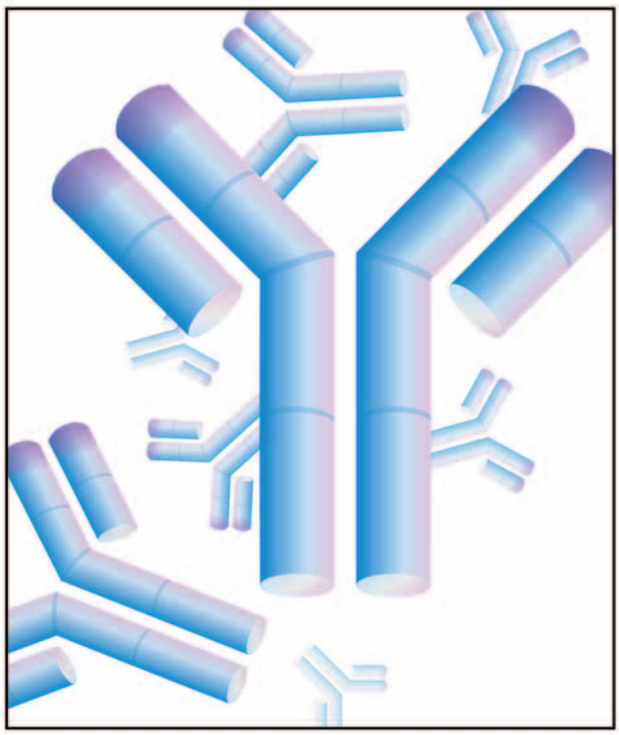
Antibody Purification

Handbook



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Handbook

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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 purification strategy, beginning with a consideration of the factors shown in Figure 1.

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.

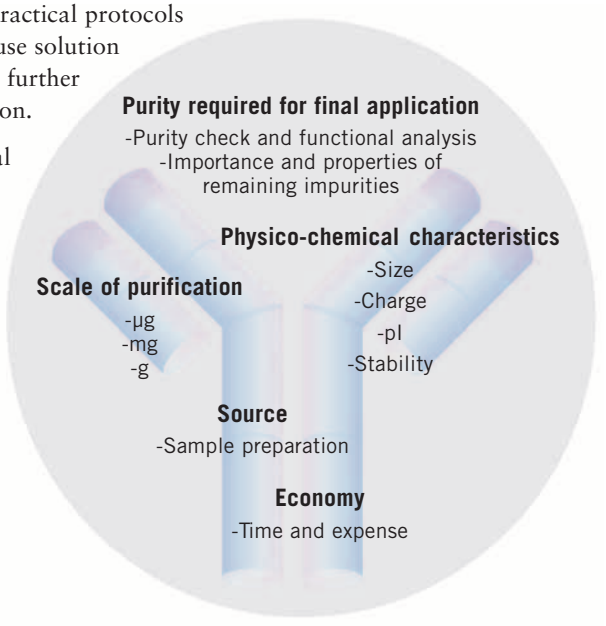


Fig. 1. Factors to consider when planning purification.

Symbols and abbreviations

this symbol indicates general advice which can improve procedures or provide recommendations for action under specific situations.

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

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

chemicals, buffers and equipment.

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, non-glycosylated light (L) chains. A disulphide bond joins a heavy chain and a light chain together. The heavy chains are also joined to each other by disulphide bonds. These disulphide 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 disulphide 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, while light chains possess a single C region. Heavy chains contain three C regions. 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 2 illustrates the basic H2L2 structure of a typical immunoglobulin.

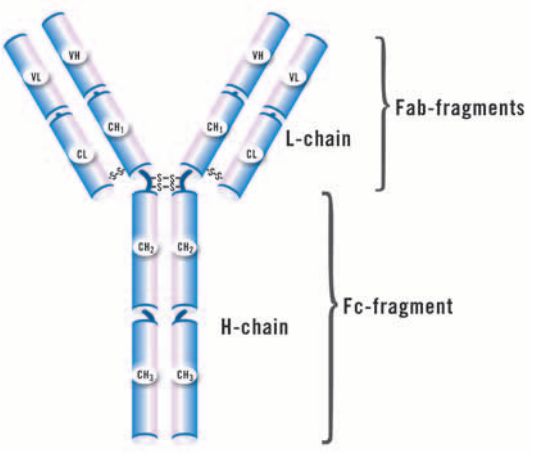


Fig. 2.

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 3 and Table 1 give a summary of human and mouse antibody classes and their physicochemical characteristics.

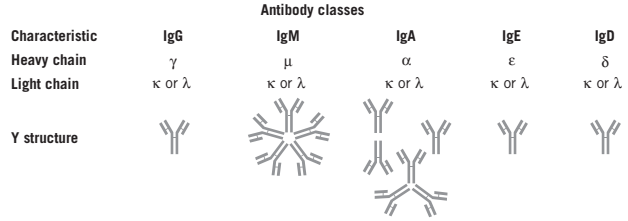


Fig. 3.

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 ten 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, 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.

Immunoglobulin	Heavy chain	Light chain	Sedimentation coefficient	Mol. Wt (M_r)	M_r heavy chain	Carbohydrate content (%)	A_{280nm}	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
IgA_3	α_1, α_2	κ, λ	11S	370 000	52–56 000	11		4.7–6.2
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 1a. Physico-chemical properties of human immunoglobulins.

Immunoglobulin	Heavy chain	Light chain	Sedimentation coefficient	Mol. Wt (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	–

Table 1b. Physico-chemical properties of mouse immunoglobulins.

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. Eggs are more easily collected than blood samples and 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 tumour therapy since they penetrate tumours more rapidly and are also cleared from the circulation more rapidly than full size antibodies. The most common types of antibody fragments are listed below. Figure 4 shows the fragments created by enzymatic cleavage.

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, VL and VH.

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.

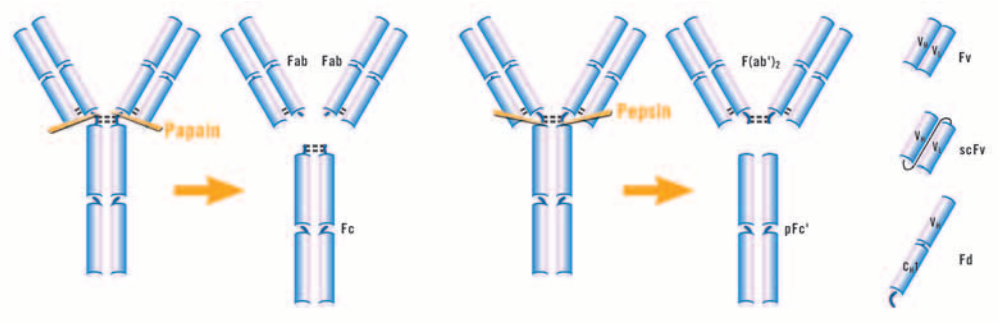


Fig. 4. Antibody fragments are created by enzymatic cleavage.

Polyclonal antibodies

Most frequently, a host will produce a large number of antibodies that recognizes 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

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 tumours in syngenic 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 non-immunogenic to humans).

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 antigen's activity. 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. Another approach to reducing immunogenicity is to produce humanized monoclonal antibodies that contain human sequences. Antibody phage libraries and breeding mice that contain parts of the human immune system provide alternative sources of therapeutic antibodies with a fully human sequence. Figure 5 illustrates various modifications to monoclonal antibodies.

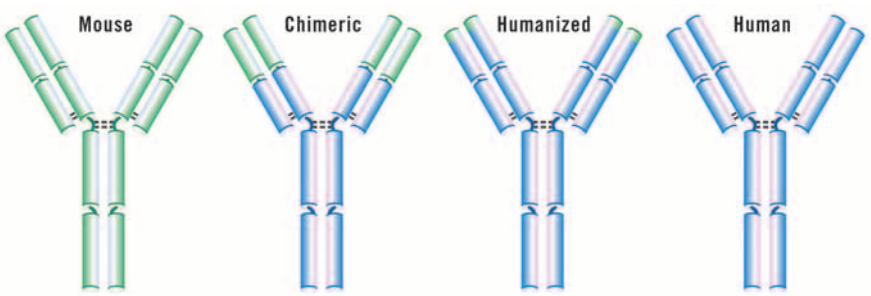


Fig. 5.

Antibody fragments

The enzymatic mechanisms used to generate antibody fragments are shown in Figure 4. Antibody fragments are also produced using recombinant technology.

Antibody fusion proteins

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 site 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 retained and a fusion partner either replaces or is linked to the Fc domain.
2. Fc fusions, also known as immunoadhesions, 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 fusion 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, (His)$_6$ or GST tags are now in common use to enable simple affinity purification at any scale. In some cases the protein yield can also be increased. Adding tags of this type is also extremely useful if the target molecule has no Fc region (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 2 reviews some of the practical advantages and disadvantages of using tagged proteins.

Advantages	Disadvantages
Cell compartments can be targeted	Tag may interfere with protein structure and affect folding and biological activity
Provide a marker for expression	Not always possible to remove the tag without modifying the sequence of interest
Simple purification using affinity chromatography under denaturing or non-denaturing conditions	
Easy detection	
Refolding achievable on a chromatography column	
Ideal for secreted proteins as the product is easily isolated from the growth medium	

Table 2.

General guidelines for the amplification and purification of recombinant proteins are covered in detail in the *Recombinant Protein Handbook: Protein Amplification and Simple Purification* and the *GST Gene Fusion System Handbook* from Amersham Biosciences.

Chapter 2
Sample preparation

Sources and their associated contaminants

Antibodies and antibody fragments are produced from native and recombinant sources. Table 3 reviews some of the most common options.

The choice of source material can affect the choice of techniques for sample preparation and purification due to the differences in specific contaminants and the quantity of target molecule required. 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.

	Molecular types	Significant contaminants	Quantity
Source: native			
Human serum	Polyclonal IgG, IgM, IgA, IgD, IgE	albumin, transferrin, α_2-macroglobulin, other serum proteins	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
Hybridoma: cell culture supernatant with 10% foetal calf serum	Monoclonal	Phenol red, water, albumin, transferrin, bovine IgG, α_2-macroglobulin, other serum proteins, viruses	Up to 1 mg/ml
Hybridoma: cell culture supernatant serum free	Monoclonal	Albumin, transferrin (often added as supplements)	Up to 0.05 mg/ml
Ascites fluid	Monoclonal	Lipids, albumin, transferrin, lipoproteins, endogenous IgG, other host proteins	1–15 mg/ml
Egg yolk	IgY	Lipids, lipoproteins and vitellin	IgY 3–4 mg/ml
Source: recombinant			
Extracellular protein expressed into supernatant	Tagged antibodies, antibody fusion proteins, Fab or F(ab')$_2$ fragments	Proteins from the host, e.g. *E. coli*. General low level of contamination	Depends upon expression system
Intracellular protein expression		Proteins from the host, e.g. *E. coli*, phage	Depends upon expression system

Table 3.

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

Extraction of recombinant antibodies and antibody fragments

The source and location of the recombinant molecule, e.g. bacterial or mammalian, inter- or intra-cellularly expressed, soluble or in the form of inclusion bodies, will determine the extraction procedure. 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 5. Buffer components should be selected to stabilize the extraction conditions. Table 4 reviews some of the substances most commonly used in buffer systems.

Use procedures that are as gentle as possible since disruption of cells or tissues leads to the release of proteolytic enzymes and general acidification.

Use additives (see Table 4) only if essential for stabilization of the product or to improve extraction. Select additives that are easily removed otherwise and additional purification step may be required.

Additives such as 8 M urea or 6 M guanidine hydrochloride can be included if solubilization of the protein is needed (e.g. if the protein is expressed as an inclusion body).

	Typical conditions for use	Purpose
Buffer components		
Tris	20 mM, pH 7.4	maintain pH minimize acidification caused by lysosomal disruption
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 non-ionic detergent		solubilize poorly soluble proteins refer to *The Recombinant Protein Handbook, Protein Amplification and Simple Purification* for details on handling inclusion bodies
DNAse and RNAse	1 µg/ml	degradation of nucleic acids, reduce viscosity of sample solution
Protease inhibitors*		**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 e.g. calcium
Reducing agents		
1, 4 dithiothreitol, DTT	1–10 mM	keep cysteine residues reduced
1, 4 dithioerythritol, DTE	1–10 mM	keep cysteine residues reduced
Mercaptoethanol	0.05%	keep cysteine residues reduced
Others		
Glycerol	5–10%	for stabilization, up to 50% can be used if required

PMSF - Phenylmethylsulphonyl fluoride.

APMSF - 4-Aminophenyl-methylsulphonyl fluoride.

