached protein A ligand from mouse IgG_{2b} on HiTrap SP HP 1 ml column. Levels of protein A leakage are usually extremely low, so the sample has been spiked with protein A to visualize the protein A peak.



Column:	HiTrap SP HP 1 ml
Sample:	Purified antibody (0.61 mg) spiked with recombinant protein A (1.8 mg)
Binding buffer:	20 mM sodium citrate, pH 5.2
Elution buffer:	20 mM sodium citrate, 1 M NaCl, pH 5.2
Flow rate:	4 ml/min
Gradient:	0% to 45% elution buffer in 15 column volumes

Fig 4.5. Removal of protein A from mouse IgG_{2b} by cation exchange chromatography on HiTrap SP HP. Recombinant protein A was spiked into mouse IgG_{2b} previously purified on rProtein A Sepharose Fast Flow.

Chapter 5
Automated purification of antibodies using ÄKTAdesign systems

Antibodies are needed for research and industrial purposes in different quantities, from microgram to kilogram scale. It is important to design and use a purification method that will yield protein of a quality and quantity that is adequate for the particular application. The number of samples to be purified is also an important consideration. For many applications, investment in a chromatography system can save valuable time, effort and sample. Manual purification techniques are discussed in Chapter 3.

A chromatography system should be used when reproducible results are important and when manual purification becomes too time-consuming and inefficient. This can be the case when processes have to be repeated in order to obtain enough purified sample, when large sample volumes have to be handled, or when there are many different samples to be purified. Chromatography systems give more reproducible results compared with manual purification, and the progress of the purification can be monitored automatically. In addition to simple step-gradient elution, high-resolution separations with accurately controlled linear-gradient elution can be performed. Systems are robust and convenient to use and can fully utilize the high flow rates that modern media can withstand. The use of ÄKTAdesign chromatography systems for purification of antibodies is described below.

ÄKTAprime plus (Fig 5.1) is a cost-effective, easy-to-learn system. Together with the appropriate columns, antibodies can be purified in microgram scale in a single chromatography step with push-button control. The system includes preprogrammed methods for purification of antibodies using different prepacked columns. Recovery is often better than when the same purification is performed manually. With prepacked columns and optimized purification protocols, yields and purity are highly consistent.

Fig 5.1. ÄKTAprime plus.

Fig 5.2. ÄKTAexplorer.

Purification of antibodies can also be performed on more advanced chromatography systems. ÄKTAexplorer (Fig 5.2) is a system that allows easy purification of proteins from microgram to gram scale. Multiple samples (up to eight) of an antibody can be automatically purified in a single step, eliminating manual work between samples.

When a single affinity step does not yield the purity required for a specific application or when a buffer exchange or polishing step is required after the affinity step, multiple chromatography steps are needed. ÄKTAxpress™ (Fig 5.3) is the system of choice when a high level of automation is required. ÄKTAxpress has a modular design where 1 to 12 modules are controlled by one computer for parallel, automated purification of up to 48 samples. ÄKTAxpress delivers the highest possible throughput for purification of monoclonal antibodies in larger scale, with no user intervention needed. Extended and automated washing procedures enable processing of a larger number of samples with minimal risk of cross-contamination. Single or two-step purifications of up to four different antibodies can be performed automatically per run and module.

Other ÄKTAdesign systems are available for the purification of antibodies. Standard ÄKTAdesign configurations are shown in Figure 5.4.

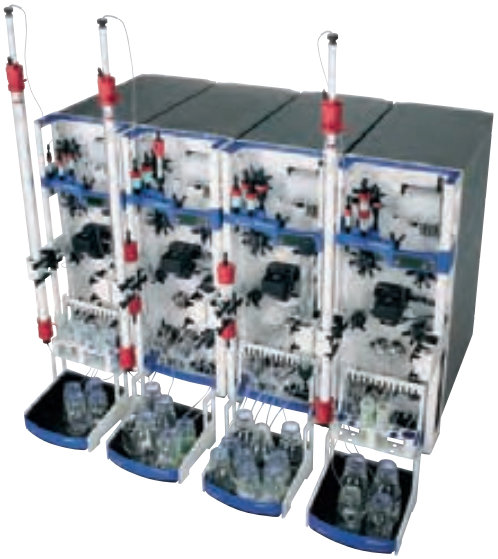


Fig 5.3. Four modules of ÄKTAxpress.

Table 5.1. Standard ÄKTAdesign configurations

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

The ◦ symbol indicates an optional feature

ÄKTAprime plus

ÄKTApurifier™

ÄKTAexplorer

AKTAxpress

ÄKTApilot™

ÄKTAcrossflow™

ÄKTAprocess™

Fig 5.4. The standard ÄKTAdesign configurations.

Chapter 6
Multistep purification strategies

As discussed in Chapter 3, a single, rapid purification step using affinity chromatography is sometimes sufficient to achieve the level of purity and quantity of a target antibody that is required for most research purposes. Unwanted small molecules, such as salts, can be removed by including desalting/buffer exchange or high-resolution gel filtration as a polishing step. When affinity chromatography (AC) cannot be used, which can be the case for antibody fragments, or if a higher degree of purity is required, alternative techniques need to be combined effectively into a multistep purification strategy.

The challenge associated with a particular purification step will depend greatly upon the properties of the starting material. Thus, the objective of a purification step will vary according to its position in the process, that is, at the beginning *capture* of product from crude sample, in the middle *intermediate purification* of partially purified sample, or at the end *polishing* of an almost pure product. A significant advantage when working with native or recombinant antibodies is that there is often considerable information available about the product as well as about major contaminants, as shown in Table 6.1 below and in Table 2.1 on page 15. Separation techniques and elution conditions can usually be selected to yield a highly pure product in as few as two purification steps.

Table 6.1. Characteristics of native IgG and IgM

Molecular weight	M_r 150 000–160 000 (IgG) M_r 900 000 (IgM)
Isoelectric point (pI)	4–9, most > 6.0, often more basic than other serum proteins
Hydrophobicity	IgG is more hydrophobic than many other proteins and so precipitates more readily in ammonium sulfate
Solubility	IgG is very soluble in aqueous buffers Lowest solubility (specific to each antibody) near pI or in very low salt concentration
Temperature stability	Relatively stable at room temperature (but specific to each antibody)
pH stability	Often stable over a wide pH interval, but unstable in very acidic buffers (specific to each antibody)
Carbohydrate content	2% to 3% for IgG, higher for IgM (12%), most carbohydrate is associated with Fc region of the heavy chains

The optimal selection and combination of purification techniques for Capture, Intermediate Purification, and Polishing is crucial for an efficient purification. These principles are described in more detail in Appendix 9.

Examples of multistep purification

The following examples demonstrate successful two-step strategies for the purification of antibodies at laboratory scale. For process-scale purification, see Chapter 7.

Example 1: Two-step purification of mouse monoclonal IgG_1 using HiTrap rProtein A FF for the capture step

This example demonstrates the effectiveness of using a high selectivity affinity purification step for initial capture. In common with most antibody preparations, IgG aggregates and/or dimers may be present, which would therefore require a second purification step. To achieve highest purity, it is therefore essential to include a gel filtration polishing step. A more detailed description of this purification can be found in *Application Note 18-1128-93*.

Target molecule: Mouse monoclonal IgG_1.

Source material: Cell culture supernatant.

Extraction and clarification: Cell culture supernatant filtered through a 0.45 µm filter.

Capture

Capture of the target protein was performed on a HiTrap rProtein A FF column. This step removes contaminating proteins, low molecular weight substances and significantly reduces sample volume.

In contrast to other IgG subclasses, most mouse monoclonal antibodies of the IgG_1 subclass require a high salt concentration to bind to rProtein A. Figure 6.1 shows the results of a scouting experiment performed to define the optimal salt concentration for binding. Scouting is also used to select the optimal pH for elution of the monoclonal antibody (pH 4.5 was selected in this example, results not shown).

Using ÄKTAdesign systems for automatic scouting of optimal binding and elution conditions can improve the recovery of a specific antibody, and the optimized purification can be automated for routine use.

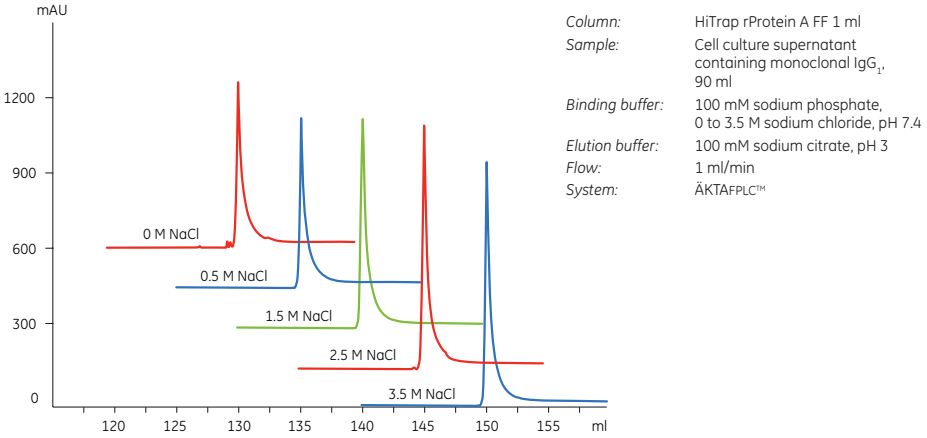
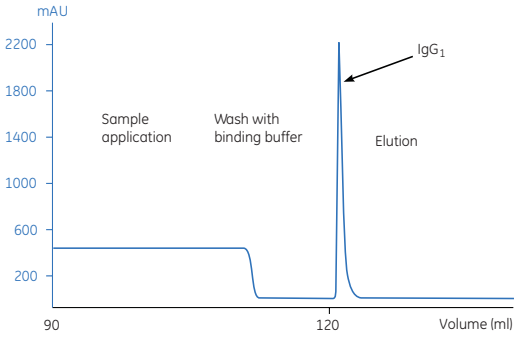


Fig 6.1. Automatic scouting of optimal salt concentration in the binding buffer on HiTrap rProtein A FF.

Optimization of binding and elution conditions gave a well-resolved peak containing IgG_1, as shown in Figure 6.2.



Column:	HiTrap rProtein A FF 1 ml
Sample:	Cell culture supernatant containing monoclonal IgG_1 (46 ml cell culture supernatant diluted to 100 ml with binding buffer)
Binding buffer:	100 mM sodium phosphate, 2.5 M sodium chloride, pH 7.4
Elution buffer:	100 mM sodium citrate, pH 4.5
Flow:	1 ml/min
System:	ÄKTAFPLC

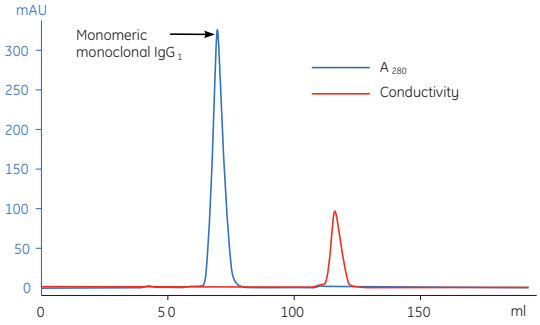
Fig 6.2. Optimized capture step on HiTrap rProtein A FF.

Intermediate purification

No intermediate step was required as the high selectivity of the capture step gave a sufficiently high level of purity so that only a final polishing step was necessary.

Polishing

HiLoad 16/60 Superdex 200 pg was used for the gel filtration (GF) polishing step to remove low or trace levels of contaminants, which in this case were IgG aggregates and/or dimers (Fig 6.3).