PMSF is a hazardous chemical. Half-life time in aqueous solution is 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.

* Protease inhibitors are available in pre-made mixes from several suppliers.

Details taken from Protein Purification, Principles and Practice, R.K. Scopes. 1994, Springer., Protein Purification, Principles, High Resolution Methods and Applications, J-C. Janson and L. Rydén, 1998, 2nd ed. Wiley Inc. and other sources.

Table 4. Common substances used in sample buffers.

Extraction process	Typical conditions	Protein source	Comment
Gentle Cell lysis (osmotic shock)	2 volumes water to 1 volume packed pre-washed cell	*E. coli* periplasm: intracellular proteins	lower product yield but reduced protease release
Enzymatic digestion	lysozyme 0.2 mg/ml, +37 °C, 15 mins.	bacteria: intracellular proteins	lab scale only, often combined with mechanical disruption
Moderate Grinding with abrasive e.g. sand	follow equipment instructions	bacteria	
Vigorous Ultrasonication or bead milling	"	cell suspensions: intracellular proteins in cytoplasm, periplasm, inclusion bodies	small-scale, release of nucleic acids may cause viscosity problems, inclusion bodies must be resolubilized
Manton-Gaulin homogenizer	"	cell suspensions	large-scale only
French press	"	bacteria	

Table 5. Common sample extraction processes for recombinant antibodies and antibody fragments.

Extraction should be performed quickly, at sub-ambient temperatures, in the presence of a suitable buffer (see Table 4) to maintain pH and ionic strength and to stabilize the sample. If lysates are too viscous to handle (caused by a high concentration of host nucleic acid), continue to sonicate on ice for a longer period, or follow one of the following procedures:

1. Add DNase I to a final concentration of 10 µg/ml.
2. 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.
3. Draw the lysate through a syringe needle several times to avoid adding enzymes.

Clarification of serum, ascitic fluid, culture supernatant or cell lysates

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

It is highly recommended to centrifuge and filter any sample immediately before chromatographic purification.

Lipids and lipoproteins can clog chromatographic columns and should be removed prior to purification. Ascitic fluid has a particularly high lipid content. See removal of specific impurities on page 17.

Phenol red is often added to cell culture supernatants as a pH indicator. Since phenol red may bind to certain chromatographic media, it is advisable to remove it prior to purification. See removal of specific impurities on page 17.

Centrifugation

Centrifugation removes lipids and particulate matter, such as cell debris. If the sample is still not clear after centrifugation, use filter paper or a 5 µm filter for the first filtration followed by a second filtration using one of the filters described in the next section.

- For small sample volumes or proteins that adsorb non-specifically to filters, centrifuge at 10 000 x g for 15 minutes.
- For cell lysates, centrifuge at 40 000–50 000 x g for 30 minutes.

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

Filtration

Filtration removes particulate matter and is suitable for small sample volumes. Membrane filters that give the least amount of non-specific binding of proteins are composed of cellulose acetate or PVDF.

For sample preparation before chromatography select a filter pore size in relation to the bead size of the chromatographic medium.

Nominal pore size of filter	Particle size of chromatographic medium
1 µm	90 µm and upwards
0.45 µm	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 non-specifically to filter surfaces.

Buffer exchange and desalting

Desalting columns are suitable for buffer exchange of any sample volume. Many smaller contaminants are removed in a single step at the same time as transferring the sample into the correct buffer conditions. Centrifugation and/or filtration of the sample before desalting is still recommended.

- Use before and/or between purification steps.
- Rapidly process small or large sample volumes. Use before and/or between purification steps, if needed (remember that each extra step can reduce yield and desalting also dilutes the sample).
- Remove salts from proteins with molecular weight M_r > 5 000.

Detailed procedures for buffer exchange and desalting are given on page 21.

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.

At laboratory scale, when samples are reasonably clean after filtration and centrifugation, the buffer exchange and desalting step can be omitted, particularly if affinity chromatography is used for purification. It may be sufficient to adjust the pH of the sample and, if necessary, dilute to reduce the ionic strength of the solution.

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. Ascitic fluid often has 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 non-specific 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 sulphate 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 sulphate solution and 1 ml 1 M $CaCl_2$ per ml of sample.
2. Mix for 15 minutes.
3. Centrifuge (10 000 x g for 10 minutes).
4. Discard precipitate.
5. Exchange sample into a suitable buffer for purification using a desalting column (see page 21).

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

1. Add solid PVP to the sample solution to a final concentration of 3% (w/v).
2. Stir for 4 hours at +4 °C.
3. Centrifuge at 17 000 x g.
4. Discard precipitate.
5. Exchange sample into a suitable buffer for purification using a desalting column (see page 21).

Phenol red

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

Use a desalting column to simultaneously remove phenol red (a low molecular weight molecule) and transfer sample to the correct buffer conditions for further purification (see page 21).

Removal of gross impurities

Low molecular weight contaminants

If samples contain a high level of low molecular weight contaminants, use a desalting column, as already described, to prepare the sample for the first chromatographic purification.

Fractional precipitation

Fractional precipitation is frequently used at laboratory scale to remove gross impurities from small sample volumes, and occasionally used in small-scale commercial production.

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

Precipitation techniques separate fractions by the principle of differential solubility. Because protein species differ in their degree of hydrophobicity, increased salt concentrations can enhance hydrophobic interactions between the proteins and cause precipitation. Fractional precipitation can be applied to remove gross impurities in three different ways, as shown in Figure 6. These techniques are reviewed in Table 6.

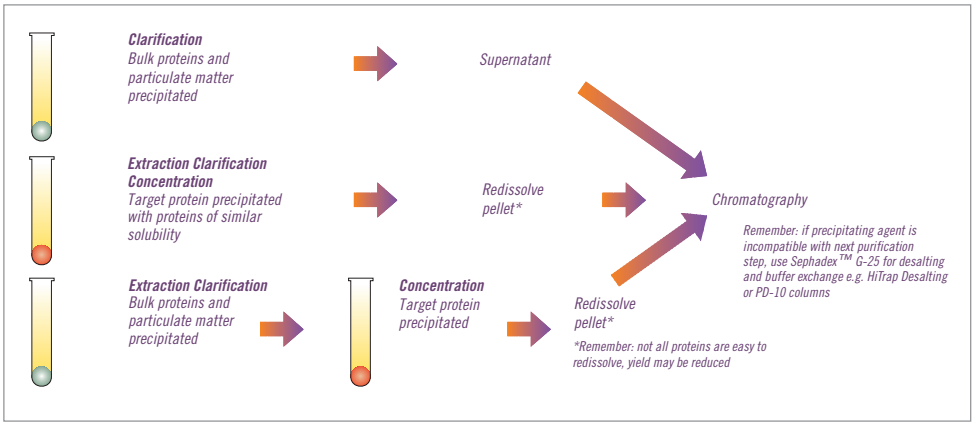


Fig. 6. Three ways to use precipitation.

Precipitation techniques are 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.

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

Precipitation agent	Comment
Ammonium sulphate	Stabilizes proteins. Helps reduce lipid content. Antibody concentration should be > 1 mg/ml. Sample can be concentrated into a pellet. Most samples retain native form. Excellent if HIC is subsequent purification step.
Caprylic acid	Sample remains in supernatant and is not concentrated. Antibody concentration should be > 1 mg/ml.
Polyethylene glycol	Stabilizes proteins. Frequently used for polyclonal antibodies and monoclonal IgM. IgMs precipitate more readily than IgGs. PEG-600 behaves as a M_r 50 000–100 000 globular protein in gel filtration and is easily separated from IgM, but difficult to remove from smaller molecules.
Ethacridine	Sample remains in supernatant and is not concentrated. Used mainly in commercial preparation. Precipitates lipids, DNA, viral particles and endotoxins. Toxic!

Table 6. Commonly used precipitation agents.

Ammonium sulphate precipitation

Ammonium sulphate is used most frequently to precipitate, and thus concentrate, immunoglobulins from a crude source.

Take care when adding crystalline ammonium sulphate: high local concentrations may cause contamination of the precipitate with unwanted proteins and some proteins may be damaged by ammonium sulphate.

For routine, reproducible purification, precipitation with ammonium sulphate should be avoided in favour of chromatography. In general, precipitation is rarely effective for protein concentrations below 1 mg/ml.

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

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

Solutions needed for precipitation:

Saturated ammonium sulphate solution (add 100 g ammonium sulphate to 100 ml distilled water, stir to dissolve).

1 M Tris-HCl, pH 8.0.

Buffer for first purification step.

1. Filter (0.45 µm) or centrifuge (10 000 × g at 4 °C) the sample.
2. Add 1 part 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently. Add saturated ammonium sulphate solution, drop by drop (solution becomes milky at about 20% saturation). Add up to 50% saturation*. Stir for 1 hour.
4. Centrifuge for 20 minutes at 10 000 x g.
5. Remove supernatant. Wash pellet twice by resuspension in an equal volume of ammonium sulphate solution of the same concentration (i.e. a solution that will not redissolve the precipitated protein or cause further precipitation). Centrifuge again, as in Step 4.

6. Dissolve pellet in a small volume of the start buffer.

7. Ammonium sulphate is removed during clarification/buffer exchange steps with Sephadex G-25, using desalting columns (see page 21), or during hydrophobic interaction purification.

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

The quantity of ammonium sulphate required to reach a given degree of saturation varies with temperature. Table 7 shows the quantities required at +20 °C.

	Final percent saturation to be obtained																
	20	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**	**65**	**70**	**75**	**80**	**85**	**90**	**95**	**100**
Starting percent saturation	**Amount of ammonium sulphate 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

Table 7. Quantities of ammonium sulphate required to reach given degrees of saturation at +20 °C.

Caprylic acid precipitation

Caprylic (octanoic) acid is as effective as ammonium sulphate and can be used to precipitate the bulk of proteins from sera and ascites. Using caprylic acid can help to avoid the formation of protein aggregates.

Unlike ammonium sulphate, 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 ascitic fluid is given here as a starting point from which other specific protocols can be developed.

1. Mix X ml ascitic fluid with 2X ml acetate buffer 50 mM, pH 4.0.
2. Adjust to pH 4.5 with 2 M HCl or NaOH.
3. Slowly add caprylic acid (X/15)g, stirring constantly.
4. Continue stirring for 30 minutes.
5. Centrifuge at 1 000 x g for 10 minutes.
6. Remove supernatant and adjust to pH 6.0 with 2 M NaOH.
7. Prepare sample for further purification as required, removing the caprylic acid using a desalting column.

Resolubilization of protein precipitates

Many proteins 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 proteins. Specific conditions will depend upon the specific protein. These agents must always be removed to allow complete refolding of the protein and to maximize recovery of mass and activity. A chromatographic step often removes a denaturant during purification. Table 8 gives examples of common denaturing agents.

Denaturing agent	Typical conditions for use	Removal/comment
Urea	2 M–8 M	Remove using Sephadex G-25.
Guanidine hydrochloride	3 M–6 M	Remove using Sephadex G-25 or during IEX.
Triton™ X-100	2%	Remove using Sephadex G-25 or during IEX.
Sarcosyl	1.5%	Remove using Sephadex G-25 or during IEX.
N-octyl glucoside	2%	Remove using Sephadex G-25 or during IEX.
Sodium dodecyl sulphate	0.1%–0.5%	Exchange for non-ionic 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 taken from:
Scopes R.K., Protein Purification, Principles and Practice, Springer, (1994), J.C. Janson and L. Rydén, Protein Purification, Principles, High Resolution Methods and Applications, 2nd ed. Wiley Inc, (1998) and other sources.

Table 8.

Buffer exchange and desalting

Dialysis is frequently mentioned in the literature as a technique to remove salt or other small molecules and to exchange buffer composition of a sample. However, dialysis is generally a slow technique, requiring large volumes of buffer. There is also a risk of losing material during handling, or because of proteolytic breakdown or non-specific binding to the dialysis membranes.

A simpler and much faster technique is to use a desalting column packed with Sephadex G-25 to performs a group separation between high and low molecular weight substances. Proteins are then separated from salts and other small molecules.

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

Desalting columns are used not only to remove low molecular weight contaminants, such as salt, but also for buffer exchange before or after different chromatographic steps and for the rapid removal of reagents to terminate a reaction.

Sample volumes up to 30% of the total volume of the desalting column can be processed. Sample concentration does not influence the separation as long as the concentration of proteins does not exceed 70 mg/ml when using normal aqueous buffers. The sample should be fully dissolved. Centrifuge or filter to remove particulate material.

For small sample volumes it may be possible to dilute the sample with the buffer that is to be used for chromatographic purification, but cell debris and particulate matter must still be removed.

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

Volatile buffers such as 100 mM ammonium acetate or 100 mM ammonium hydrogen carbonate can be used if it is necessary to avoid the presence of NaCl.

Figure 7 shows a typical buffer exchange and desalting separation. The process can be monitored by following changes in UV absorption and conductivity.

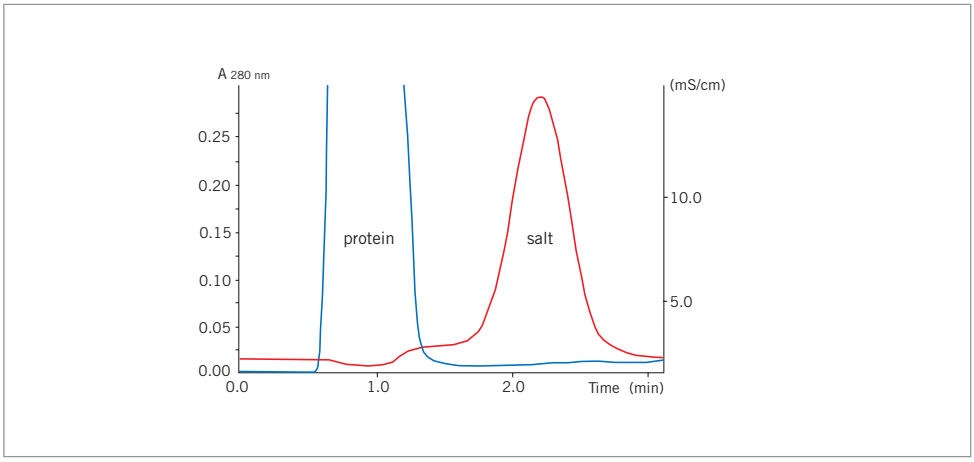


Fig 7. Buffer exchange of mouse plasma (10 ml) on HiPrep™ 26/10 Desalting.

For normal laboratory scale operations Table 9 shows a selection guide for prepacked, ready to use desalting and buffer exchange columns.

Column	Sample volume	Sample elution volume
PD-10 (gravity feed column)	1.5–2.5 ml	2.5–3.5 ml
HiTrap Desalting 5 ml	0.25–1.5 ml	1.0–2.0 ml
HiPrep 26/10 Desalting	2.5–15 ml	7.5–20 ml

Table. 9. Selection guide for desalting and buffer exchange.

To desalt larger sample volumes:

-connect up to 5 HiTrap Desalting columns 5 ml in series to increase the sample volume capacity, e.g. 2 columns: sample volume 3 ml, 5 columns: sample volume 7.5 ml.

-connect up to 4 HiPrep 26/10 Desalting columns in series to increase the sample volume capacity, e.g. 2 columns: sample volume 30 ml, 4 columns: sample volume 60 ml. Even with 4 columns in series, the sample can be processed in 20 to 30 minutes at room temperature in aqueous buffers.

Alternative 1. Manual desalting with HiTrap Desalting 5 ml using a syringe or pipette

1. Fill the syringe with buffer. Remove the stop plug. To avoid introducing air into the column, connect the column "drop to drop" to the syringe (via the adapter).
2. Remove the twist-off end.
3. Wash the column with 25 ml buffer at 5 ml/min to remove completely the 20% ethanol (supplied as storage buffer). If air is trapped in the column, wash with degassed buffer until the air disappears. Air bubbles introduced onto the column by accident during sample application do not influence the separation.
4. Apply the sample using a 2–5 ml syringe at a flow rate between 1–10 ml/min. Discard the liquid eluted from the column.
5. 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.
6. Elute the protein with the appropriate volume selected from Table 10. Collect the desalted protein in the volume indicated.

Note: 5 ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column.
Note: A simple peristaltic pump can also be used to apply sample and buffers.

A multi-dispensing pipette (Eppendorf™ model 4780 Multipipette™) can also be used. To deliver more precise volumes for sample application and elution, use the M6 threaded stopper from the HiTrap column as an adapter by piercing a hole through the bottom of the stopper. Connect the modified "stopper" to the top of the column and, by using gentle force, drive the pipette tip (Combitip with a pipette tip mounted) into the stopper.

When dispensing liquid with the Multipipette, do not exceed the maximum flow rate for the column. Take care that all liquid is dispensed for each stroke before a new stroke is delivered.

The maximum recommended sample volume is 1.5 ml. If the sample volume is less than 1.5 ml, add buffer until a total of 1.5 ml buffer is eluted. Discard the eluted liquid (see Table 10).

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	2.0	> 95	< 0.2	1.3

Table 10. Recommended sample and elution volumes using a syringe or Multipipette.

Desalting larger sample volumes using HiTrap Desalting 5 ml

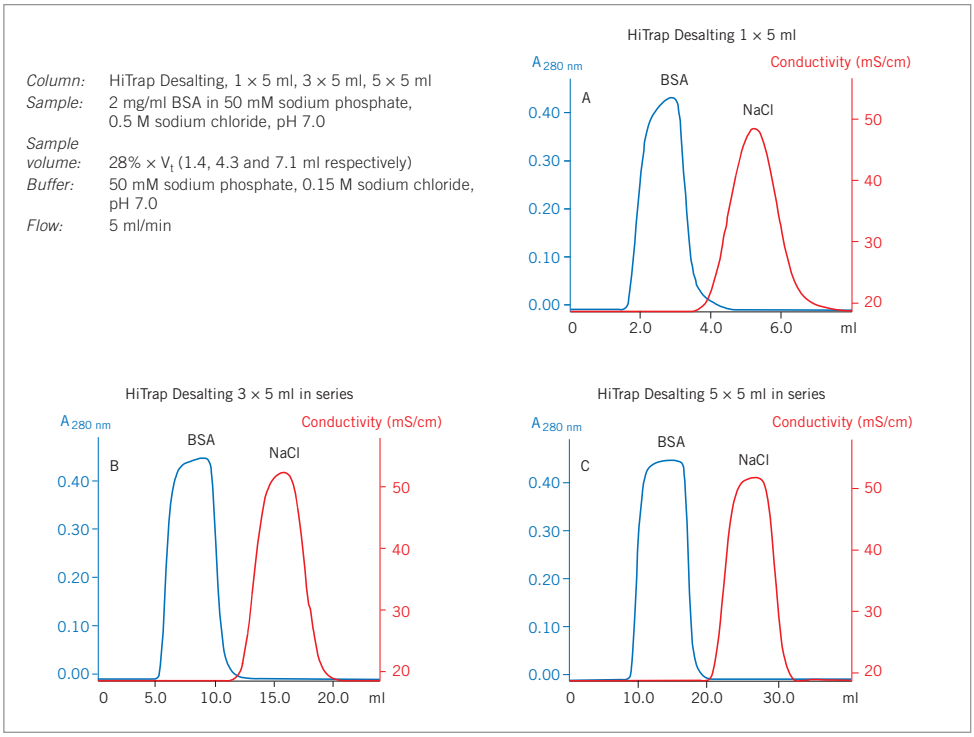


Fig. 8. Increasing sample loading capacity from 1.5 ml up to 7.5 ml.

Alternative 2. Simple desalting with ÄKTAprime

ÄKTA™prime contains pre-programmed templates for individual HiTrap Desalting 5 ml and HiPrep 26/10 Desalting columns.

Buffer Preparation

Prepare at least 500 ml of the required buffer

1. Follow the instructions supplied on the ÄKTAprime cue card to connect the column and load the system with buffer.
2. Select the Application Template.
3. Start the method.
4. Enter the sample volume and press OK.

Figure 9 shows a typical result obtained from using ÄKTAprime. The UV (protein) and conductivity (salt) traces enable pooling of the desalted fractions.

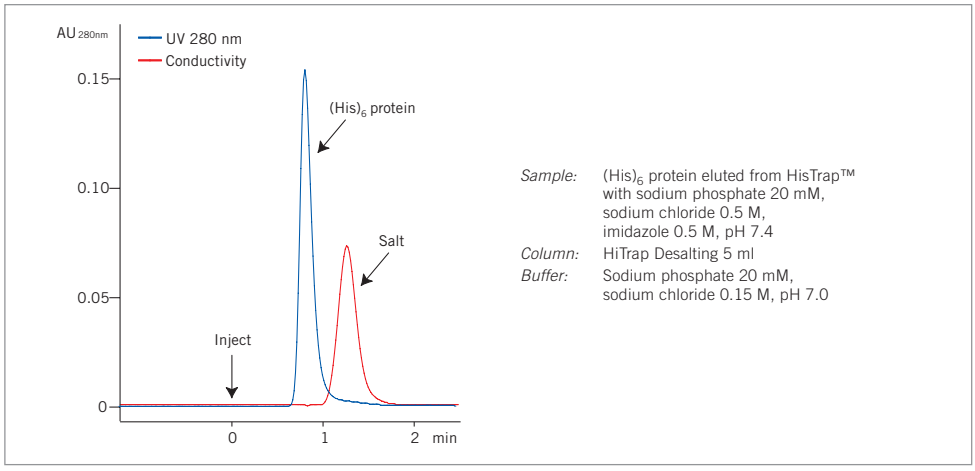


Fig. 9. Desalting of a $(His)_6$ fusion protein on ÄKTAprime.

Alternative 3. Scale up and processing large sample volumes

Connecting columns in series increases the effective column volume and so increases sample loading capacity. Table 11 shows the sample loading capacities and dilution factors when using prepacked desalting columns alone or in series. With up to four HiPrep 26/10 Desalting columns connected in series (Figure 10), flow rates can be maintained without causing back pressure difficulties to enable up to 60 ml of sample to be processed in 20–30 minutes. See also Figure 8 for HiTrap application example.

Column	Loaded volume (ml)	Eluted volume (ml)	Dilution factor	Operation
HiPrep 26/10 Desalting	10	10–15	1–1.5	pump
	15 (max)	15–20	1–1.3	pump
2 x HiPrep 26/10 Desalting	30 (max)	30–40	1–1.3	pump
3 x HiPrep 26/10 Desalting	45 (max)	45–55	1–1.2	pump
4 x HiPrep 26/10 Desalting	60 (max)	60–70	1–1.2	pump
HiTrap Desalting	0.25	1.0	4	syringe/pump
	0.5	1.5	3	syringe/pump
	1.0	2.0	2	syringe/pump
	1.5 (max)	2.0	1.3	syringe/pump
2 x HiTrap Desalting	3.0	4–5	1.3–1.7	syringe/pump
3 x HiTrap Desalting	4.5 (max)	6–7	1.3–1.7	syringe/pump
PD-10 Desalting columns	1.5	3.5	2.3	gravity
	2.0	3.5	1.7	gravity
	2.5 (max)	3.5	1.4	gravity

Table 11. Selection guide for desalting/buffer exchange columns

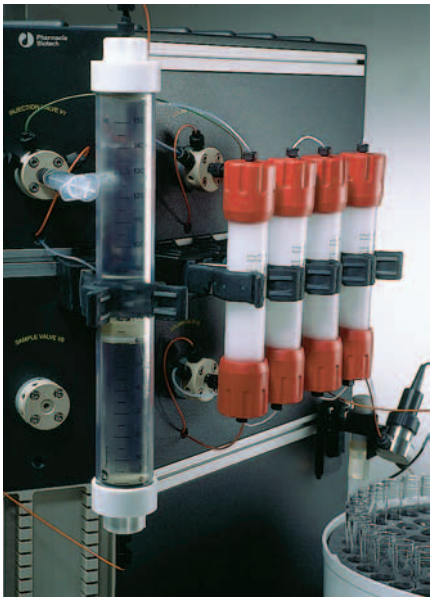


Fig. 10. Four HiPrep 26/10 Desalting columns connected in series.

Chapter 3
Simple, rapid 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 Table 3, page 13 and Table 17, page 55).

When there is an immunospecific interaction affinity chromatography is often the first, and frequently the only, step required. Affinity purification offers high selectivity, hence high resolution, and, usually, high capacity for the target protein(s). The target molecule is concentrated into a smaller volume and purity levels as high as 99% are achievable in one step. This chapter focuses specifically on the solutions available for simple, rapid affinity purification in the laboratory.

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 thus providing an effective affinity purification.

For additional information on the purification of recombinant proteins, including purification of GST and $(His)_6$ tagged proteins, please refer to *The Recombinant Protein Handbook: Protein Amplification and Simple Purification,* the *GST Gene Fusion System Handbook* and *Affinity Chromatography: Principles and Methods* from Amersham Biosciences.

Further details on the purification of protein A fusion proteins can be found in the handbook *Affinity Chromatography: Principles and Methods* from Amersham Biosciences.