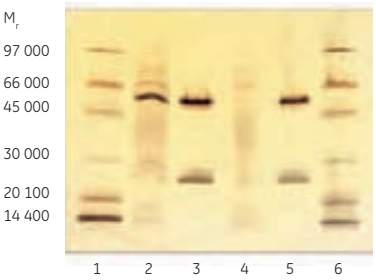
Column:	HiLoad 16/60 Superdex 200 pg
Sample:	Fraction from HiTrap rProtein A FF column containing monoclonal IgG_1 (3 ml)
Buffer:	50 mM sodium phosphate, 0.15 M sodium chloride, pH 7.4
Flow:	1 ml/min
System:	ÄKTAFPLC

Fig 6.3. Polishing on HiLoad 16/60 Superdex 200 pg.

Affinity purification reduces sample volume and concentrates the sample. Gel filtration is the slowest of all chromatography techniques, and the size of the column determines the volume of sample that can be applied. Therefore, it is most logical to use gel filtration after techniques that reduce sample volume.

Yield and analysis

Approximately 1.2 mg monoclonal antibody was recovered from about 50 ml of cell culture supernatant. The recovery from the capture and polishing steps was above 95%. Figure 6.4 shows the purity analysis by SDS-PAGE of selected fractions.



Lane

1. Low molecular weight markers
2. Starting material (diluted 2-fold)
3. Eluted IgG_1 peak from HiTrap rProtein A FF column (diluted 10-fold)
4. Flowthrough, HiTrap rProtein A FF
5. Eluted IgG_1 peak from HiLoad 16/60 Superdex 200 pg (diluted 6-fold)
6. LMW markers

Fig 6.4. Purity analysis by SDS-PAGE, reducing conditions, using a PhastGel Gradient 10–15 gel on PhastSystem.

Example 2: Two-step purification of mouse monoclonal IgG_1 using HiTrap Protein G HP for the capture step

This case shows a purification method for mouse monoclonal IgG from cell culture supernatant using HiTrap Protein G HP for the initial capture. Polishing was performed in the second, gel filtration step on HiLoad Superdex 16/60 200 pg. The capture and polishing steps were performed on ÄKTAprime plus. Monoclonal mouse IgG_1 was captured in the first step and eluted using a low pH buffer.

Target molecule: Mouse monoclonal IgG_1.

Source material: Cell culture supernatant.

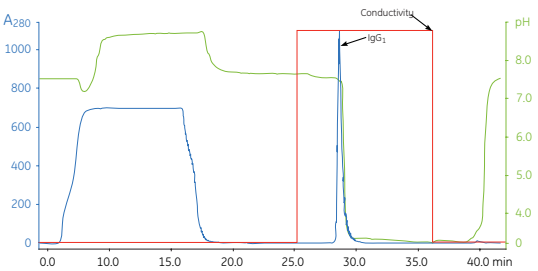
Extraction and clarification: Cell culture supernatant filtered through a 0.45 µm filter.

Capture

Binding buffer: 20 mM potassium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

1. Equilibrate column with 5 column volumes of binding buffer.
2. Apply sample.
3. Wash the column with 10 column volumes binding buffer or until the absorbance at 280 nm has returned to baseline.
4. Elute with 5 to 10 column volumes of elution buffer.
5. Re-equilibrate with 5 column volumes of binding buffer.



Column:	HiTrap Protein G HP 1 ml
Sample:	10 ml cell culture supernatant containing mouse monoclonal IgG_1
Binding buffer:	20 mM potassium phosphate, pH 7.0.
Elution buffer :	0.1 M glycine-HCl, pH 2.7
Flow rate:	1 ml/min
System:	ÄKTAprime plus

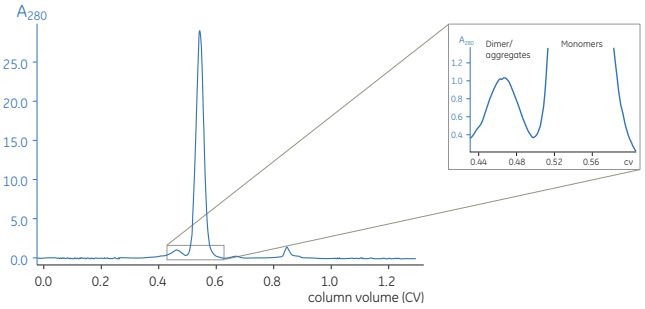
Fig 6.5. Capture step in a two-step purification of mouse monoclonal IgG_1 using HiTrap Protein G HP. The curves shown are absorbance (blue), pH (green), and conductivity (red).

Intermediate purification

No intermediate step was required as the high selectivity of the capture step gave a sufficiently high level of purity so that only a final polishing step was necessary.

Polishing

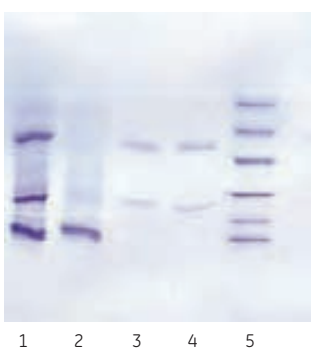
1. Equilibrate the column with phosphate buffered saline, pH 7.4 (see Table A3.1, page 140).
2. Apply sample (maximum sample volume 1% to 2% of total column volume).
3. Elute sample in one column volume of buffer. Collect fractions.
4. Wash with 2 to 3 column volumes of buffer.



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

Fig 6.6. Note the separation between dimers and monomers (magnified).

Affinity purification reduces sample volume and concentrates the sample. Gel filtration is the slowest of all chromatography techniques and the size of the column determines the volume of sample that can be applied. Therefore, it is most logical to use gel filtration after techniques that reduce sample volume.



Lane
1. Mouse monoclonal IgG_1 (cell culture supernatant)
2. Flowthrough (capture step)
3. Eluted fractions (capture step)
4. Eluted fractions (polishing step)
5. LMW markers

Fig 6.7. Purity analysis of mouse monoclonal IgG_1 by SDS-PAGE, reducing conditions.

Purity was controlled by SDS-PAGE under reducing conditions, which showed that the antibody was highly pure already after the first affinity step. The gel filtration step further improved target quality by separating the dimer and monomer of the antibody. Note that both dimers and monomers run as heavy and light chains under the reducing conditions used.

Example 3: Unattended two-step purification of antibodies

In this application, ÄKTAxpress was used for automated two-step purification of antibodies at milligram scale. One- and two-step protocols including cleaning-in-place (CIP) procedures can be easily generated by a method wizard in UNICORN™. This example demonstrates automated capture by affinity chromatography followed by desalting.

Target molecule: Human monoclonal antibody.

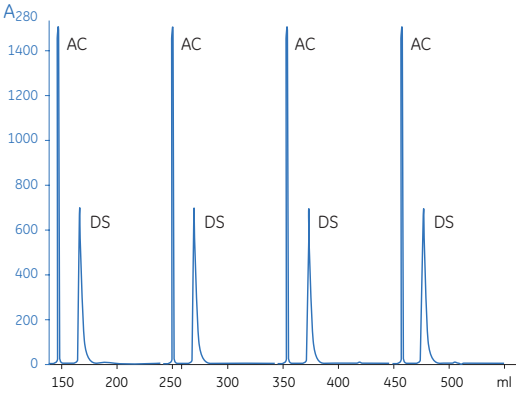
Source material: Cell culture supernatant.

Extraction and clarification: Cell culture supernatant filtered through a 0.45 µm filter.

Binding buffer: 20 mM phosphate, 150 mM NaCl, pH 7.0

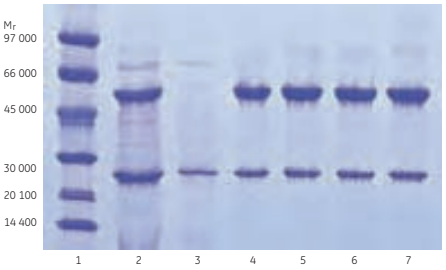
Elution buffer: 100 mM sodium citrate, pH 3.0

Desalting buffer: 50 mM phosphate buffer, 150 mM NaCl, pH 7.2



Columns: AC: HiTrap MabSelect SuRe 1 ml
DS: HiPrep 26/10 Desalting
Sample: 20 ml human monoclonal antibody in culture supernatant, ~0.5 mg/ml
Sample volume: 20 ml
Flow rate: 1 ml/min
System: ÄKTAxpress

Fig 6.8. Chromatogram of four repetitive runs showing purification of human monoclonal antibody from cell culture by affinity chromatography (AC) and desalting (DS).



Lane
1. LMW markers
2. Starting material
3. Flowthrough
4. Run 1
5. Run 2
6. Run 3
7. Run 4

Fig 6.9. SDS-PAGE purity analysis (reducing conditions) of human monoclonal antibody purified by affinity chromatography using ÄKTAxpress. Lanes 4–7 are fractions from the final desalting steps shown in Figure 6.8. Data kindly provided by G.J. Perdock, T. Verhagen and P.H.C. van Berkel, Genmab BV, Utrecht, Netherlands, and used with permission.

The desalting step is important for the preservation of physiological conditions and activity. On average, 8.3±0.17 mg of highly pure target antibody was recovered after the two-step purification.

Example 4: Two-step purification of a mouse monoclonal IgG_1 for diagnostic use

The goal of this example was purification of a monoclonal antibody to achieve a level of purity sufficient for *in vitro* diagnostic use. The two-step procedure combined hydrophobic interaction chromatography (HIC) for the capture step and gel filtration for polishing.

Target molecule: Mouse monoclonal IgG_1 anti-IgE.

Source material: Hybridoma cell culture.

Clarification: Sample was filtered and ammonium sulfate added to 0.05 M. This is to enhance binding to the HIC column, not to precipitate the monoclonal antibody.

Capture

HIC purification was chosen for the capture step because the antibody binds very strongly to the medium (Phenyl Sepharose High Performance) and most fetal calf serum proteins pass through the column as shown in Figure 6.10. The sample was concentrated into a smaller volume for polishing.

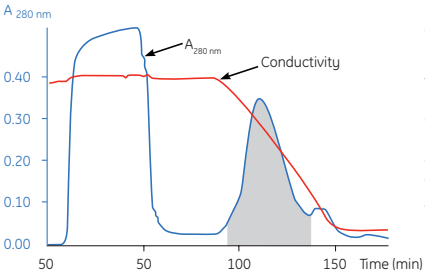
Screening of HIC media, using HiTrap HIC Selection Kit or RESOURCE HIC Test Kit, is recommended to select the medium that gives the best results. See Ordering information or refer to *Hydrophobic Interaction and Reversed Phase Chromatography: Principles and Methods*, code number 11-0012-69 AA for more information.

Buffer conditions should be checked to select the concentration of ammonium sulfate that gives the highest binding selectivity for the antibody and avoids binding albumin.

Binding buffer: 20 mM potassium phosphate, 500 mM ammonium sulfate, pH 7.0

Elution buffer: 20 mM potassium phosphate, pH 7.0

1. Equilibrate column in binding buffer.
2. Apply sample.
3. Wash the column with binding buffer until the absorbance at 280 nm has returned to baseline.
4. Use the elution buffer to create a linear gradient (10 column volumes) from 0.5 to 0 M ammonium sulfate.
5. Wash with 2 to 3 column volumes of 100% elution buffer.
6. Re-equilibrate with 2 to 3 column volumes of binding buffer.