Affinity chromatography isolates a specific protein or a group of proteins with similar characteristics. The technique separates proteins on the basis of a reversible interaction between the protein(s) and a specific ligand coupled to a chromatography matrix. The basic principles of affinity chromatography are outlined in Appendix 9.

It is generally recommended to follow any affinity step with a second purification technique, such as a high resolution gel filtration. For example, Superdex™ 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 gel filtration step is often used to separate dimeric and monomeric forms of the molecule, as shown in Figure 11.

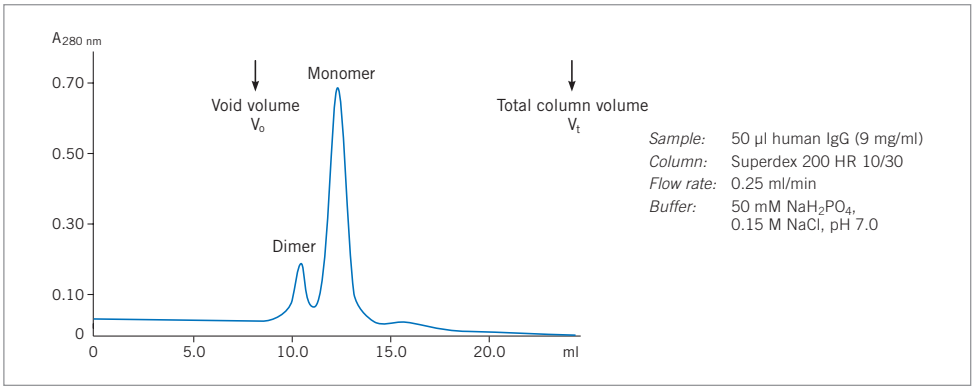


Fig. 11.

Alternatively, a desalting column that gives a low resolution separation, but has high sample capacity, can be used to transfer the sample into storage buffer and remove excess salt (see page 21).

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 page 21) or dilute in binding buffer.

Ready to use media are supplied in a range of prepacked column formats or as loose media, to suit the needs of a specific purification step (e.g. scale, resolution, speed). Table 12 presents recommendations for the most useful products for a laboratory scale purification of target molecules containing a protein A- or protein G binding region.

For laboratory scale affinity purification, a wide range of HiTrap columns is available. All columns are supplied with a detailed protocol that outlines the buffers and steps required for optimal results. Purification on a HiTrap column is used as a typical example for many of the applications described in this handbook and the buffers and procedures presented can be used for guidance when scaling up.

HiTrap Protein A HP, HiTrap rProtein A FF and HiTrap Protein G HP are designed for the isolation and purification of monoclonal and polyclonal IgG from serum, cell culture supernatants and ascites.

HiTrap columns can be used with a syringe, a peristaltic pump or a liquid chromatography system such as ÄKTAprime. General instructions for use are given in Appendix 3. To increase capacity HiTrap columns can be linked in series.

Most media are available for packing larger columns. Always check availability if the intention is to scale up. Custom-designed affinity media can be produced.

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

Step	Increasing scale →	
Capture	HiTrap Protein G HP, 1 ml or 5 ml HiTrap Protein A HP, 1 ml or 5 ml HiTrap rProtein A FF, 1 ml or 5 ml	Protein G Sepharose Fast Flow Protein A Sepharose Fast Flow rProtein A Sepharose Fast Flow MabSelect™
Polishing (select medium according to size of target molecule and contaminants)	Superdex 200 HR 10/30 Superdex 75 HR10/30	HiLoad™ 16/60 Superdex 200 pg HiLoad 26/60 Superdex 200 pg HiLoad 16/60 Superdex 75 pg HiLoad 26/60 Superdex 75 pg HiLoad 16/60 Superdex 30 pg HiLoad 26/60 Superdex 30 pg
Buffer exchange/desalting	HiTrap Desalting 5 ml	HiPrep 26/10 Desalting

Table 12.

The goal of a purification and the nature of the target molecules and contaminants may require the use of other purification techniques, such as ion exchange (IEX), hydrophobic interaction (HIC) and gel filtration (GF) chromatography. The strategy of Capture, Intermediate Purification and Polishing (CIPP) that is used to develop a multi-step purification protocol is explained more fully in Chapter 5.

IgG, IgG classes, fragments and subclasses

The high affinity of protein A and protein G for the Fc region of polyclonal and monoclonal IgG-type antibodies forms the basis for purification IgG, IgG fragments and subclasses. Protein A and protein G are bacterial proteins from *Staphylococcus aureus* and *Streptococcus*, (respectively) which, when coupled to Sepharose, create extremely useful, easy to use media for many routine applications. Examples include the purification of monoclonal IgG-type antibodies, purification of polyclonal IgG and its subclasses, and the adsorption and purification of immune complexes involving IgG. IgG subclasses can be isolated from ascites fluid, cell culture supernatants and serum.

Table 13 shows a comparison of the relative binding strengths of protein A and protein G to different immunoglobulins. Information has been compiled from various publications.

Binding strengths are tested with free protein A or protein G and can be used as guidelines to predict the binding behaviour to a protein A or protein G 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.

Species	Subclass	Protein A binding	Protein G 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	++++	++
	IgG_2	++++	++
Hamster		+	++
Horse		++	++++
Koala		–	+
Llama		–	+
Monkey (rhesus)		++++	++++
Mouse	IgG_1	+	++++
	IgG_{2a}	++++	++++
	IgG_{2b}	+++	+++
	IgG_3	++	+++
	IgM*	variable	–
Pig		+++	+++
Rabbit	no distinction	++++	+++
Rat	IgG_1	–	+
	IgG_{2a}	–	++++
	IgG_{2b}	–	++
	IgG_3	+	++
Sheep		+/–	++

** Purify using HiTrap IgM Purification HP columns.*
*** Purify using HiTrap IgY Purification HP columns.*

Table 13. Relative binding strengths of protein A or protein G.

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 as the ligand to remove host IgG or target specific antigen to avoid binding host IgG), ion exchange or hydrophobic interaction chromatography (see Chapter 6).

Both protein A and a recombinant protein A are available, with similar specificities for the Fc region of IgG. The recombinant protein A has been engineered to include a C-terminal cysteine that enables a single-point coupling to Sepharose. Single point coupling often results in an enhanced binding capacity.

Using Protein G Sepharose media

Protein G, a cell surface protein from Group G *streptococci*, is a type III Fc-receptor. Protein G binds through a non-immune mechanism. Like protein A, protein G binds specifically to the Fc region of IgG, but it binds more strongly to several polyclonal IgGs (Table 13) and to 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 weakly to protein A.

Amersham Biosciences offers 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.

Protein G Sepharose is a better choice for general purpose capture of antibodies since it binds a broader range of IgG from eukaryotic species and binds more classes of IgG. Usually protein G has a greater affinity than protein A for IgG 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, although some do bind weakly to protein A.

Leakage of ligands from an affinity medium is always a possibility, especially if harsh elution conditions are used. The multi-point attachment of protein G to Sepharose media results in very low ligand leakage over a wide range of elution conditions.

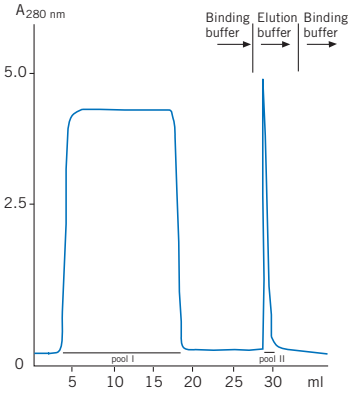
Purification options

	Binding capacity/ml medium	Comments
HiTrap Protein G HP	Human IgG > 25 mg	Purification of IgG, fragments and subclasses, including human IgG_3. Strong affinity to monoclonal mouse IgG_1 and rat IgG. Prepacked 1 ml or 5 ml columns.
Protein G Sepharose 4 Fast Flow	Human IgG > 20 mg Cow IgG 23 mg Goat IgG 19 mg Guinea pig IgG 17 mg Mouse IgG 10 mg Rat IgG 7 mg	Supplied as a suspension ready for column packing.
MAbTrap™ Kit	Human IgG > 25 mg	Purification of IgG, fragments and subclasses, including human IgG_3. Strong affinity for monoclonal mouse IgG_1 and rat IgG. Complete kit contains HiTrap Protein G HP (1 × 1 ml), accessories, pre-made buffers for 10 purifications and detailed experimental protocols.

Purification

Figure 12 shows the purification of mouse monoclonal IgG_1 on HiTrap Protein G HP 1 ml. The monoclonal was purified from a hybridoma cell culture supernatant.

Sample: 12 ml mouse IgG_1 hybridoma cell culture fluid
Column: HiTrap Protein G HP, 1 ml
Flow rate: 1.0 ml/min
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
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



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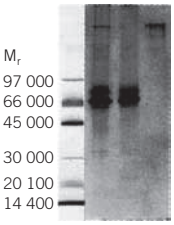


Lane 1. Low Molecular Weight Calibration Kit, reduced
Lane 2. Mouse hybridoma cell culture fluid, non-reduced, diluted 1:10
Lane 3. Pool I, unbound material, non-reduced, diluted 1:10
Lane 4. Pool II, purified mouse IgG_1, non-reduced, diluted 1:10

Immunodiffusion

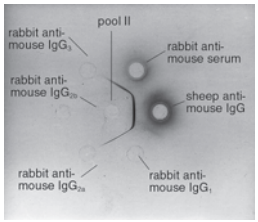


Fig. 12. Purification of monoclonal mouse IgG_1 on HiTrap Protein G HP, 1 ml.

Performing a separation

Column:	HiTrap Protein G HP, 1 ml or 5 ml.
Recommended flow rates:	1 ml/min (1 ml column) or 5 ml/min (5 ml column).
Binding buffer:	0.02 M sodium phosphate, pH 7.0.
Elution buffer:	0.1 M glycine-HCl, pH 2.7.
Neutralization buffer:	1 M Tris-HCl, pH 9.0.

Centrifuge samples (10 000 x g for 10 minutes) to remove cells and debris. Filter through a 0.45 µm filter. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see page 21) or dilution and pH adjustment.

1. Equilibrate column with 5 column volumes of binding buffer.
2. Apply sample.
3. Wash with 5–10 column volumes of the binding buffer to remove impurities and unbound material. Continue until no protein is detected in the eluent (determined by UV absorbance at 280 nm).
4. Elute with 5 column volumes of elution buffer**.
5. Immediately re-equilibrate with 5–10 column volumes of binding buffer.

**Since elution conditions are quite harsh, it is recommended to collect fractions into a neutralization buffer (60–200 µl 1 M Tris-HCl, pH 9.0 per ml fraction), so that the final pH of the fractions will be approximately neutral.

IgGs 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, it is worth consulting the most recent literature. Avoid excessive washing if the interaction between the protein and the ligand is weak, since this may decrease the yield.

Most immunoglobulin species do not elute from Protein G Sepharose until pH 2.7 or less. 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 pH may be less harsh.

Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see page 21).

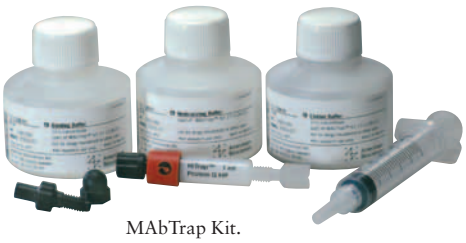
To increase capacity, connect several HiTrap Protein G HP columns (1 ml or 5 ml) in series. HiTrap columns can be used with a syringe, a peristaltic pump or connected to a liquid chromatography system, such as ÄKTAprime. For greater capacity pack a larger column with Protein G Sepharose 4 Fast Flow.

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

Storage

Wash media and columns with 20% ethanol (5 column volumes for packed media) and store at +4 to +8 °C.

Using MAbTrap Kit



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. The kit contains sufficient material for up to 20 purifications of monoclonal or polyclonal IgG from serum, cell culture supernatant or ascitic fluid, when using a syringe. The column can also be connected to a peristaltic pump if preferred. Figure 13 shows the purification of mouse monoclonal IgG_1 from cell culture supernatant with syringe operation and a similar purification with pump operation. Eluted fractions were analyzed by SDS-PAGE as shown in Figure 14.

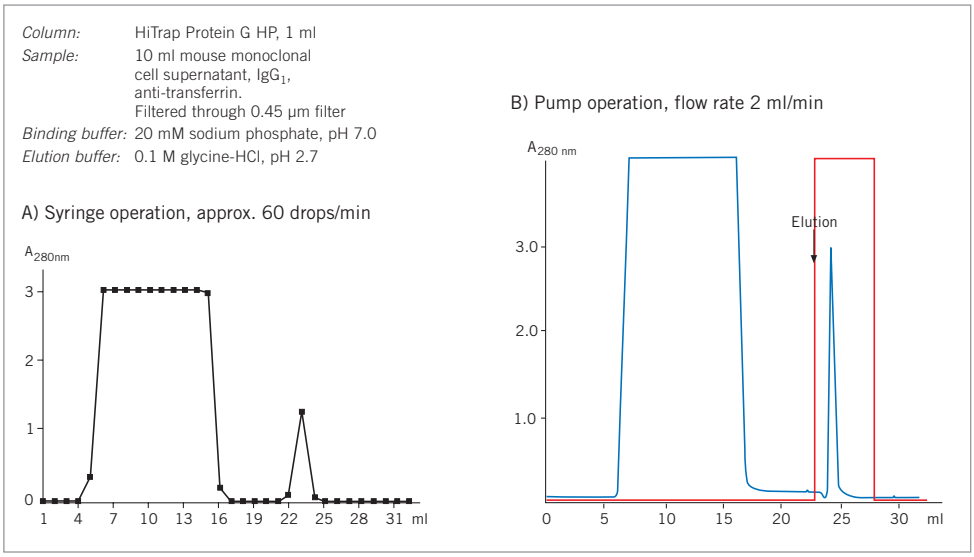


Fig. 13. Purification of mouse monoclonal IgG_1 from cell culture supernatant A. with syringe operation. B. with pump operation.

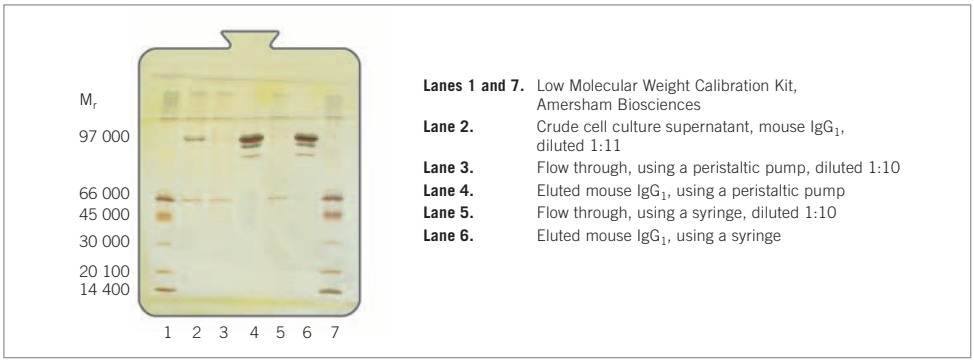


Fig. 14. SDS-PAGE on PhastSystem using PhastGel 10–15, non-reduced, and silver staining.

Performing a separation

Column:	HiTrap Protein G HP, 1 ml.
Binding buffer:	Dilute buffer concentrate 10-fold.
Elution buffer:	Dilute buffer concentrate 10-fold.
Neutralization buffer:	Add 60–200 µl of neutralization buffer per ml fraction to the test tubes in which IgG will be collected.

Centrifuge samples (10 000 x g for 10 minutes) to remove cells and debris. Filter through a 0.45 µm filter. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see page 21) or by dilution and pH adjustment.

1. Allow the HiTrap Protein G HP column and buffers to warm to room temperature.
2. Dilute the buffers.
3. Connect the syringe to the column using the luer adapter supplied.
4. Equilibrate the column with 5 ml distilled water, followed by 3 ml diluted binding buffer.
5. Apply the sample.
6. Wash with 5–10 ml diluted binding buffer until no material appears in the eluent.
7. Elute with 3–5 ml diluted elution buffer. Collect fractions into tubes containing neutralization buffer.
8. Immediately re-equilibrate the column with 5 ml diluted binding buffer.

Storage

Wash media and columns with 20% ethanol (use approximately 5 column volumes for packed media) and store at +4 to +8 °C.

Using Sepharose media coupled to native or recombinant protein A

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 and a recombinant protein A are available from Amersham Biosciences. 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. Single-point coupling often results in an enhanced binding capacity.

The binding strength of protein A for IgG depends upon the source species of the immunoglobulin. The dynamic binding capacity depends upon the binding strength as well as factors, like flow rate during sample application.

Although IgG is the major human immunoglobulin, some other types have also been demonstrated to bind with protein A (see IgA and IgM page 40).

Leakage of ligands from an affinity matrix is always a possibility, especially if harsh elution conditions are used. The multi-point attachment of protein A to Sepharose media results in very low ligand leakage over a wide range of elution conditions.

Purification options

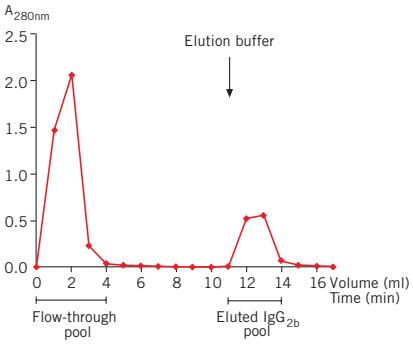
	Binding capacity/ml medium	Comments
HiTrap Protein A HP	Human IgG > 20 mg	Purification of IgG, fragments and sub-classes. Prepacked 1 ml or 5 ml columns.
Protein A Sepharose 4 Fast Flow*	Human IgG > 35 mg Mouse IgG 3–10 mg	Supplied as a suspension ready for column packing.
HiTrap rProtein A FF	Human IgG > 50 mg	Purification of IgG, fragments and sub-classes. Enhanced binding capacity. Prepacked 1 ml or 5 ml columns.
rProtein A Sepharose 4 Fast Flow*	Human IgG > 50 mg Mouse IgG 8–20 mg	Enhanced binding capacity. Supplied as a suspension ready for column packing.
Protein A Sepharose 6MB	Human IgG > 5 mg	For purification of cells coated with antibodies.
MabSelect (recombinant protein A ligand)	Human IgG, approx. 30 mg	Purification of IgG, IgG fragments and sub-classes. Fast processing of large sample volumes. Retains high binding capacity at high flow rates. Supplied as a suspension ready for column packing.

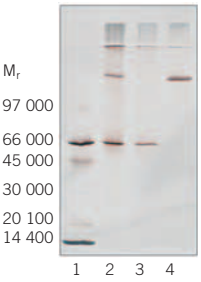
**Protein A Sepharose 4 Fast Flow and rProtein A Sepharose Fast Flow have a higher binding capacity, a more rigid matrix and provide more convenient alternatives to Protein A Sepharose CL-4B which must be rehydrated prior to column packing.*

Purification

Figure 15 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 IgG_{2b} and the silver stained SDS-PAGE gel confirmed a purity level of over 95%.

Sample: 1 ml of 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
Column: HiTrap rProtein A FF 1 ml
Binding buffer: 0.02 M sodium phosphate, pH 7.0
Elution buffer: 0.1 M sodium citrate, pH 3.0
Flow rate: ~ 1 ml/min





SDS-PAGE on PhastSystem using PhastGel Gradient 10–15, silver staining
Lane 1. Low Molecular Weight Calibration Kit, Amersham Biosciences
Lane 2. Starting material, diluted 10-fold
Lane 3. Flow-through pool
Lane 4. Eluted IgG_{2b} pool

Fig. 15. Purification of mouse IgG_{2b} from ascites.

Performing a separation

Column:	HiTrap Protein A HP, 1 ml or 5 ml, or HiTrap rProtein A FF, 1 ml or 5 ml.
Recommended flow rates:	1 ml/min (1 ml columns) or 5 ml/min (5 ml columns).
Binding buffer:	0.02 M sodium phosphate, pH 7.0.
Elution buffer:	0.1 M citric acid, pH 3–6.
Neutralization buffer:	1 M Tris-HCl, pH 9.0.

Centrifuge samples (10 000 x g for 10 minutes) to remove cells and debris. Filter through a 0.45 µm filter. If needed, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see page 21) or by dilution and pH adjustment.

1. Equilibrate the column with 5 column volumes of binding buffer.
2. Apply sample.
3. Wash with 5–10 column volumes of the binding buffer to remove impurities and unbound material. Continue until no protein is detected in the eluent (determined by UV absorbance at 280 nm).
4. Elute with 5 column volumes of elution buffer**.
5. Re-equilibrate with 5–10 column volumes of binding buffer.

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

Table 14 gives examples of some typical binding and elution conditions that have been used with Protein A Sepharose.

Species	Subclass	Protein A binding	Protein A binding condition	Protein A elution pH
				Usually elutes by pH 3
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	use step elution
Cow	IgG_2	+ +		2
Goat	IgG_2	+		5.8
Guinea pig	IgG_1	+ +		4.8
	IgG_2	+ +		4.3
Mouse	IgG_1	+	8.0–9.0	5.5–7.5
	IgG_{2a}	+	7.0–8.0	4.5–5.5
	IgG_{2b}	+	7	3.5–4.5
	IgG_3	+	7	4.0–7.0
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	3–4 (using thiocyanate)

Table 14.

Binding strengths are tested with free protein A and can be used as guidelines to predict the binding behaviour 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, such as mouse IgG_1, it might be necessary to add sodium chloride up to a concentration of 3 M in the binding buffer to achieve efficient binding when using protein A. Use for example 1.5 M glycine, 3 M NaCl, pH 8.9.

IgGs from most species 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 the 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 or 0.1 M glycine-HCl, pH 3.0 or 3 M potassium isothiocyanate.

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 page 21).

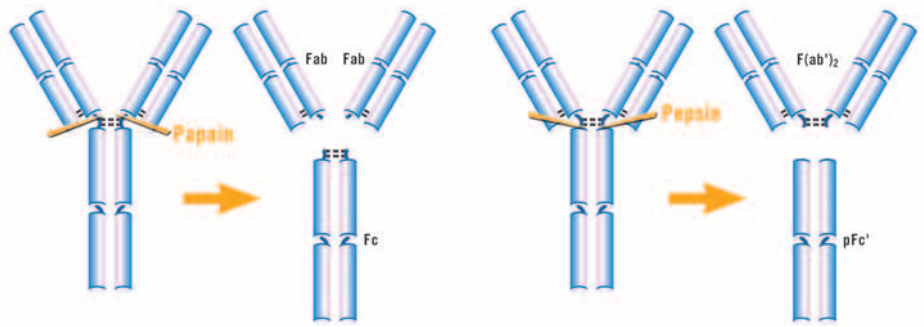
To increase capacity, connect several HiTrap Protein A HP or HiTrap rProtein A FF columns (1 ml or 5 ml) in series. Alternatively pack a larger column with Protein A Sepharose 4 Fast Flow or rProtein A Sepharose 4 Fast Flow (see Appendix 4). When working with large scale fermentation, consider using MabSelect. 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.

Storage

Wash media and columns with 20% ethanol (5 column volumes for packed media) and store at +4 to +8 °C.

Fab, F(ab')$_2$ fragments



Protein G has a low affinity site for the Fab region (binding to C-$_H$1 domains of heavy chains bound to C_κ light chains). Consequently, Protein G affinity purification can sometimes be used for the purification of Fab and F(ab')$_2$ fragments. Figure 16 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.

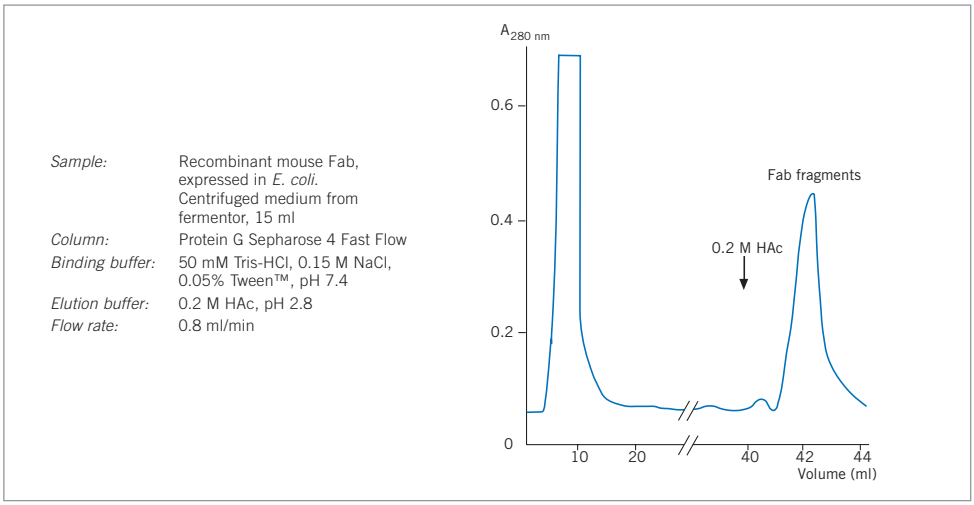


Fig. 16. Purification of recombinant mouse Fab fragments, expressed in *E. coli* using Protein G Sepharose 4 Fast Flow.