Column:	HiLoad 16/10 Phenyl Sepharose High Performance
Sample:	Hybridoma cell culture supernatant, mouse IgG_1, anti-IgE. Ammonium sulfate added to 0.5 M
Binding buffer:	20 mM potassium phosphate, 500 mM ammonium sulfate, pH 7.0
Elution buffer:	20 mM potassium phosphate, pH 7.0
Gradient:	0% to 100% elution buffer, 10 column volumes
Flow rate:	3.3 ml/min

Fig 6.10. Capture of mouse IgG_1 on HiLoad 16/10 Phenyl Sepharose High Performance.

Intermediate purification

No intermediate step is required as the capture step gives a purity level >95%.

Polishing

A final purity of > 99% was achieved using Superdex 200 prep grade (Fig 6.11).

1. Equilibrate column in phosphate buffered saline, pH 7.5.
2. Apply sample (maximum sample volume 1% to 2% of total column volume).
3. Elute sample in one column volume of buffer. Collect fractions.
4. Wash with 2 to 3 column volumes of buffer.

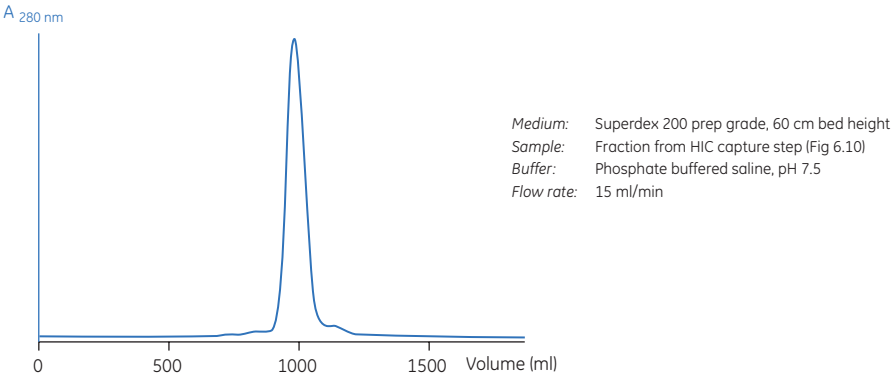


Fig 6.11. Polishing of mouse monoclonal IgG_1 anti-IgE using Superdex 200 prep grade.

Chapter 7
Large-scale purification

The clinical success of monoclonal antibodies (MAbs) is one of the most exciting achievements in the biopharmaceutical industry, resulting in annual production requirements of, in some cases, several tonnes – joining insulin and plasma proteins in sheer scale of bulk production. MAbs are currently the second largest category of biotech drugs on the market after growth factors, but are by far the fastest growing category and are believed by many to be the future of the biotechnology industry. To meet this demand, cell culture capacity is increasing with reactors of up to 25 000 l. In addition, expression levels, currently in the range of 3 to 5 g/l, are expected to further increase, which will put additional demand on the development of purification tools such as high-throughput media and process solutions.

Key concerns in large-scale purification (downstream processing) differ from those typical at laboratory scale; the emphasis in large-scale purification is developing robust and cost-effective protocols and decreasing the number of unit operations in order to improve overall process economy. Current trends in antibody production show that affinity chromatography using Protein A media (e.g., MabSelect chromatography media family) is the most cost-effective alternative for capture of antibodies.

Platform technologies in MAb purification

Platform technologies in MAb purification refers to a standard set of unit operations, conditions, and methods applied to the purification of molecules of a given class, in order to facilitate rapid and economical process development and scale-up. For MAb purification at large scale, the platform recommended by GE Healthcare consists of a protein A-based capture step followed by one or two additional chromatography steps. The protein A step is a rapid, robust unit operation yielding highly pure MAbs (typically > 99%) with high recovery. Additional chromatography unit operations such as cation exchange chromatography (CIEX), anion exchange chromatography (AIEX), and hydrophobic interaction chromatography (HIC) can be considered for the intermediate and polishing steps. A suitable purification process can be designed based on properties of the individual MAb, such as pI, hydrophobicity, stability, glycosylation pattern, impurity profile, and tendency to form aggregates.

Affinity chromatography

The principles of affinity chromatography using protein A are discussed in Chapter 3.

Ion exchange chromatography

In general, a MAb has a higher pI than most host cell proteins (HCP). This gives a good opportunity to use ion exchange chromatography (IEX) for purification. A CIEX step can be designed for the MAb to bind to the chromatography medium while most impurities like DNA, endotoxins, and HCP flow through the column or are washed away with a washing buffer. Alternatively, an AIEX step can be designed for use under nonbinding conditions, allowing the MAb to pass in the flowthrough (often described as flowthrough mode), while impurities such as DNA, endotoxins, leached protein A ligand, and HCP remain bound to the medium. When used as a polishing step, the AIEX alternatives provide an advantage in terms of capacity since only impurities are adsorbed. In addition, AIEX is a generally good technique for removal of viruses.

New types of IEX media that have a ligand with additional interaction mechanisms in combination with ion charges, commonly called multimodal media, are now also available. Capto adhere is one example of a multimodal medium that effectively removes DNA, viruses, endotoxins, leached protein A ligand, and HCP. Capto adhere is a multimodal strong anion exchanger, and is designed for operation in flowthrough mode for the MAb.

Hydrophobic interaction chromatography

Many antibodies form dimers or aggregates, in particular at high expression levels. The aggregates are more hydrophobic and will bind more strongly to HIC media compared with the corresponding monomer. Therefore, HIC is an efficient tool for aggregate and dimer removal in flowthrough mode. Aggregates bind to the medium while the antibody passes straight through. HIC is also useful for removing HCP and endotoxins.

Process design

In designing a platform technology for purification of monoclonal antibodies, the properties of individual antibodies determine which unit operations (chromatographic steps) should be used and in what order (see Fig 7.1 for an overview of recommended platform technologies).

Usually, three chromatography unit operations are used, with Protein A as the first capture step (Fig 7.1, strategies 1 to 3). For an antibody with low aggregate level, the process often has two IEX steps after affinity chromatography: either CIEX in binding mode (strategy 1); or AIEX in flowthrough mode (strategies 2 and 3). For an antibody that has formed significant amounts of aggregates, the protein A step can be followed by an intermediate/polishing step using AIEX to remove DNA, endotoxins, HCP, and viruses before a HIC flowthrough step for aggregate removal. Alternatively, a well-optimized chromatography step with Capto adhere can often eliminate an entire step of the process, allowing the development of a two-step strategy (Fig 7.1, strategy 4). This two-step strategy decreases production costs significantly by reducing process time, buffer consumption, media costs, while at the same time increases product yield.

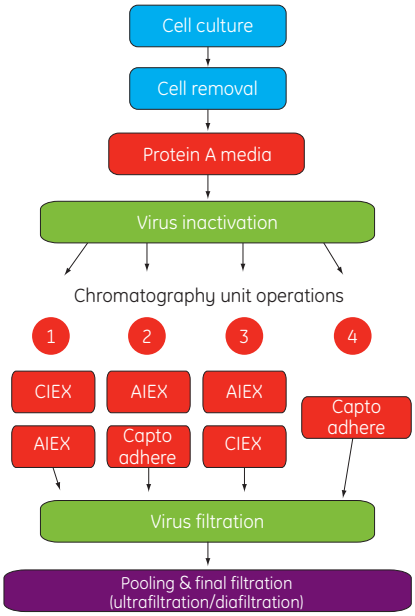


Fig 7.1. Overview of different combinations of chromatography unit operations in a MAb purification process.

High productivity media for MAb purification

GE Healthcare has been the leading supplier of chromatography media for downstream processing of a broad range of biomolecules since the late 1950's. The latest additions to the portfolio include the MabSelect range of products, for MAb capture and the Capto family, which has been specifically designed to meet the increasing demand for high capacity and high throughput.

Table 7.1 briefly describes each of the products recommended for efficient and cost-effective large-scale purification of MAbs.

Table 7.1. Overview of recommended media for downstream purification of monoclonal antibodies

Purification step	Chromatography method	Product features
Capture		
MabSelect SuRe	Affinity (protein A)	Increased alkali stability facilitates cleaning-in-place; high throughput and purity
MabSelect	Affinity (protein A)	High throughput and purity
MabSelect Xtra	Affinity (protein A)	High binding capacity; high purity
Intermediate purification and polishing		
Capto Q	Anion exchange	High capacity and throughput
Capto S	Cation exchange	High capacity and throughput
SP Sepharose Fast Flow	Cation exchange	High selectivity
Capto adhere	Multimodal anion exchange	Enables a two-step process; removes host cell proteins, leached protein A ligand, and dimers/aggregates
Phenyl Sepharose Fast Flow	Hydrophobic interaction	High capacity and selectivity; removes dimers/aggregates

Prepacked, disposable solutions speed up the downstream process

In addition to a wide range of industrial-scale columns and bulk media for purification of MAbs, GE Healthcare now offers large-scale, disposable ReadyToProcess™ columns. These columns are prepacked, prequalified, and presanitized process chromatography columns available with a range of BioProcess media: MabSelect SuRe, Capto Q, Capto S, Capto adhere, and Phenyl Sepharose 6 Fast Flow (low sub). ReadyToProcess columns are available in different sizes (Fig 7.2), are ready-to-use, and the design makes them easy to connect to chromatography systems and to dispose of after completed production.

ReadyToProcess columns are designed for purification of biopharmaceuticals (e.g., proteins and antibodies, vaccines, plasmids, and viruses) for clinical phase I and II studies. Depending on the scale of operations, the columns can also be used for manufacturing, as well preclinical studies. As ReadyToProcess columns make column packing, column qualification, and sanitization redundant in the purification process, significant time savings can be achieved in the downstream process.

Fig 7.2. ReadyToProcess columns are easily connected to the system and can be disposed after completed production.

Custom Designed Media and columns

Custom Designed Media (CDM) can be produced for specific industrial process separations when suitable media are not available from the standard range. The Custom Designed Media group (CDM group) works in close collaboration with the user to design, manufacture, test, and deliver media for specialized purification requirements. When a chromatographic step is developed to be an integral part of a manufacturing process, the choice of column is important to ensure consistent performance and reliable operation. GE Healthcare provides a wide range of columns that ensures the highest performance from all our purification media and meets the demands of modern pharmaceutical manufacturing. In addition, prepacked columns, made according to the client's choice from the GE Healthcare range of columns and media, can be supplied by the CDM group.

Appendix 1
Characteristics of Protein G and Protein A Sepharose products

The basis for antibody affinity purification is the high affinity and specificity of protein G and protein A for the Fc region of IgG from a variety of species. These proteins have been immobilized to several different matrixes resulting in an excellent means of isolating IgG and IgG subclasses from ascites, cell culture supernatants, and serum.

Tables A1.1–A1.3 summarize key characteristics of bulk Protein G and Protein A Sepharose media. Tables A1.4-A1.6 summarize the characteristics of these media in prepacked columns and 96-well plates.