Performing a separation

Column:	Protein G Sepharose Fast Flow.
Recommended flow rate:	0.8 ml/min.
Binding buffer:	50 mM Tris-HCl, 0.15M NaCl, 0.05% Tween, pH 7.4.
Elution buffer:	0.2 M HAc, pH 2.8.

Centrifuge samples (10 000 x g for 10 minutes) to remove cells and debris. Filter through a 0.45 µm filter. If needed, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see page 21) or dilution and pH adjustment.

1. Equilibrate column with 2–3 column volumes of binding buffer.
2. Apply sample.
3. Wash with 5–10 column volumes of the binding buffer to remove impurities and unbound material. Continue until no protein is detected in the eluent (determined by UV absorbance at 280 nm).
4. Elute with 1–3 column volumes of elution buffer**.
5. Re-equilibrate column with 5–10 column volumes of binding buffer.

** Since elution conditions are quite harsh, it is recommended to collect fractions into a neutralization solution (60–200 µl 1 M Tris-HCl, pH 9.0 per ml fraction) so that the final pH of the fractions will be approximately neutral.

Although protein A has no affinity for Fab regions, protein A affinity purification can sometimes be used to separate whole or partially digested IgG from $F(ab')_2$ after proteolytic cleavage.

Protein A does have an affinity for the variable region in the human heavy chain subgroup III so that $F(ab')_2$ derived from this group can occasionally be purified with protein A affinity chromatography, although protein G is preferred.

Alternatively, produce the Fab or F(ab')$_2$ fragment with a (His)$_6$ or GST tag and purify using HisTrap Kit, HiTrap Chelating HP, GSTrap™ FF or GSTPrep™ FF 16/10. For detailed protocols please refer to *The Recombinant Protein Handbook: Protein Amplification and Simple Purification* and the *GST Gene Fusion System Handbook* from Amersham Biosciences.

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, high selectivity affinity purification medium (see page 45). Alternatively, a multi-step purification strategy could be employed (see Chapter 5).

IgD

Protein A and protein G 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, high selectivity affinity purification medium (see page 45). Alternatively, a multi-step purification strategy could be employed (see Chapter 5).

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 A and protein G do not bind to IgE (see Table 13, page 30).

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, high selectivity affinity purification medium (see page 45).

Alternatively, and for any initial purification, a multi-step purification strategy should be employed (see Chapter 5). Use ion exchange chromatography or hydrophobic interaction in the first step as this will purify and concentrate the IgE.

IgM

The technique described in this section 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 other IgM preparations.

Purification options

	Binding capacity/ml medium	Comments
HiTrap IgM Purification HP	Human IgM – 5 mg	Purification of monoclonal and human IgM. Prepacked 1 ml column.

HiTrap IgM Purification HP columns are packed 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 hydrophilic-hydrophobic interaction.

Purification

Figures 17 and 18 show results from the purification of monoclonal α-Shigella IgM from hybridoma cell culture supernatant. SDS-PAGE analysis demonstrated a purity level of over 80%. Results from an ELISA (not shown) indicated high activity in the purified fraction.

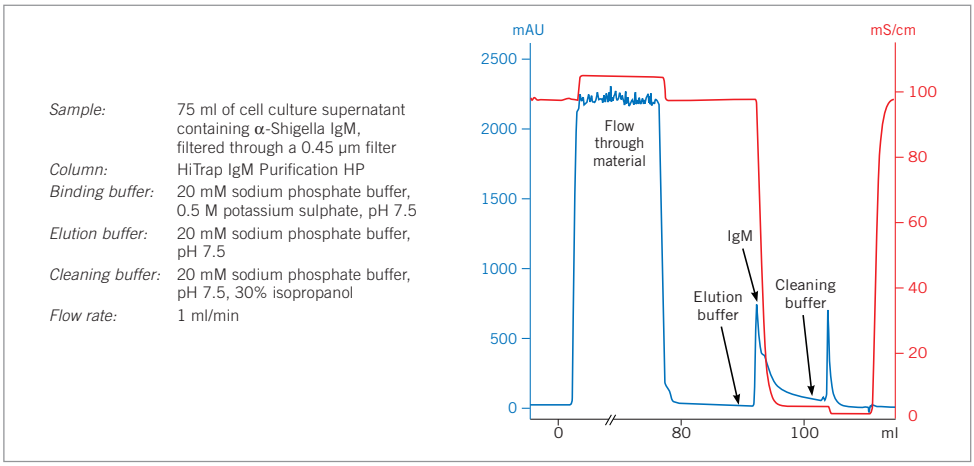


Fig. 17. Purification of α-Shigella IgM on HiTrap IgM Purification HP.

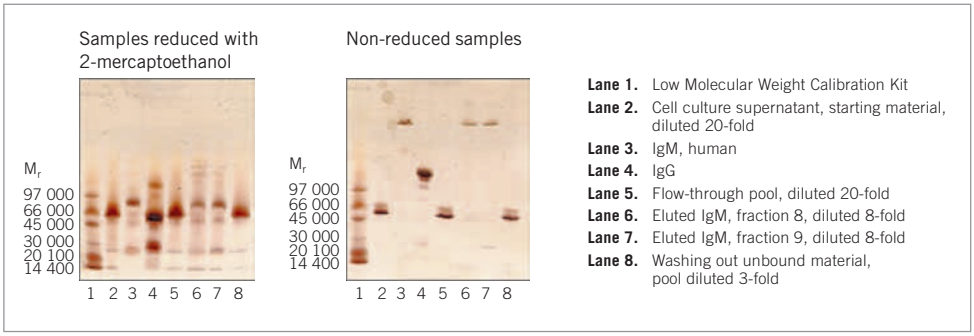


Fig. 18. SDS-PAGE on PhastSystem, using PhastGel 4–15, silver staining.

Performing a separation

Column:	HiTrap IgM Purification HP.
Recommended flow rate:	1 ml/min.
Binding buffer:	20 mM sodium phosphate, 0.8 M $(NH_4)_2SO_4$, 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 sulphate as the binding buffer. Slowly add small amounts of solid ammonium sulphate 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 might not bind to the column at 0.8 M ammonium sulphate. Binding can be improved by increasing the ammonium sulphate concentration to 1.0 M.

To avoid precipitation of IgM, it is important to add the ammonium sulphate slowly. An increased concentration of ammonium sulphate 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.

Purification

1. Wash column with 5 column volumes of each buffer.
2. Equilibrate column with 5 column volumes of binding buffer.
3. Apply the sample.
4. Wash out unbound sample with 15 column volumes of binding buffer or until no material appears in the eluent (monitored at A_{280}).
5. Elute the IgM with 12 column volumes of elution buffer.
6. Wash the column with 7 column volumes of cleaning buffer.
7. Re-equilibrate the column with 5 column volumes of binding buffer.

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

Some monoclonal IgM may bind too tightly to the column for complete elution. The remaining IgM will be eluted during cleaning, but the high content of isopropanol will cause precipitation of IgM. Perform an immediate buffer exchange (see page 21) 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 lgM 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, such as ÄKTAprime.

Storage

Wash the column with 5 column volumes 20% ethanol and store at +4 to +8 °C.

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

Avian IgY from egg yolk

Purification option

	Binding capacity	Comments
HiTrap IgY Purification HP	100 mg pure IgY per 5 ml column	Purification of IgY from egg yolk. Prepacked 5 ml column.

HiTrap IgY Purification HP columns are packed 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.

Purification

Figures 19 and 20 show 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 indicating a purity of over 70%.

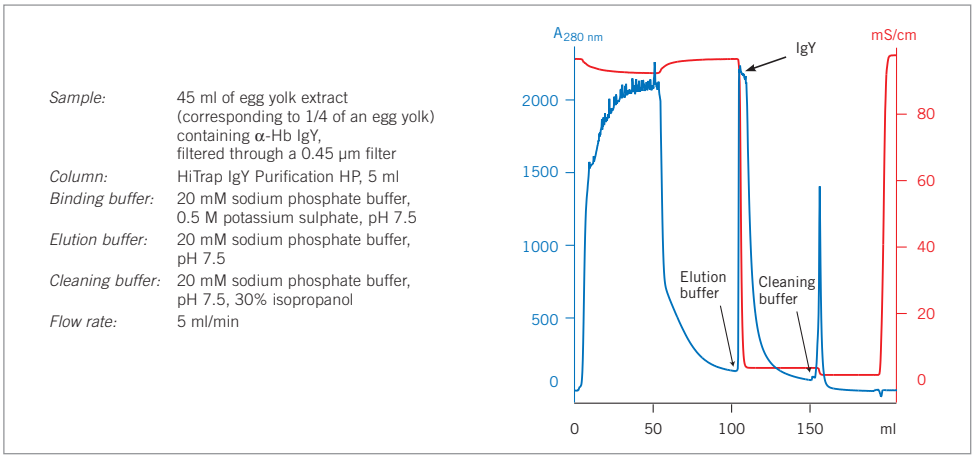


Fig. 19. Purification of IgY on HiTrap IgY Purification HP.

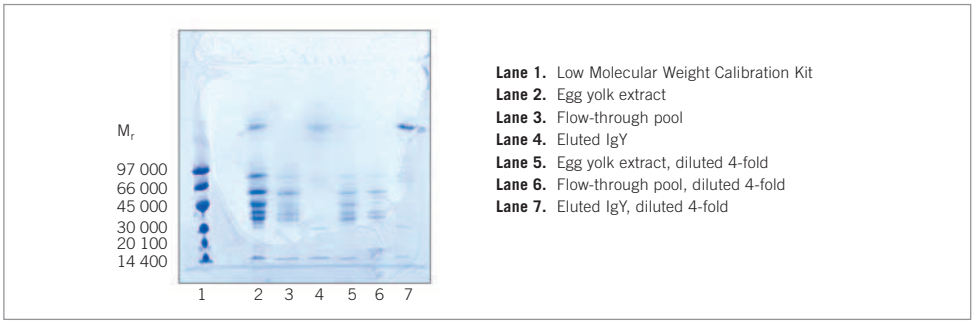


Fig. 20. SDS-PAGE of non-reduced samples on PhastSystem, using PhastGel 4–15%, Coomassie™ staining.

Performing a separation

Column:	HiTrap IgY Purification HP.
Recommended flow rate:	5 ml/min.
Binding buffer:	20 mM sodium phosphate, 0.5 M K_2SO_4, pH 7.5.
Elution buffer:	20 mM sodium phosphate, pH 7.5.
Wash buffer:	20 mM sodium phosphate, pH 7.5 with 30% isopropanol.

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.

Precipitation of the egg yolk lipid using water

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 hours at +4 °C.
4. Centrifuge at 10 000 x g, at +4 °C for 25 minutes to precipitate the lipids.
5. Collect the supernatant containing the IgY.
6. Slowly add K_2SO_4 to the sample, stirring constantly, to reach a 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

1. Wash the column with at least 5 column volumes of binding, elution and wash buffer.
2. Equilibrate with 5 column volumes of binding buffer.
3. Apply the sample.
4. Wash with at least 10 column volumes of binding buffer or until no material appears in the eluent, as monitored at A_{280}.
5. Elute the IgY with 10 column volumes of elution buffer.
6. Wash the column with 8 column volumes of cleaning buffer.
7. Re-equilibrate the column with 5 column volumes of binding buffer.

To improve recovery of total IgY or a specific IgY antibody, replace 0.5 M K_2SO_4 with 0.6–0.8 M Na_2SO_4 in the binding buffer. The sample should have the same concentration of Na_2SO_4 as the binding buffer. Using more than the recommended salt concentration in the binding buffer will reduce the purity of the eluted IgY.

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

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

Wash the column with 5 column volumes 20% ethanol and store at +4 to +8 °C.

Making immunospecific purification media

If an affinity medium is not available, a ligand (such as a pure antigen or an anti-antibody) can be 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 handbook describes the simplest coupling method i.e. when a ligand is coupled via its primary amine group to a pre-activated medium, NHS-activated Sepharose. The immunospecific medium created is used for the initial capture step and most frequently followed by a gel filtration polishing step (for more details see Chapter 5 Mutli-step purification strategies). Table 15 presents recommendations for the products that would be most useful for laboratory scale purification.

Affinity chromatography is used to achieve a high resolution purification in the first capture step. Gel filtration is used to separate dimers from monomers and remove any leached ligand in the polishing step.