Table A1.1. Characteristics of Protein G Sepharose 4 Fast Flow

Characteristics	Protein G Sepharose 4 Fast Flow
Ligand	Recombinant protein G lacking albumin-binding region
Ligand coupling method	Cyanogen bromide activation
Matrix	Highly cross-linked agarose, 4%
Binding capacity	> 20 mg human IgG/ml medium
Average particle size	90 µm
Ligand density	~ 2 mg protein G/ml medium
Recommended flow rate	50–300 cm/h
Chemical stability	Stable in all commonly used aqueous buffers - 1 M acetic acid, 1% SDS, and 6 M guanidine-HCl (tested at 37°C for 7 d), as well as 0.1 M glycine-NaOH, pH 11, 1 M HCl, and 8 M urea (stable for at least 2 h at room temperature)
pH stability[1]	
Long term	3–9
Short term	2–10
Storage	20% ethanol
Storage temperature	4ºC to 8ºC

[1]*pH below 3 is sometimes required to elute strongly bound IgG species. Note that protein ligands may hydrolyze at very low pH*

Table A1.2. Characteristics of nProtein A Sepharose 4 Fast Flow

Characteristics	nProtein A Sepharose 4 Fast Flow
Ligand	Native protein A
Ligand coupling method	Cyanogen bromide activation
Matrix	Highly cross-linked agarose, 4%
Binding capacity	> 30 mg human IgG/ml medium
Average particle size	90 µm
Ligand density	~ 6 mg native protein A/ml medium
Recommended flow rate	50–300 cm/h
Chemical stability	Stable in all commonly used aqueous buffers - 1 M acetic acid, 1% SDS, and 6 M guanidine-HCl (tested at 37°C for 7 d), as well as 0.1 M glycine-NaOH, pH 11, 1 M HCl, and 8 M urea (stable for at least 2 h at room temperature)
pH stability[1]	
Long term	3–9
Short term	2–10
Storage	20% ethanol
Storage temperature	4ºC to 8ºC

[1]*pH below 3 is sometimes required to elute strongly bound IgG species. Note that protein ligands may hydrolyze at very low pH*

Table A1.3. Characteristics of rProtein A Sepharose Fast Flow

Characteristics	rProtein A Sepharose Fast Flow
Ligand	Recombinant protein A (*E. coli)*
Ligand coupling method	Epoxy activation, thioether coupling
Matrix	Highly cross-linked agarose, 4%
Dynamic binding capacity	~ 50 mg human IgG/ml medium
Average particle size	90 µm
Ligand density	~6 mg recombinant protein A/ml medium
Recommended flow rate	50–300 cm/h
Chemical stability	Stable in all aqueous buffers, cleaning, and preservation solutions commonly used in protein A chromatography: 10 mM HCl (pH 2), 10 mM NaOH, pH 11, 0.1 M sodium citrate-HCl, pH 3, 6 M guanidine-HCl, 20% ethanol
pH stability[1]	
Long term	3–10
Short term	2–11
Storage	20% ethanol
Storage temperature	4ºC to 8ºC

[1]*pH below 3 is sometimes required to elute strongly bound IgG species. Note that protein ligands may hydrolyze at very low pH*

Table A1.4. Characteristics of HiTrap Protein G HP, MAb Trap Kit, HiTrap Protein A HP, and HiTrap rProtein A FF

Characteristics	HiTrap Protein G HP /MAb Trap Kit[1]	HiTrap Protein A HP	HiTrap rProtein A FF
Ligand	Recombinant protein G lacking albumin-binding region	Native protein A	Recombinant protein A (*E. coli*)
Ligand coupling method	N-hydroxysuccinimide activation	N-hydroxysuccinimide activation	Epoxy activation, thioether coupling
Ligand density	~ 2 mg protein G/ml medium	~ 3 mg protein A/ml medium	~ 6 mg recombinant protein A/ml medium
Matrix	Highly cross-linked agarose, 6%	Highly cross-linked agarose, 6%	Highly cross-linked agarose, 4%
Prepacked medium	Protein G Sepharose High Performance	Protein A Sepharose High Performance	rProtein A Sepharose Fast Flow
Average particle size	34 µm	34 µm	90 µm
Binding capacity	~ 25 mg human IgG/ml medium	~ 20 mg human IgG/ ml medium	~ 50 mg human IgG/ml medium
Average particle size	34 µm	34 µm	90 µm
Recommended flow rate			
1 ml column	1 ml/min	1 ml/min	1 ml/min
5 ml column	5 ml/min	5 ml/min	5 ml/min
Maximum flow rate			
1 ml column	4 ml/min	4 ml/min	4 ml/min
5 ml column	20 ml/min	20 ml/min	20 ml/min
pH stability[2]			
Long term	3–9	3–9	3–10
Short term	2–9	2–9	2–11
Storage	20% ethanol	20% ethanol	20% ethanol
Storage temperature	4ºC to 8ºC	4ºC to 8ºC	4ºC to 8ºC

[1]*The kit includes: HiTrap Protein G HP (1 ml column), stock solutions of binding buffer (50 ml), elution buffer (15 ml), neutralizing buffer (25 ml), Luer connector, stop plugs female (1/16"), syringe, and instructions*

[2]*pH below 3 is sometimes required to elute strongly bound antibody species. Note that protein ligands may hydrolyze at very low pH*

Table A1.5. Characteristics of Ab SpinTrap/Protein G HP SpinTrap/Protein G HP MultiTrap

Characteristics	Ab SpinTrap/ Protein G HP SpinTrap	Protein G HP MultiTrap
Ligand	Recombinant protein G lacking albumin-binding region	Recombinant protein G lacking albumin-binding region
Ligand coupling method	N-hydroxysuccinimide activation	N-hydroxysuccinimide activation
Ligand density	~ 2 mg protein G/ml medium	~ 2 mg protein G/ml medium
Matrix	Highly cross-linked agarose, 6%	Highly cross-linked agarose, 6%
Medium	Protein G Sepharose High Performance	Protein G Sepharose High Performance
Binding capacity	> 1 mg human IgG/column	> 0.5 mg human IgG/well
Average particle size	34 µm	34 µm
pH stability[1]		
Long term	3–9	3–9
Short term	2–9	2–9
Well volume	-	800 µl
Medium volume	100 µl	50 µl
Max. sample loading volume	600 µl	600 µl
Column/multiwell plate material	Polypropylene barrel and polyethylene frits	Polypropylene barrels and frits, polyethylene bottom
Storage	20% ethanol	20% ethanol
Storage temperature	4°C to 8°C	4°C to 8°C
Filter plate size[2]	-	127.8 × 85.5 × 30.6 mm

[1]*pH below 3 is sometimes required to elute strongly bound antibody species. Note that protein ligands may hydrolyze at very low pH*

[2]*According to American National Standards Institute (ANSI) and Society for Biomolecular Screening (SBS) standards 1-2004, 3-2004, and 4-2004*

Table A1.6. Characteristics of Protein A HP SpinTrap/Protein A HP MultiTrap

Characteristics	Protein A HP SpinTrap	Protein A HP MultiTrap
Ligand	Native protein A	Native protein A
Ligand coupling method	N-hydroxysuccinimide activation	N-hydroxysuccinimide activation
Ligand density	~ 3 mg protein A/ml medium	~ 3 mg protein A/ml medium
Matrix	Highly cross-linked agarose, 6%	Highly cross-linked agarose, 6%
Medium	Protein A Sepharose High Performance	Protein A Sepharose High Performance
Binding capacity	> 1 mg human IgG/column	> 0.5 mg human IgG/well
Average particle size	34 µm	34 µm
pH stability[1] Long term Short term	 3–9 2–9	 3–9 2–9
Well volume	-	800 µl
Medium volume	100 µl	50 µl
Max. sample loading volume	600 µl	600 µl
Column/multiwell plate material	Polypropylene barrel and polyethylene frits	Polypropylene barrels and frits, polyethylene bottom
Storage	20% ethanol	20% ethanol
Storage temperature	4°C to 8°C	4°C to 8°C
Filter plate size[2]	-	127.8 × 85.5 × 30.6 mm

[1]*pH below 3 is sometimes required to elute strongly bound antibody species. Note that protein ligands may hydrolyze at very low pH*

[2]*According to American National Standards Institute (ANSI) and Society for Biomolecular Screening (SBS) standards 1-2004, 3-2004, and 4-2004*

Cleaning of Protein G and Protein A Sepharose products

After purification, the medium should be regenerated as follows:

1 After elution, wash with 2 to 3 column volumes of elution buffer.

2 Immediately re-equilibrate by washing with 2 to 3 column volumes of binding buffer.

Cleaning-in-place

When an increase in backpressure is seen, the medium should be cleaned. In some applications, substances like denatured proteins or lipids do not elute in the regeneration procedure.

To remove precipitated or denatured proteins:

1 Wash the medium with 2 column volumes of 6 M guanidine hydrochloride.

2 Immediately wash with at least 5 column volumes of binding buffer.

To remove strongly bound hydrophobic proteins, lipoproteins and lipids:

1 Wash with a nonionic detergent, e.g., 0.1% Triton X-100 at 37°C for 1 min.

2 Immediately wash with a least 5 column volumes of sterile binding buffer.

2a Alternatively, wash the column with 70% ethanol and let it stand for 12 h. After treatment, wash with at least 5 column volumes of binding buffer.

Reversed flow may improve the efficiency of the cleaning-in-place procedure. After cleaning, store in 20% ethanol.

Washing with 70% ethanol will increase backpressure. Use a lower flow rate when cleaning with 70% ethanol.

Characteristics of MabSelect media

MabSelect media have been designed for capturing monoclonal antibodies from large volumes of feed. The recombinant protein A ligand of MabSelect is engineered to favor an oriented coupling that delivers enhanced binding capacity. MabSelect SuRe uses an alkali-tolerant recombinant protein A ligand that is resistant to harsh cleaning agents (e.g., 0.1 to 0.5 M NaOH). MabSelect Xtra uses the same recombinant protein A ligand as MabSelect, but the medium has a smaller particle size and greater porosity for increased dynamic binding capacity. Table A1.7 summarizes key characteristics of bulk MabSelect media. Table A1.8 summarizes the characteristics of these media in prepacked columns.

Table A1.7. Characteristics of MabSelect, MabSelect Xtra, and MabSelect SuRe

Characteristics	MabSelect	MabSelect Xtra	MabSelect SuRe
Ligand	Recombinant protein A (*E. coli*)	Recombinant protein A (*E. coli*)	Alkali-tolerant protein A (*E. coli*)
Ligand coupling method	Epoxy activation	Epoxy activation	Epoxy activation
Matrix	Rigid, highly cross-linked agarose	Rigid, highly cross-linked agarose	Rigid, highly cross-linked agarose
Binding capacity	~ 30 mg human IgG/ml medium	~ 40 mg human IgG/ml medium	~ 30 mg human IgG/ml medium
Average particle size	85 µm	75 µm	85 µm
Recommended flow rate	100–500 cm/h	100–300 cm/h	100–500 cm/h
Chemical stability	No significant change in chromatographic performance after 1 week storage using 8 M urea, 6 M guanidine-HCl, 2% benzyl alcohol, or 20% ethanol	No significant change in chromatographic performance after 1 week storage using 8 M urea, 6 M guanidine-HCl, 2% benzyl alcohol, or 20% ethanol	No significant change in chromatographic performance after 1 week storage using 8 M urea, 6 M guanidine-HCl, 2% benzyl alcohol, or 20% ethanol
pH stability[1]			
Long term	3–10	3–10	3–12
Short term	2–12	2–12	2–14
Storage	20% ethanol or 2% benzyl alcohol	20% ethanol or 2% benzyl alcohol	20% ethanol or 2% benzyl alcohol
Storage temperature	4°C to 8°C	4°C to 8°C	4°C to 8°C

[1]*pH below 3 is sometimes required to elute strongly bound antibody species. Note that protein ligands may hydrolyze at very low pH*

Table A1.8. Characteristics of HiTrap MabSelect, HiTrap MabSelect Xtra, and HiTrap MabSelect SuRe

Characteristics	HiTrap MabSelect	HiTrap MabSelect Xtra	HiTrap MabSelect SuRe
Ligand	Recombinant protein A (*E. coli*)	Recombinant protein A (*E. coli*)	Alkali-tolerant protein A (*E. coli*)
Coupling chemistry	Epoxy activation	Epoxy activation	Epoxy activation
Matrix	Rigid, highly cross-linked agarose	Rigid, highly cross-linked agarose	Rigid, highly cross-linked agarose
Medium	MabSelect	MabSelect Xtra	MabSelect SuRe
Average particle size	85 µm	75 µm	85 µm
Binding capacity	~ 30 mg human IgG/ml medium	~ 40 mg human IgG/ml medium	~ 30 mg human IgG/ml medium
Column volumes	1 ml or 5 ml	1 ml or 5 ml	1 ml or 5 ml
Recommended flow rate			
1 ml column	1 ml/min	1 ml/min	1 ml/min
5 ml column	5 ml/min	5 ml/min	5 ml/min
Maximum flow rate			
1 ml column	4 ml/min	4 ml/min	4 ml/min
5 ml column	20 ml/min	20 ml/min	20 ml/min
pH stability[1]			
Long term	3-10	3-10	3-12
Short term	2-12	2-12	2-14
Storage	20% ethanol	20% ethanol	20% ethanol
Storage temperature	4ºC to 8ºC	4ºC to 8ºC	4ºC to 8ºC

[1]*pH below 3 is sometimes required to elute strongly bound antibody species. However protein ligands may hydrolyze at very low pH*

Cleaning of MabSelect products

All MabSelect media can be cleaned using the following procedures:

To remove precipitated or denatured substances:

1. Wash the medium with 2 column volumes of 50 mM NaOH in 0.5 M Na_2SO_4, or 50 mM NaOH in 1.0 M NaCl, or 0.1 M H_3PO_4, or 6 M guanidine hydrochloride in 10 mM NaOH. Contact time: at least 10 min.
2. Immediately wash with at least 5 column volumes of sterile filtered binding buffer at pH 7 to 8.