Step	Increasing scale	
Capture	Specific ligand bound to HiTrap NHS-activated HP, 1 ml or 5 ml	Specific ligand bound to NHS-activated Sepharose Fast Flow
Polishing (select medium according to size of target molecule and contaminants)	Superdex 200 10/300 GL (Tricorn™) Superdex 75 10/300 GL (Tricorn)	HiLoad 16/60 Superdex 200 pg HiLoad 26/60 Superdex 200 pg HiLoad 16/60 Superdex 75 pg HiLoad 26/60 Superdex 75 pg HiLoad 16/60 Superdex 30 pg HiLoad 26/60 Superdex 30 pg
Buffer exchange/desalting	HiTrap Desalting	HiPrep 26/10 Desalting

Table 15.

If there is no primary amine available (e.g. this group may be required for the specific interaction), then pre-activated media for ligand attachment via carboxyl, thiol or hydroxyl groups can be considered. These are described in the handbook, *Affinity Chromatography: Principles and Methods* from Amersham Biosciences. Pre-activated media are supplied in different prepacked column formats, in suspension or as dry medium.

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. Too low or too high affinity will result in poor yields after purification. The target protein may wash through or leak from the column, or the target molecule may not dissociate from the ligand during elution.

Immunospecific interactions often require harsh elution conditions. It is recommended to collect fractions into a neutralization buffer, such as 60–200 µl 1 M Tris-HCl, pH 9.0 per ml fraction.

Purification examples

Figure 21 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.

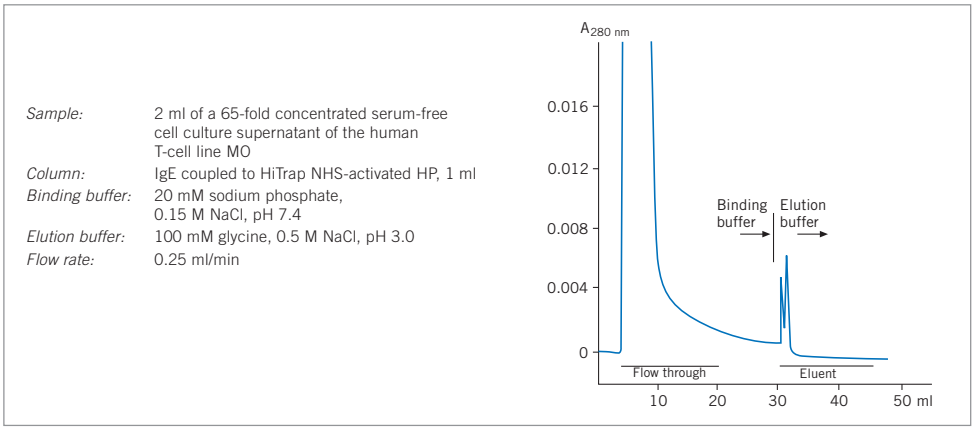


Fig. 21. Purification of an IgE-stimulating factor from a human T-cell line.

Figure 22 shows an example of the purification of anti-mouse Fc-IgG from sheep serum using mouse IgG_1 coupled to HiTrap NHS-activated HP 1 ml column.

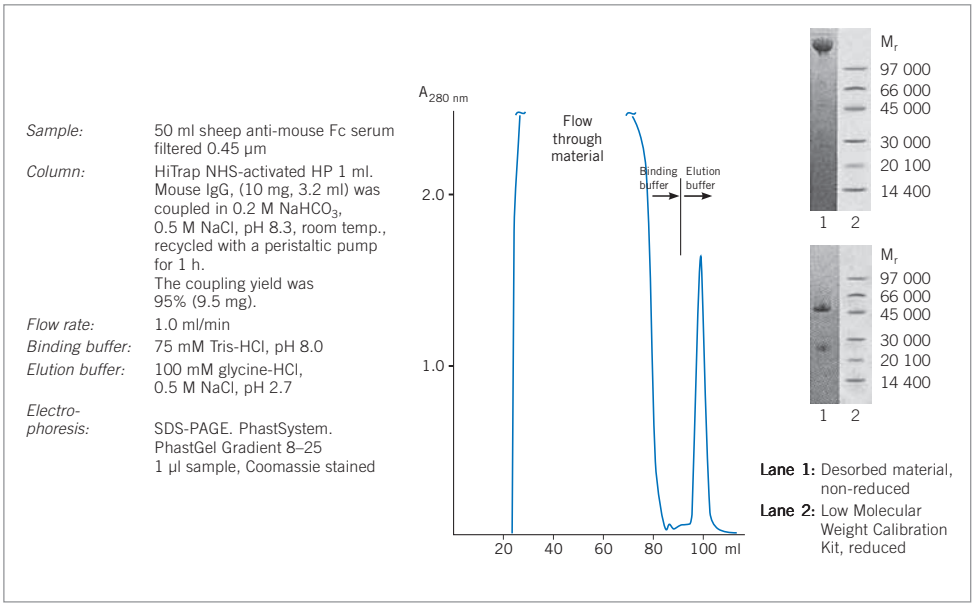


Fig. 22. Purification of anti-mouse Fc-IgG from sheep antiserum.

Preparing NHS-activated media

Product	Comments
HiTrap NHS-activated HP	Pre-activated medium for coupling via primary amine group of a ligand. Prepacked 1 ml and 5 ml columns.
NHS-activated Sepharose 4 Fast Flow	Supplied as a suspension ready for column packing.

NHS-activated Sepharose media are chromatographic matrices specifically designed to allow the covalent coupling of ligands (often antigens or antibodies) containing primary amino groups (the most common form of attachment). The excellent hydrophilic properties of the base matrix minimize non-specific adsorption of proteins that can reduce the binding capacity of the target protein. Fifteen atoms spacer arms make the matrix suitable for the coupling of smaller molecules. The pH range for coupling is well suited to the stability characteristics of many immunoglobulins. The media are stable at high pH to allow stringent washing procedures (subject to the pH stability of the coupled ligand).

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. A general column packing procedure is described in Appendix 4. Coupling can take place within the pH range 6.5–9 with a maximum yield achieved at around pH 8.

Buffer Preparation

Acidification solution: 1 mM HCl (kept on ice).

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

Blocking buffer: 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3

Wash buffer: 0.1 M acetate, 0.5 M NaCl, pH 4.0

Use high quality water and chemicals. Filtration through 0.45 µm filters is recommended.

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 ligand in the coupling buffer to a final concentration of 0.5–10 mg/ml (for protein ligands) or perform a buffer exchange using a desalting column (see page 21). The optimal concentration depends on the ligand. Dissolve the ligand in one column volume of buffer.
2. Remove the top cap and apply a drop of ice cold 1 mM HCl to the top of the column to avoid air bubbles.
3. Connect the top of the column to the syringe or pump.
4. Remove the twist-off end.

Ligand coupling

1. Wash out the isopropanol with 3 x 2 column volumes of ice-cold 1 mM HCl.
2. Inject one column volume of ligand solution onto the column.
3. Seal the column and leave for 15–30 minutes at +25 °C (or 4 hours at +4 °C).

Do not use excessive flow rates (maximum recommended flow rates are 1 ml/min, equivalent to approximately 30 drops/min when using a syringe, with HiTrap 1 ml and 5 ml/min, equivalent to approximately 120 drops/min when using a syringe, with HiTrap 5 ml). The column contents can be irreversibly compressed.

Re-circulate the solution if larger volumes of ligand solution are used. For example, when using a syringe, connect a second syringe to the outlet of the column and gently pump the solution back and forth for 15–30 minutes or, if using a peristaltic pump, simply re-circulate the sample through the column.

If required, the coupling efficiency can be measured after this step. Procedures are supplied with each HiTrap NHS-activated HP column.

Washing and deactivation

This procedure deactivates any excess active groups that have not coupled to the ligand and washes out non-specifically bound ligand.

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

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

1. Inject 3 × 2 column volumes of blocking buffer.
2. Inject 3 × 2 column volumes of wash buffer.
3. Inject 3 × 2 column volumes of blocking buffer.
4. Let the column stand for 15–30 min.
5. Inject 3 × 2 column volumes of wash buffer.
6. Inject 3 × 2 column volumes of blocking buffer.
7. Inject 3 × 2 column volumes of wash buffer.
8. Inject 2–5 column volumes of a buffer with neutral pH.

The column is now ready for use.

A HiTrap column can be used with a syringe, a peristaltic pump or connected to a liquid chromatography system, such as ÄKTAprime.

Storage

Store the column in a solution that maintains the stability of the ligand and contains a bacteriostatic agent, for example phosphate-buffered saline, 0.05% NaN_3, pH 7.2.

pH stability of the media when coupled to a chosen ligand will depend upon the stability of the ligand. Sodium azide can interfere with many coupling methods and some biological assays. It can be removed by using a desalting column.

Performing a separation on coupled NHS-activated media

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 elution buffer suggestions). Literature references and textbooks may give good guidelines. The general protocol given here can be used as a guideline for a preliminary separation.

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 page 21) or dilute the sample in binding buffer.

1. Prepare the column (blank run).
   a. Wash with 2 column volumes of binding buffer.
   b. Wash with 3 column volumes of elution buffer.
2. Equilibrate with 10 column volumes of binding buffer.
3. Apply sample. The optimal flow rate depends on the binding constant of the ligand, but a recommended flow rate range is, for example, 0.5–1 ml/min on a HiTrap NHS-activated HP 1 ml column.
4. Wash with 5–10 column volumes of binding buffer, or until no material appears in the eluent as monitored by absorption at A_{280}.
5. Elute with 1–3 column volumes of elution buffer (larger volumes may be necessary).
6. If required, purified fractions can be desalted and transferred into the buffer of choice using prepacked desalting columns (see page 21).
7. Re-equilibrate the column immediately by washing with 5–10 column volumes of binding buffer.

Avoid excessive washing if the interaction between the protein of interest and the ligand is weak, since this may decrease the yield.

If elution conditions are quite harsh, collect fractions into a neutralization buffer (60–200 µl 1 M Tris-HCl, pH 9.0 per ml fraction), so that the final pH of the fractions 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%).

NHS-activated Sepharose is the first choice for the preparation of immunospecific media. CNBr-activated Sepharose media offer a well-established option for the attachment of larger ligands and can be an alternative to NHS-activated Sepharose.

Chapter 4
Immunoprecipitation

Immunoprecipitation is a highly specific technique for the analysis of target antigens from crude cell lysates. In combination with other techniques, such as SDS-PAGE and immunoblotting, immunoprecipitation can be used to detect and quantify 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 from a wide range of mammalian species, Protein A Sepharose 4 Fast Flow and Protein G Sepharose 4 Fast Flow media offer effective and rapid removal of immune complexes formed between an antigen and its specific antibody in the immunoprecipitation reaction.

The Immunoprecipitation Starter Pack from Amersham Biosciences is ideal to begin working with immunoprecipitation. The pack includes Protein 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.

When using immunoprecipitation, procedures must often be optimized empirically to obtain satisfactory results. For example, determining cell lysis conditions is critical with regard to cell type and how the antigen is to be used and, 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.

The protocol presented here describes a generic step-by-step method for immunoprecipitation with Protein A Sepharose Fast Flow and Protein G Sepharose Fast Flow.

Refer to Table 13 on page 30 to see which medium is likely to be suitable for the antibody source and sub-type, or test using the Immunoprecipitation Starter Pack.

Immunoprecipitation protocol

Preparation of Protein A Sepharose 4 Fast Flow or Protein G Sepharose 4 Fast Flow

1. Wash the medium three times with lysis buffer to remove 20% ethanol.
2. Between the washes centrifuge at 12 000 x g for 20 seconds and discard the supernatant.
3. Mix equal volumes of medium and lysis buffer to prepare a 50% slurry.
4. Store at +4 °C and mix well before use.

Cell lysis

Adherent cells:
Step 1. Remove all culture medium and wash twice with ice-cold PBS. Discard the supernatants and drain well.

Step 2. Place the tissue culture dish on ice. Add ice-cold lysis buffer to a concentration of 10^6–10^7 cells/ml (1 ml to a cell culture plate, Ø 10 cm). Incubate on ice for 10–15 minutes with occasional agitation.

Cells in suspension:
Step 1. Collect cells by centrifugation at 1 000 x g for 5 minutes and discard the culture medium supernatant. Resuspend the pellet in ice-cold PBS, centrifuge and discard the supernatant. Repeat the wash.

Step 2. Suspend the washed pellet in ice-cold lysis buffer at a concentration of 10^6–10^7 cells/ml (approximately 10 cell volumes lysis buffer). Incubate on ice for 10–15 minutes, mixing gently.

3) Transfer the cells to a homogenization tube.
4) Disrupt the cells by sonication, Dounce homogenization or passage through a 21 Gauge needle. Keep the cells on ice to prevent an increase in temperature.
5) Centrifuge at 12 000 x g for 10 minutes at +4 °C to remove particulate matter.
6) Transfer the lysate (the supernatant) to a fresh tube. Keep on ice.