MabSelect SuRe is alkali-tolerant, allowing the use of more concentrated solutions of NaOH:

1. Wash with 3 column volumes of binding buffer.
2. Wash with at least 2 column volumes of 0.1 to 0.5 M NaOH. Contact time: 10 to 15 min.
3. Immediately wash with at least 5 column volumes of sterile and filtered binding buffer at pH 7 to 8.

To remove strongly bound hydrophobic proteins, lipoproteins, and lipids:

1. Wash with 2 column volumes of a nonionic detergent (e.g., 0.1% solution).
2. Immediately wash with at least 5 column volumes of sterile filtered binding buffer at pH 7 to 8.

2a Alternatively, wash with 3 to 4 column volumes of 70% ethanol or 30% 2-propanol. Wash immediately with at least 5 column volumes of sterile filtered binding buffer at pH 7 to 8. Apply increasing gradients to avoid air bubble formation when using high concentrations of organic solvents.

Washing with 70% ethanol and 30% 2-propanol will increase backpressure. Use a lower flow rate when cleaning with 70% ethanol or 30% 2-propanol.

Thiophilic adsorption media

HiTrap IgY Purification HP and HiTrap IgM Purification HP are packed with a thiophilic adsorption medium, 2-mercaptopyridine coupled to Sepharose High Performance. Table A1.9 summarizes the characteristics of 2-mercaptopyridine media used for purification of IgY and IgM.

Table A1.9. Characteristics of HiTrap IgY Purification HP and HiTrap IgM Purification HP

Characteristics	HiTrap IgY Purification HP	HiTrap IgM Purification HP
Ligand	2-mercaptopyridine	2-mercaptopyridine
Ligand density	~ 3 mg /ml medium	~ 2 mg /ml medium
Matrix	Highly cross-linked agarose, 6%	Highly cross-linked agarose, 6%
Medium	2-mercaptopyridine Sepharose High Performance	2-mercaptopyridine Sepharose High Performance
Binding capacity	100 mg pure IgY or ¼ egg yolk/5 ml column	5 mg human IgM/ml medium
Average particle size	34 µm	34 µm
Recommended flow rate	5 ml/min	1 ml/min
Maximum flow rate	20 ml/min	4 ml/min
pH stability[1]		
Long term	3–11	3–11
Short term	2–13	2–13
Column volume	5 ml	1 ml
Storage	20% ethanol	20% ethanol
Storage temperature	4°C to 8°C	4°C to 8°C

[1]*pH below 3 is sometimes required to elute strongly bound antibody species. Note that protein ligands may hydrolyze at very low pH*

Appendix 2
Analytical assays during purification

Analytical assays are essential to follow the progress of purification. They are used to assess the effectiveness of each step in terms of yield, biological activity, recovery, and for optimization of experimental conditions. The importance of a reliable assay for the target molecule cannot be over-emphasized.

When testing chromatographic fractions, ensure that the buffers used for purification do not interfere with the assay.

Total protein determination

Lowry or Bradford assays are the most common assays for total protein content determination. The Bradford assay is particularly suited to samples where there is a high lipid content that may interfere with the Lowry assay.

Purity determination

Purity is most often estimated by SDS-PAGE. Alternatively, isoelectric focusing, capillary electrophoresis, reversed phase chromatography, or mass spectrometry may be used.

SDS-PAGE analysis

Reagents required

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

1. Add 2 µl of 6× SDS loading buffer to 5 to 10 µl of supernatant from crude extracts, cell lysates, or purified fractions as appropriate.
2. Vortex briefly and heat for 5 min at between 90°C and 100°C.
3. Load the samples onto an SDS-polyacrylamide gel.
4. Run the gel and stain with Coomassie Blue (Coomassie Blue R Tablets) or silver stain (PlusOne Silver Staining Kit).

The percentage of acrylamide in the SDS-gel should be selected according to the expected molecular weight of the protein of interest (see Table A2.1).

Table A2.1. Relationship between percentage acrylamide in SDS gel and molecular weight of the protein of interest to be analyzed

Percentage acrylamide in resolving gel		Separation size range ($M_r \times 10^3$)
Single percentage:	5%	36–200
	7.5%	24–200
	10%	14–200
	12.5%	14–100
	15%	14–60*
Gradient:	5–15%	14–200
	5–20%	10–200
	10–20%	10–150

**Larger proteins fail to move significantly into the gel*

For information and advice on electrophoresis techniques, please refer to the Protein Electrophoresis Technical Manual, code number 80-6013-88.

Functional assays

Immunospecific interactions have enabled the development of many alternative assay systems for the assessment of active concentration of target molecules.

- Western blot analysis is used when the sensitivity of SDS-PAGE with Coomassie Blue or silver staining is insufficient.

1. Separate the protein samples by SDS-PAGE.
2. Transfer the separated proteins from the gel to an appropriate membrane, depending on the choice of detection reagents. For chemiluminescent detection, such as ECL™, ECL Plus, or ECL Advance™, Hybond™ ECL, or Hybond P membranes are recommended. For fluorescent detection with ECL Plex™, Hybond LFP™ is recommended.
3. Develop the membrane with the appropriate specified reagents.

Electrophoresis and protein transfer may be accomplished using a variety of equipment and reagents. For further details, refer to the *Protein Electrophoresis Technical Manual*, code number 80-6013-88, and the instruction manuals supplied with the detection kits.

- ELISAs are most commonly used as activity assays.
- Functional assays using the phenomenon of surface plasmon resonance to detect immunospecific interactions (e.g., using Biacore™ systems) enable the determination of active concentration, epitope mapping, and studies of interaction kinetics.

Appendix 3
Immunoprecipitation techniques

Target proteins can be isolated and enriched from crude cell lysates by immunoprecipitation (also known as immunoaffinity or pull-down techniques). An antibody selected for its specificity is first affinity captured onto Protein A Sepharose or Protein G Sepharose media. In a second step, the immobilized antibody is used for capture and enrichment of the protein of interest (i.e., antigen). The target protein can be enriched several hundredfold, depending of the specificity of the antibody. In combination with other techniques, such as SDS-PAGE and immunoblotting, immunoprecipitation can be used to detect and quantitate antigens, determine relative molecular weights, monitor protein turnover and post-translational modifications, and check for enzyme activity.

By using the high specificity of protein A and protein G for the Fc regions of IgG molecules from a wide range of mammalian species, Protein A Sepharose and Protein G Sepharose media offer effective and rapid isolation and enrichment of such immune complexes.

Protein A Sepharose High Performance and Protein G Sepharose High Performance media are available prepacked both in convenient columns and in 96-well format. nProtein A Sepharose 4 Fast Flow and Protein G Sepharose 4 Fast Flow are available as bulk media. The binding capacity for Protein A Sepharose media is between 20 and 30 mg human IgG/ml and the binding capacity for Protein G Sepharose media is >25 mg human IgG/ml medium.

Immunoprecipitation Starter Pack (Fig A3.1) from GE Healthcare is an ideal starting point for immunoprecipitation work. The pack includes nProtein A Sepharose 4 Fast Flow (2 ml) and Protein G Sepharose 4 Fast Flow (2 ml) to enable work with a wide range of antibody species and selection of the optimal medium.

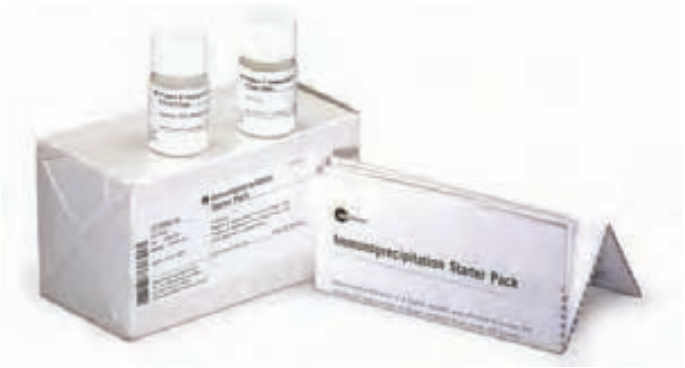


Fig A3.1. Immunoprecipitation Starter Pack for immunoprecipitation of a wide range of antibodies with different binding selectivities.

Procedures for immunoprecipitation must often be optimized empirically to obtain satisfactory results. For example, the choice of cell lysis conditions is critical with regard to cell type and how the antigen is to be used. Whereas cells without cell walls (e.g., animal cells) are easily disrupted by treatment with mild detergent, other cells may need some type of mechanical shearing, such as sonication or Dounce homogenization.

Refer to Table 3.2 in Chapter 3 to see which medium is likely to be suitable for the antibody source and subtype, or test using Immunoprecipitation Starter Pack.

Cell lysis conditions

Cell lysis must be harsh enough to release the target antigen, but mild enough to maintain its immunoreactivity. Some commonly used lysis buffers are listed in Table A3.1.

NP-40 (IGEPAL™ CA-630) and RIPA buffer release most soluble cytoplasmic or nuclear proteins without releasing chromosomal DNA and are a good choice for initial experiments.

Parameters that affect the extraction of an antigen include salt concentration (0 to 1 M), nonionic detergents (0.1% to 2%), ionic detergents (0.01% to 0.5%), and pH (6 to 9).

Table A3.1. Common lysis buffers

Buffers and solutions	Contents	Ability to disrupt cells
Lysis buffers		
Low salt	1% IGEPAL CA-630, 50 mM Tris, pH 8.0, 1 mM PMSF	+
NP-40 (IGEPAL CA-630)	150 mM NaCl, 1% IGEPAL CA-630, 50 mM Tris, pH 8.0, 1 mM PMSF	++
RIPA	150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM PMSF	+++
High salt	500 mM NaCl, 1% IGEPAL CA-630, 50 mM Tris, pH 8.0, 1 mM PMSF	++++
Other buffers and solutions		
PBS	1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4	
Wash buffer	50 mM Tris, pH 8.0	
Sample buffer (reducing)	1% SDS, 100 mM DTT, 50 mM Tris, pH 7.5	

Choice of antibody

Polyclonal serum contains antibodies against multiple epitopes of an antigen. These antibodies help to stabilize the antigen-antibody-medium complexes, but can also create problems with high background during analysis.

Monoclonal antibodies (MAbs) are more specific, which reduces background, but may lead to the formation of less stable immune complexes due to lower affinity. This can be overcome by using pools of different MAbs.

Protein enrichment

Fig A3.2. Protein A HP SpinTrap spin columns and Protein A HP MultiTrap 96-well plates for the enrichment of proteins from a variety of biological samples.

Trap protein A and G products (Protein A HP SpinTrap, Protein G HP SpinTrap, Protein A HP MultiTrap, and Protein G HP MultiTrap) are designed for small-scale protein enrichment, for example for use upstream of gel electrophoresis, liquid chromatography, and mass spectrometry (Fig A3.2). There are two alternative protocols for protein enrichment using these products: the cross-link protocol and classic protocol.

In the cross-link protocol, antigen-capturing antibodies are covalently bound to the Protein A or Protein G Sepharose High Performance matrixes by a cross-linking agent. The antigen is enriched from the sample, purified through washings, and eluted from the column whereas the antibody remains bound to the matrix.

Use the crosslink protocol if the desired protein/antigen has similar molecular weight as the heavy or light chain of the antibody, which causes problems with co-migration in SDS-PAGE analysis or if the antibody interferes with downstream analysis.

In the classic protocol, antigen-capturing antibodies are immobilized by binding to protein A or protein G coupled to the Protein A or Protein G Sepharose High Performance matrixes, respectively. The bound antibody is then used for capture of the antigen of interest. The classic protocol requires that the capturing antibody used binds to protein A or protein G. The antigen of interest is enriched from the sample, purified through washings and eluted from the column together with the antibody.

The optimal parameters for protein enrichment are dependent on the specific antibody-antigen combination. Optimization may be required for each specific antibody-antigen combination to obtain the best results. Examples of parameters that may require optimization are sample pretreatment, amount of protein to be enriched, incubation time, choice of buffers, and number of washes.

For complete protocols for immunoprecipitation using Immunoprecipitation Starter Pack or protein enrichment using Protein A and Protein G HP SpinTrap or MultiTrap, please refer to the Instructions that are delivered with each respective product or download at www.gelifesciences.com/protein-purification.

Appendix 4
General instructions for affinity purification using HiTrap columns

Alternative 1. Manual purification with a syringe

Fig A4.1. 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.

1. Fill the syringe with binding buffer. Remove the stopper and connect the column to the syringe (use the connector supplied) "drop to drop" to avoid introducing air into the column.
2. Remove the snap-off end at the column outlet.
3. Equilibrate the column with 5 column volumes of binding buffer.
4. Apply the pretreated sample using a syringe fitted to the Luer connector on the column. For best results, use a flow rate of 0.2 to 1 ml/min (1 ml column) and 0.5 to 5 ml/min (5 ml column) during sample application*.
5. Wash with 5 to 10 column volumes of binding buffer or until no material appears in the effluent. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for washing. Optional: collect the flowthrough (in 1 ml fractions for the 1 ml column and 2 ml fractions for the 5 ml column) and reserve until the procedure has been successfully completed. Retain a sample for analysis by SDS-PAGE to measure the efficiency of protein binding to the medium.
6. Elute with 5 to 10 column volumes of elution buffer. Maintain a flow rate of 0.2 to 1 ml/min (ml column) and 0.5 to 5 ml/min (5 ml column) for elution.
7. After elution, regenerate the column by washing it with 3 to 5 column volumes of binding buffer. The column is now ready for a new purification.

   **1 ml/min corresponds to approximately 30 drops/min when using a syringe with a HiTrap 1 ml column; 5 ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column*

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

Alternative 2. Simple purification with ÄKTAprime plus

ÄKTAprime plus contains pre-programmed templates for purification of IgG, IgM and IgY using the appropriate HiTrap columns.

Prepare at least 500 ml of each buffer.

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


A


B


D


C

Fig A4.2. Typical procedures using ÄKTAprime plus. (**A**) Prepare the buffers. (**B**) Connect the column. (**C**) Prepare the fraction collector. (**D**) Load the sample.

Appendix 5
Column packing and preparation

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

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

Efficient column packing is essential for a good separation, especially when using gradient elution. A poorly packed column gives rise to poor and uneven flow, peak broadening, and loss of resolution. If column packing is required, the following guidelines will apply at all scales of operation:

- When using a binding technique, use short, wide columns (typically 5 to 15 cm bed height) for rapid purification, even with low linear flow.
- The amount of medium required will depend on the binding capacity of the medium and the amount of sample. The binding capacity of a medium is always significantly influenced by the nature of the sample as well as the medium itself and must be determined empirically. Estimate the amount of medium required to bind the sample of interest and use five times this amount to pack the column. The amount of medium required can be reduced if resolution is satisfactory.
- Once separation parameters have been determined, scale up a purification by increasing the diameter of the column to increase column volume. Avoid increasing the length of the column as this will alter separation conditions.

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

Fig A5.1. "Column Packing — The Movie" provides a step-by-step demonstration of column packing.

1. Equilibrate all materials to the temperature at which the separation will be performed.
2. Eliminate air by flushing column end pieces with the recommended buffer. Ensure no air is trapped under the column net. Close column outlet leaving 1 to 2 cm of buffer in the column.
3. Gently resuspend the medium.

Note that affinity media from GE Healthcare are supplied ready to use. Decanting of fines that could clog the column is unnecessary.

Avoid using magnetic stirrers because they may damage the matrix.

4. Estimate the amount of slurry (resuspended medium) required on the basis of the recommendations supplied 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 (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 medium must be thoroughly washed to remove the storage solution, usually 20% ethanol. Residual ethanol may interfere with subsequent procedures.

Many media equilibrated with sterile phosphate-buffered saline containing an antimicrobial agent may be stored at 4°C for up to 1 month, but always follow the specific storage instructions supplied with the product.

Column selection

Tricorn and XK columns are fully compatible with the high flow rates allowed by with modern media, and a broad range of column dimensions are available (see Table A5.1). In most cases the binding capacity of the medium and the amount of sample to be purified will determine the column size required. Also, Empty Disposable PD-10 Columns are available for single-use applications using gravity flow. For a complete listing of available columns, refer to the GE Healthcare Products for Life Sciences catalog, or www.gelifesciences.com/protein-purification.

Table A5.1. Column bed volumes and heights[1]

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

[1] *All Tricorn and XK column specifications apply when one adapter is used*

[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*

Appendix 6
Storage of biological samples

The advice given here is of a general nature and cannot be applied to every biological sample. Always consider the properties of the specific sample and its intended use before following any of these recommendations.

General recommendations

- Add stabilizing agents when necessary. Stabilizing agents are often required for storage of purified proteins.
- Serum, culture supernatants, and ascites should be kept frozen at -20°C or -70°C, in small aliquots.
- Avoid repeated freeze/thawing or freeze drying/redissolving that may reduce biological activity.
- Avoid conditions close to stability limits, in terms of for example pH or salt concentrations, reducing or chelating agents.
- Keep refrigerated at 4°C in a closed vessel to minimize bacterial growth and protease activity. For prolonged storage at 4°C (more than 24 h), add a preserving agent (e.g., merthiolate 0.01%).

Sodium azide can interfere with coupling methods, and some biological assays, and can be a health hazard. It can be removed by using a desalting column (see Chapter 2).

Common storage conditions for purified proteins

- Store as a precipitate in a high concentration of ammonium sulfate, for example 4.0 M.
- Freeze in 50% glycerol, especially suitable for enzymes.
- Avoid using preserving agents if the product is to be used for a biological assay. Preserving agents should not be added if *in vivo* experiments are to be performed. Store samples in small aliquots and keep frozen.
- Sterile filter to prolong storage time.
- Add stabilizing agents, for example, glycerol (5% to 20%), serum albumin (10 mg/ml), ligand (concentration is selected based on concentration of active protein) to maintain biological activity. Remember that any additive will reduce the purity of the protein and may need to be removed at a later stage.
- Avoid repeated freeze/thawing or freeze drying/redissolving that may reduce biological activity.

Certain proteins, including some mouse antibodies of the IgG_3 subclass, should not be stored at 4°C as they precipitate at this temperature (cryoproteins). Store at room temperature in the presence of a preserving agent.

Appendix 7
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

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

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

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 8
Conversion data: proteins, column pressures

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

Protein	A_{280} for 1 mg/ml
IgG	1.35
IgM	1.20
IgA	1.30
Protein A	0.17
Avidin	1.50
Streptavidin	3.40
Bovine Serum Albumin	0.70

1 kb of DNA = 333 amino acids of coding capacity

= 37 000 g/mol

270 bp DNA = 10 000 g/mol

1.35 kb DNA = 50 000 g/mol

2.70 kb DNA = 100 000 g/mol

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

Column pressures

The maximum operating backpressure refers to the pressure above which the column contents may begin to compress.

Pressure units may be expressed in megaPascal (MPa), bar, or pounds per square inch (psi) and can be converted as follows: 1 MPa = 10 bar = 145 psi

Appendix 9
Principles and standard conditions for different purification techniques

Affinity chromatography (AC)

AC separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand attached to a chromatographic matrix. The technique is well-suited for a capture or intermediate step and can be used whenever a suitable ligand is available for the protein(s) of interest. AC offers high selectivity, hence high resolution, and usually high capacity. Affinity chromatography is frequently used as the first step (capture step) of a two-step purification protocol, followed by a second chromatographic step (polishing step) to remove remaining impurities.

The target protein(s) is/are specifically and reversibly bound by a complementary binding substance (ligand). The sample is applied under conditions that favor specific binding to the ligand. Unbound material is washed away, and bound target protein is recovered by changing conditions to those favoring desorption. Desorption is performed specifically, using a competitive ligand, or nonspecifically, by changing the pH, ionic strength, or polarity. Samples are concentrated during binding, and the target protein is collected in purified and concentrated form. The key stages in an affinity chromatography separation are shown in Figure A9.1. AC is also used to remove specific contaminants; for example, Benzamidine Sepharose 4 Fast Flow can remove serine proteases.

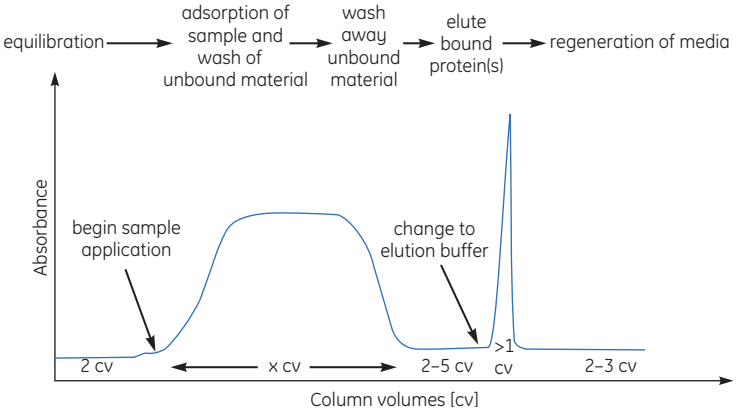


Fig A9.1. Typical affinity purification.

Further information

Protein Purification Handbook (Code No. 18-1132-29)
Affinity Chromatography Handbook: Principles and Methods (Code No. 18-1022-29)
Chapter 3 in this handbook for the purification of antibodies.

Ion exchange chromatography (IEX)

IEX separates proteins with differences in surface charge to give a very high resolution separation with high sample loading capacity. The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatography

medium. Proteins bind as they are loaded onto a column. Conditions are then altered so that bound substances are eluted differentially. Elution is usually performed by increasing salt concentration or changing pH. Changes are made stepwise or with a continuous gradient. Most commonly, samples are eluted with salt (NaCl), using a gradient elution (Fig A9.2). Target proteins are concentrated during binding and collected in a purified, concentrated form.

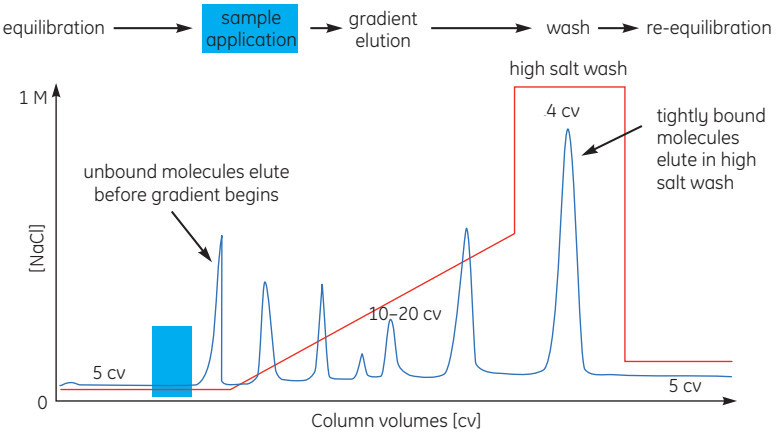


Fig A9.2. Typical IEX gradient elution.

The net surface charge of proteins varies according to the surrounding pH. Typically, when above its isoelectric point (pI) a protein will bind to an anion exchanger; when below its pI a protein will bind to a cation exchanger. However, it should be noted that binding depends on charge and that surface charges may thus be sufficient for binding even on the other side of the pI. Typically IEX is used to bind the target molecule, but it can also be used to bind impurities if required. IEX can be repeated at different pH values to separate several proteins that have distinctly different charge properties, as shown in Figure A9.3.

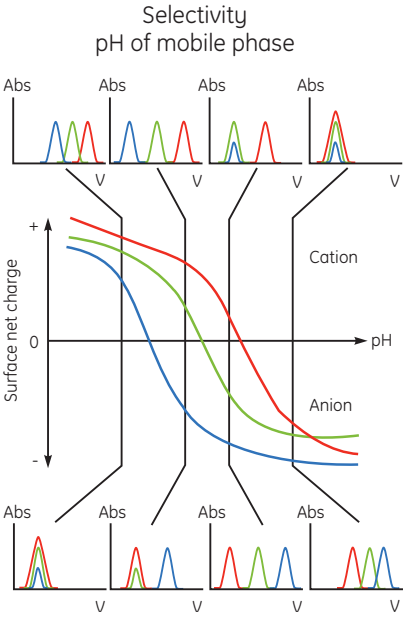


Fig A9.3. Effect of pH on protein elution patterns.

Method development (in priority order)

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

To reduce separation times and buffer consumption, transfer to a step elution after method optimization as shown in Figure A9.4. It is often possible to increase sample loading when using step elution.

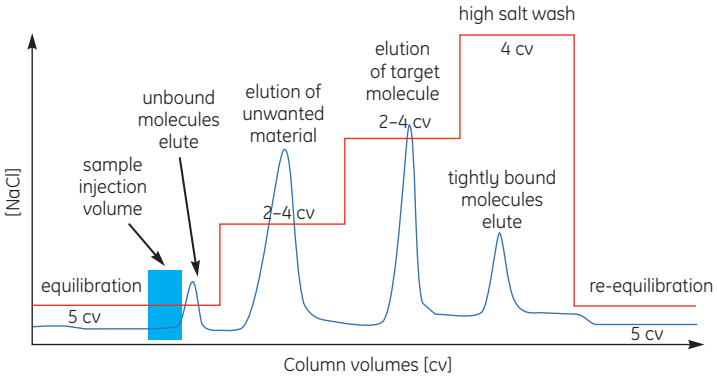


Fig A9.4. Step elution.

Further information

Protein Purification Handbook (Code No. 18-1132-29)
Ion Exchange Chromatography and Chromatofocusing Handbook: Principles and Methods (Code No. 11-0004-21)

Hydrophobic interaction chromatography (HIC)

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

Elution is usually performed by decreases in salt concentration (Fig A9.5). Changes are made stepwise or with a continuous decreasing salt gradient. Most commonly, samples are eluted with a decreasing gradient of ammonium sulfate. Target proteins are concentrated during binding and collected in a purified and concentrated form. Other elution procedures include reducing eluent polarity (ethylene glycol gradient up to 50%), adding chaotropic species (urea, guanidine hydrochloride) or detergents, changing pH or temperature.

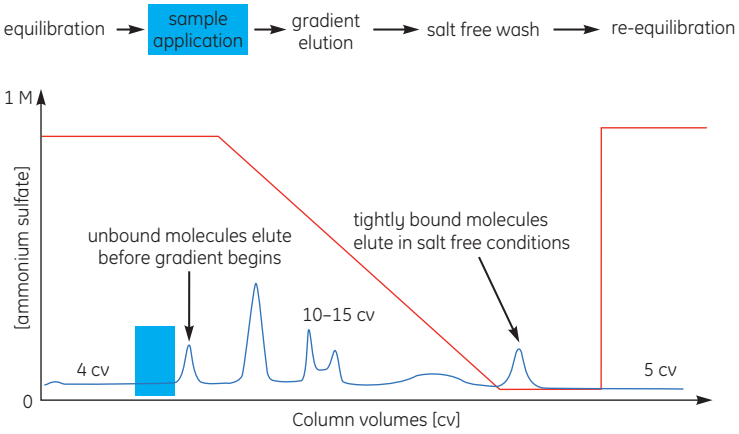


Fig A9.5. Typical HIC gradient elution.

Method development (in priority order)

1. The hydrophobic behavior of a protein is difficult to predict, and binding conditions must be studied carefully. Use HiTrap HIC Selection Kit or RESOURCE HIC Test Kit to select the medium that gives optimal binding and elution over the required range of salt concentration. For proteins with unknown hydrophobic properties begin with 0% to 100% B (0% B, e.g., 1 M ammonium sulfate). Knowledge of the solubility of protein in the binding buffer is important because high concentrations of, for example, ammonium sulfate may precipitate proteins.
2. Select a gradient that gives acceptable resolution.
3. Select the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.
4. If samples adsorb strongly to a medium, separation conditions such as pH, temperature, chaotropic ions, or organic solvents may have caused conformational changes and should be altered. Conformational changes are specific to each protein. Use screening procedures to investigate the effects of these agents. Alternatively, change to a less hydrophobic medium.

To reduce separation times and buffer consumption, transfer to a step elution after method optimization, as shown in Figure A9.6. It is often possible to increase sample loading when using step elution.

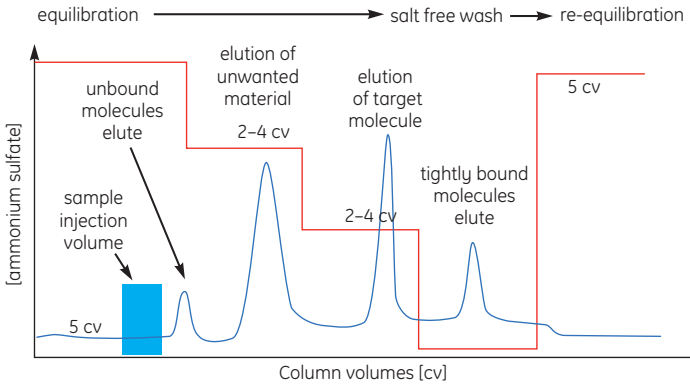


Fig A9.6. Step elution.

Further information

Protein Purification Handbook (Code No. 18-1132-29)
Hydrophobic Interaction Chromatography and Reversed Phase Handbook: Principles and Methods (Code No. 11-0012-69)

Gel filtration (GF)

GF separates proteins with differences in molecular size. The technique is well-suited for the final polishing steps in purification when sample volumes have been reduced (sample volume significantly influences speed and resolution in gel filtration). Samples are eluted isocratically (single buffer, no gradient, Fig A9.7). Buffer conditions are varied to suit the sample type or the requirements for further purification, analysis, or storage, because buffer composition usually does not have major effects on resolution. Proteins are collected in purified form in the chosen buffer.

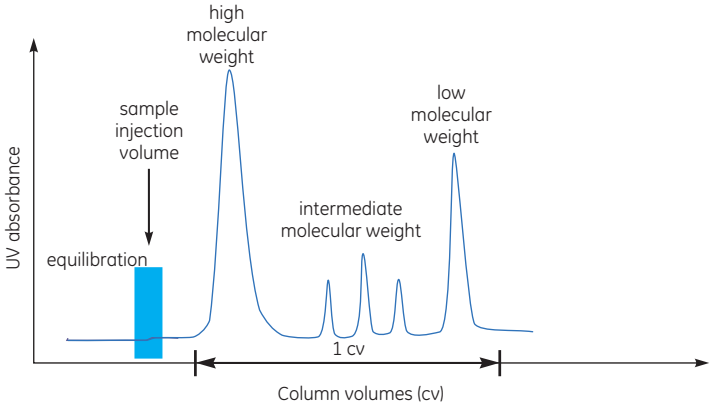


Fig A9.7. Typical GF elution.

Further information

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

Reversed phase chromatography (RPC)

RPC separates proteins and peptides with differing hydrophobicity based on their reversible interaction with the hydrophobic surface of a chromatographic medium. Samples bind as they are loaded onto a column. Conditions are then altered so that the bound substances are eluted differentially. Due to the nature of the reversed phase matrixes, binding is usually very strong. Binding may be modulated by the use of organic solvents and other additives (ion pairing agents). Elution is usually performed by increases in organic solvent concentration, most commonly acetonitrile. Samples that are concentrated during the binding and separation process are collected in a purified, concentrated form. The key stages in a separation are shown in Figure A9.8.

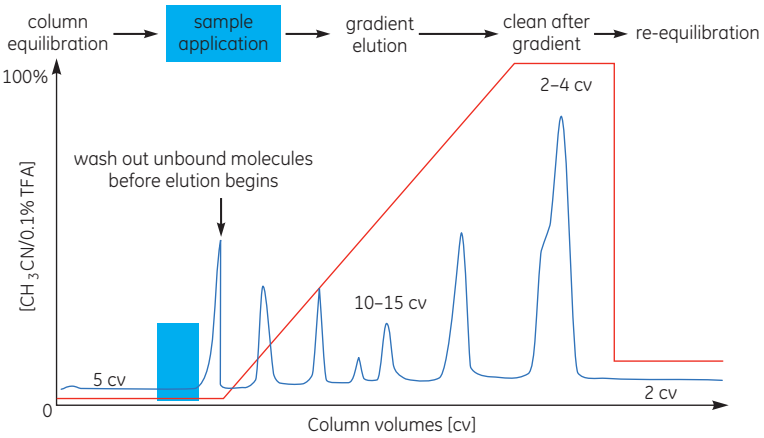


Fig A9.8. Typical RPC gradient elution.

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

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

Method development

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

Further information

Protein Purification Handbook (Code No. 18-1132-29)
Hydrophobic Interaction and Reversed Phase Chromatography Handbook: Principles and Methods (Code No. 11-0012-69)

Product index

Related literature

	Code No.
Handbooks	
Affinity Chromatography Handbook: Principles and Methods	18-1022-29
Purifying Challenging Proteins: Principles and Methods	28-9095-31
Recombinant Protein Purification Handbook: Principles and Methods	18-1142-75
GST Gene Fusion System Handbook	18-1157-58
Gel Filtration: Principles and Methods	18-1022-18
Hydrophobic Interaction and Reversed Phase Chromatography: Principles and Methods	11-0012-69
Ion Exchange Chromatography and Chromatofocusing: Principles and Methods	11-0004-21
Protein Purification	18-1132-29
2-D Electrophoresis: Principles and Methods	80-6429-60
Protein Electrophoresis Technical Manual	80-6013-88
Selection guides/brochures	
Affinity Columns and Media, selection guide	18-1121-86
Convenient Protein Purification, HiTrap column guide	18-1129-81
Gel Filtration Columns and Media, selection guide	18-1124-19
Ion Exchange Columns and Media, selection guide	18-1127-31
Prepacked Chromatography Columns with ÄKTAdesign Systems, selection guide	18-1173-49
Years of Experience in Every Column, brochure	28-9090-94
CD	
Column Packing CD—The Movie	18-1165-33
The Protein Purifier—Software-based learning aid for purification strategies	18-1155-49
Data files	
Ab SpinTrap/Ab Buffer Kit	28-9020-30
Protein A HP MultiTrap/Protein A HP SpinTrap/Buffer kit	28-9067-89
Protein G HP MultiTrap/Protein G HP SpinTrap/Buffer kit	28-9067-90
HiTrap rProtein A FF/HiTrap Protein A HP/HiTrap Protein G HP	11-0035-58
MabTrap Kit	18-1034-14
HiTrap MabSelect Sure/HiTrap MabSelect Xtra/HiTrap MabSelect	11-0034-90
nProtein A Sepharose 4 Fast Flow	18-1125-19
rProtein A Sepharose Fast Flow	18-1113-94
Protein G Sepharose 4 Fast Flow	18-1012-91
HiTrap IgM Purification HP	18-1127-43
HiTrap IgY Purification HP	18-1127-42
Immunoprecipitation Starter Pack	18-1141-04
HiPrep Desalting/HiTrap Desalting	28-9137-87
PD-10 Desalting Columns/PD MidiTrap G-25/PD MiniTrap G-25, PD SpinTrap G-25, and PD MultiTrap G-25	28-9267-48

Ordering information

Product	Quantity	Code No.
Affinity chromatography		
Prepacked columns		
Protein G HP MultiTrap	4 × 96-well plates	28-9031-35
Protein G HP SpinTrap	16 columns	28-9031-34
Ab SpinTrap	50 columns	28-4083-47
HiTrap Protein G HP	5 × 1 ml 2 × 1 ml 1 × 5 ml 5 × 5 ml	17-0404-01 17-0404-03 17-0405-01 17-0405-03
MAbTrap Kit	HiTrap Protein G HP (1 × 1 ml), accessories, pre-made buffers for 10 purifications	17-1128-01
Protein A HP MultiTrap	4 × 96-well plates	28-9031-33
Protein A HP SpinTrap	16 columns	28-9031-32
HiTrap Protein A HP	5 × 1 ml 2 × 1 ml 1 × 5 ml 5 × 5 ml	17-0402-01 17-0402-03 17-0403-01 17-0403-03
HiTrap rProtein A FF	5 × 1 ml 2 × 1 ml 1 × 5 ml 5 × 5 ml	17-5079-01 17-5079-02 17-5080-01 17-5080-02
HiTrap MabSelect	5 × 1 ml 1 × 5 ml 5 × 5 ml	28-4082-53 28-5042-55 28-4082-56
HiTrap MabSelect SuRe	5 × 1 ml 1 × 5 ml 5 × 5 ml	11-0034-93 11-0034-94 11-0034-95
HiTrap MabSelect Xtra	5 × 1 ml 1 × 5 ml 5 × 5 ml	28-4082-58 28-4082-60 28-4082-61
Immunoprecipitation Starter Pack Protein A Sepharose 4 Fast Flow Protein G Sepharose 4 Fast Flow	2 × 2 ml	17-6002-35
HiTrap IgY Purification HP	1 × 5 ml	17-5111-01
HiTrap IgM Purification HP	5 × 1 ml	17-5110-01
HiTrap NHS-activated HP	5 × 1 ml 1 × 5 ml	17-0716-01 17-0717-01
HiTrap Blue HP	5 × 1 ml 1 × 5 ml	17-0412-01 17-0413-01
Companion product		
Ab Buffer Kit	10 × stock solutions of binding, buffer, elution buffer, and neutralization buffer	28-9030-59

Product	Quantity	Code No.
Lab packs of bulk media*		
Protein G Sepharose 4 Fast Flow	5 ml 25 ml	17-0618-01 17-0618-02
nProtein A Sepharose 4 Fast Flow	5 ml 25 ml	17-5280-01 17-5280-04
rProtein A Sepharose 4 Fast Flow	5 ml 25 ml	17-1279-01 17-1279-02
Protein A Sepharose CL 4B	1.5 g	17-0780-01
MabSelect	25 ml	17-5199-01
MabSelect SuRe	25 ml	17-5438-01
MabSelect Xtra	25 ml	17-5269-07
NHS-activated Sepharose Fast Flow	25 ml	17-0906-01
CNBr-activated Sepharose 4 Fast Flow	10 g	17-0981-01
Chelating Sepharose Fast Flow	50 ml	17-0575-01
Blue Sepharose 6 Fast Flow	50 ml	17-0948-01
Benzamidine Sepharose 4 Fast Flow (high sub)	25 ml	17-5123-10

Ion exchange chromatography

Prepacked columns		
HiTrap IEX Selection Kit HiTrap Q XL 1 ml HiTrap SP XL 1 ml HiTrap ANX FF (high sub) 1 ml HiTrap DEAE FF 1 ml HiTrap CM FF 1 ml HiTrap Q FF 1 ml HiTrap SP FF 1 ml	7 × 1 ml	17-6002-33
HiTrap Q HP	5 × 1 ml 5 × 5 ml	17-1153-01 17-1154-01
HiTrap SP HP	5 × 1 ml 5 × 5 ml	17-1151-01 17-1152-01
Lab packs of bulk media*		
SP Sepharose Fast Flow	25 ml	17-0729-10
Capto Q	25 ml	17-5316-10
Capto S	25 ml	17-5441-10
Capto MMC	25 ml	17-5317-10
Capto adhere	25 ml	17-5444-10

**Larger quantities available on request. Please contact GE Healthcare for more information*

Product	Quantity	Code No.
Hydrophobic interaction chromatography		
Prepacked columns		
HiTrap HIC Selection Kit HiTrap Phenyl HP HiTrap Phenyl FF (low sub) HiTrap Phenyl FF (high sub) HiTrap Butyl HP HiTrap Butyl FF HiTrap Butyl-S FF HiTrap Octyl FF	7 × 1 ml	28-4110-08
RESOURCE HIC Test Kit SOURCE 15 ETH SOURCE 15ISO SOURCE 15 PHE	3 × 1 ml	17-1187-01
HiTrap Phenyl FF (high sub)	5 × 1 ml 5 × 5 ml	17-1355-01 17-5193-01
HiTrap Phenyl FF (low sub)	5 × 1 ml 5 × 5 ml	17-1353-01 17-5194-01
HiTrap Phenyl HP	5 × 1 ml 5 × 5 ml	17-1351-01 17-5195-01
HiTrap Butyl FF	5 × 1 ml 5 × 5 ml	17-1357-01 17-5197-01
HiTrap Octyl FF	5 × 1 ml 5 × 5 ml	17-1359-01 17-5196-01
HiLoad 16/10 Phenyl Sepharose HP	1 × 20 ml	17-1085-01
HiLoad 26/10 Phenyl Sepharose HP	1 × 53 ml	17-1086-01
Lab packs of bulk media*		
Phenyl Sepharose 6 Fast Flow (high sub)	25 ml	17-0973-10
Phenyl Sepharose 6 Fast Flow (low sub)	25 ml	17-0965-10
Phenyl Sepharose High Performance	75 ml	17-1082-01
Gel filtration (desalting and buffer exchange)		
Prepacked columns		
HiTrap Desalting	5 × 5 ml	17-1408-01
Disposable PD-10 Desalting Columns	30 columns	17-0851-01
PD SpinTrap G-25	50 columns	28-9180-04
PD MultiTrap G-25	4 × 96-well plates	28-9180-06
PD MiniTrap G-25	50 columns	28-9180-07
PD MidiTrap G-25	50 columns	28-9180-08
HiPrep 26/10 Desalting	1 × 53 ml	17-5087-01
Companion products		
MiniSpin Adapter	10	28-9232-43
MidiSpin Adapter	10	28-9232-44
PD-10 Spin Adapter	10	28-9232-45
LabMate PD-10 Buffer Reservoir	10	18-3216-03
Collection plate 500 µl (V-bottom)	5 × 96 well plates	28-4039-43

**Larger quantities available on request. Please contact GE Healthcare for more information*

Product	Quantity	Code No.
Gel filtration (high resolution)		
Prepacked columns		
Superdex 200 10/300 GL	1 × 24 ml	17-5175-01
Superdex 75 10/300 GL	1 × 24 ml	17-5174-01
Superdex 200 5/150 GL	1 × 3 ml	28-9065-61
Superdex 75 5/150 GL	1 × 3 ml	28-9205-04
HiLoad 16/60 Superdex 200 pg	1 × 120 ml	17-1069-01
HiLoad 26/60 Superdex 200 pg	1 × 320 ml	17-1071-01
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 30 pg	1 × 120 ml	17-1139-01
HiLoad 26/60 Superdex 30 pg	1 × 320 ml	17-1140-01
Western blotting		
Hybond P, 20 × 20 cm	10 sheets	RPN2020F
Hybond ECL, 20 × 20 cm	10 sheets	RPN2020D
Hybond LFP, 20 × 20 cm	10 sheets	RPN2020LFP
ECL Western Blotting Detection Reagents	for 1000 cm^2	RPN2109
ECL Plus Western Blotting Detection Reagents	for 1000 cm^2	RPN2132
ECL Advance Western Blotting Detection Kit	for 1000 cm^2	RPN2135
ECL Plex Western Blotting Combination Pack (Cy™3, Cy5, Hybond ECL)	for 1000 cm^2	RPN998
ECL Plex Western Blotting Combination Pack (Cy3, Cy5, Hybond ELFP)	for 1000 cm^2	RPN999
Empty columns		
Tricorn 5/20	1	28-4064-08
Tricorn 5/50	1	28-4064-09
Tricorn 10/20	1	28-4064-13
Tricorn 10/50	1	28-4064-14
Tricorn 10/100	1	28-1065-15
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, and instructions		
XK 16/20	1	18-8773-01
XK 16/40	1	18-8774-01
XK 26/20	1	18-1000-72
XK 26/40	1	18-8768-01
XK 50/20	1	18-1000-71
XK 50/30	1	18-8751-01
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		
Empty Disposable PD-10 Desalting columns	50	17-0435-01

Product	Quantity	Code No.
Accessories and spare parts		
Tricorn 10 Coarse Filter Kit	1	11-0012-54
Tricorn Packing Connector 10-10	1	18-1153-23
Glass Tube 10/100	1	18-1153-15
Glass Tube 10/300	1	18-1153-18
Tricorn 10 Bottom Unit	1	18-1153-10
Packing Connector XK 16	1	18-1153-44
Packing Connector XK 26	1	18-1153-45

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imagination at work

18-1037-46 AD 12/2007