Pre-clearing (optional)

1) Add 50–100 µl of prepared Protein A Sepharose 4 Fast Flow or Protein G Sepharose 4 Fast Flow suspension (50% slurry) to 1 ml cell lysate in an Eppendorf tube.
2) Gently mix for 1 hour at +4 °C.
3) Centrifuge at 12 000 x g for 20 seconds. Save the supernatant.

Couple antigen to antibody

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

Precipitation of the immune complexes

1) Add 50 µl of prepared Protein A Sepharose 4 Fast Flow or Protein G Sepharose 4 Fast Flow suspension (50% slurry).
2) Mix gently for 1 hour at +4 °C.
3) Centrifuge at 12 000 x g for 20 seconds and save the pellet.
4) Wash the pellet three times with 1 ml lysis buffer and once with wash buffer. Centrifuge at 12 000 x g for 20 seconds between each wash and discard the supernatants.

Be very careful when removing the supernatants to avoid loss of the immunocomplexes.

Dissociation and analysis

1) Suspend the final pellet in 30 µl SDS-PAGE sample buffer.
2) Heat to +95 °C for 3 minutes.
3) Centrifuge at 12 000 x g for 20 seconds to remove the Sepharose. Carefully remove the supernatant.
4) Add 1 µl 0.1% bromophenol blue.
5) Analyse the supernatant by SDS-PAGE, followed by protein staining and/or immunoblotting for detection. Radiolabelled antigens are detected by autoradiography.

Buffers and solutions

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 16.

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 (0–1 M), non-ionic detergents (0.1–2%), ionic detergents (0.01–0.5%) and pH (6–9).

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	
Sample buffer (reducing)	1% SDS, 100 mM DTT, 50 mM Tris, pH 7.5	

Table 16.

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 a problem of high background during analysis.
- Monoclonal antibodies are more specific, which reduces background, but may mean that less stable immune complexes are formed due to lower affinity. This can be over-come by using pools of different monoclonal antibodies.

Troubleshooting

Target antigen cannot be detected due to incomplete release during lysis

Try harsher lysis conditions.

High levels of background proteins on SDS-PAGE

Specific cause: polyclonal serum may contain antibodies that recognize other antigens.

Purify the antibody by affinity purification using a specific purification column.

Try a different antibody.

Non-specific cause: proteins binding to medium and/or to the plastic tubes or the presence of protein aggregates that co-precipitate with the immune complex.

Pre-coat plastic tube with lysis buffer prior to addition of cell lysate.

Add saturating amount of competitive protein (i.e. BSA, gelatine, acetone powders).

Spin the lysate at 100 000 x g for 30 minutes to remove aggregated proteins before addition of the antibody.

Spin the antibody at 100 000 x g for 30 minutes to remove particulate matter.

Spin the antigen-antibody complex at 10 000 x g for 10 minutes prior to addition of medium to remove protein aggregates.

Try a different antibody.

Use more stringent washing conditions, for example: 1 M sodium chloride, 1 M potassium thiocyanate, 0.5 M lithium chloride, 0.2% SDS or 1% Tween 20. Alternate between high and low salt wash buffer, or wash the beads with distilled water. Prolong washing times and/or increase the number of washes.

Titrate the optimal amounts of cell lysate, antibody and Protein A Sepharose 4 Fast Flow/ Protein G Sepharose 4 Fast Flow.

Chapter 5
Multi-step purification strategies

As discussed in Chapter 3, a single, rapid purification step using affinity chromatography is often sufficient to achieve the level of purity and quantity of product required for research purposes. Antibodies or their fragments can be adequately purified for further use, and a polishing step (desalting/buffer exchange or high resolution gel filtration) is sufficient to remove unwanted small molecules such as salts. If affinity chromatography cannot be used, or if a higher degree of purity is required, alternative techniques need to be combined effectively into a multi-step purification strategy.

A significant advantage when working with native or recombinant antibodies or fragments is that there is often considerable information available about the product and contaminants, as shown in Table 17 below and in Table 3 on page 13.

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 sulphate.
Solubility	IgG 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	2–3% for IgG, higher for IgM (12%), most carbohydrate is associated with Fc region of the heavy chains.

Table 17. Characteristics of native IgG and IgM.

With this information, and with detection assays and sample preparation and extraction procedures in place, a purification strategy of Capture, Intermediate Purification and Polishing (CIPP) can be applied (Figure 23). This strategy is used in both the pharmaceutical industry and in the research laboratory to ensure faster method development, a shorter time to pure product and good economy. This section gives a brief overview of the approach recommended for any multi-step protein purification and some typical examples. The *Protein Purification Handbook* from Amersham Biosciences is highly recommended as a guide to planning efficient and effective protein purification strategies and for the selection of the correct medium for each step and scale of purification.

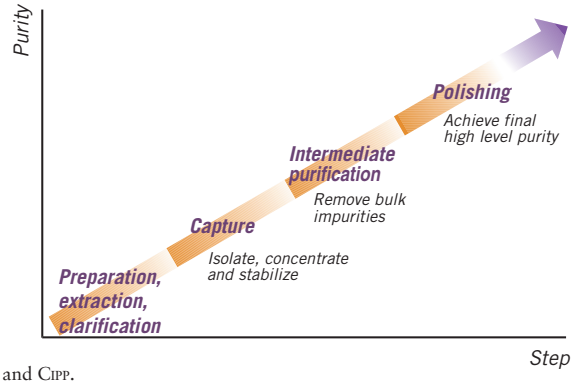


Fig. 23. Preparation and CIPP.

Applying CIPP:

Imagine the purification has three phases: Capture, Intermediate Purification and Polishing. Assign a specific objective to each step within the purification process.

The purification problem associated with a particular step will depend greatly upon the properties of the starting material. Thus, the objective of a purification step will vary according to its position in the process.

As shown in Figure 23, an important step for any purification is correct *sample preparation* and this is covered in detail in Chapter 2.

In the *capture phase* the objectives are to *isolate, concentrate and stabilize* the target product. The product should be concentrated and transferred to an environment that will conserve potency/activity.

During the *intermediate purification phase* the objective is to *remove most of the bulk impurities,* such as other proteins and nucleic acids, endotoxins and viruses.

In the *polishing phase* most impurities have already been removed except for trace amounts or closely related substances. The objective is to *achieve final purity* by removing any remaining trace impurities or closely related substances.

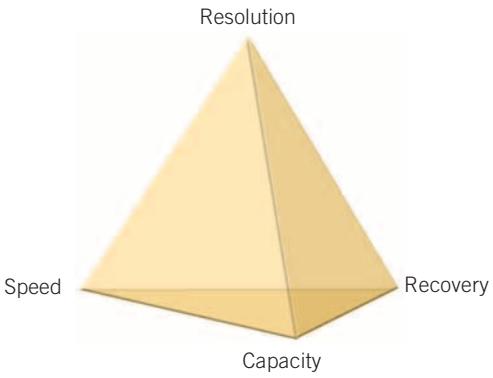
The optimal selection and combination of purification techniques for *Capture, Intermediate Purification and Polishing* is crucial for an efficient purification.

Selection and combination of purification techniques

Proteins are purified using purification techniques that separate according to differences in specific properties, as shown in Table 18.

Protein property	Technique
Charge	Ion exchange (IEX)
Size	Gel filtration (GF)
Hydrophobicity	Hydrophobic interaction (HIC), Reversed phase (RPC)
Biorecognition (ligand specificity)	Affinity (AC)
Charge, ligand specificity or hydrophobicity	Expanded bed adsorption (EBA) follows the principles of AC, IEX or HIC

Table 18. Protein properties used during purification.



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

Capacity, in the simple model shown, refers to the amount of target protein loaded during purification. In some cases the amount of sample that can be loaded will be limited by volume (as in gel filtration) or by large amounts of contaminants rather than the amount of the target protein.

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

Recovery becomes increasingly important as the purification proceeds because of the increased value of the purified product. Recovery is influenced by destructive processes in the sample and by unfavorable conditions on the column.

Resolution is achieved by the selectivity of the technique and the efficiency of the chromatography matrix in producing narrow peaks. In general, resolution is most difficult to achieve in the final stages of purification when impurities and target protein are likely to have very similar properties.

Select a technique to meet the objectives for the purification step.

Choose logical combinations of purification techniques based on the main benefits of the technique and the condition of the sample at the beginning or end of each step.

A guide to the suitability of each purification technique for the stages in CIPP is shown in Table 19.

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

Table 19. Suitability of purification techniques for the CIPP.

Minimize sample handling between purification steps by combining techniques to avoid the need for sample conditioning. The product should be eluted from the first column in conditions suitable for the start conditions of the next column (see Table 19).

Ammonium sulphate, often used for clarification and concentration of antibodies (see page 19), leaves the sample in high salt consequently HIC, which requires high salt to enhance binding to the media, is ideal as the capture step. The salt concentration and the total sample volume will be significantly reduced after elution from the HIC column. Dilution of the fractionated sample or rapid buffer exchange using a desalting column will prepare it for the next IEX or AC step.

Gel filtration is a non-binding technique unaffected by buffer conditions, but with limited volume capacity. GF is well suited for use after any of the concentrating techniques (IEX, HIC, AC, EBA) since the target protein will be eluted in a reduced volume and the components from the elution buffer will not affect the gel filtration process.

Selection of the final strategy will always depend upon specific sample properties and the required level of purification. Logical combinations of techniques are shown in Figure 24.

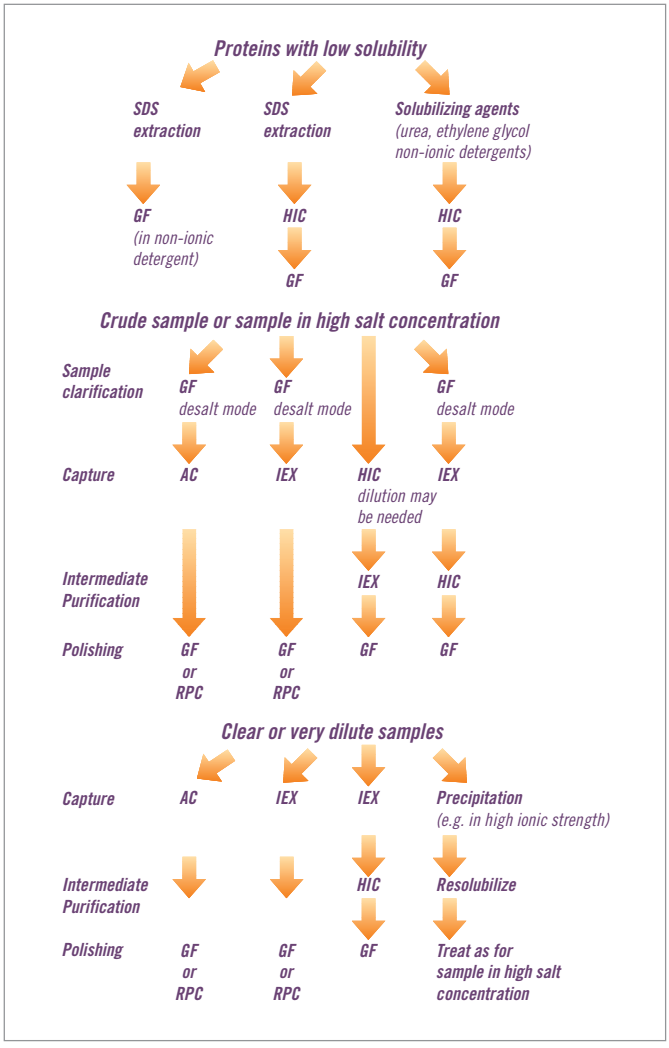


Fig. 24. Logical combinations of chromatography techniques.

For any capture step, select the technique showing the most effective binding to the target protein while binding as few of the contaminants as possible, i.e. the technique with the highest selectivity and/or capacity for the target protein.

A sample is purified using a combination of techniques and alternative selectivities. For example, in an IEX-HIC-GF strategy, the capture step selects according to differences in charge (IEX), the intermediate purification step according to differences in hydrophobicity (HIC) and the final polishing step according to differences in size (GF).

If nothing is known about the target protein use IEX-HIC-GF. This combination of techniques can be regarded as a standard protocol. Consider the use of both anion and cation exchange chromatography to give different selectivities within the same purification strategy.

IEX is a technique which offers different selectivities using either anion or cation exchangers. The pH of the purification can be modified to alter the charge characteristics of the sample components. It is therefore possible to use IEX more than once in a purification strategy, for capture, intermediate purification or polishing. IEX can be used effectively in the same purification scheme for rapid purification in low resolution mode during capture and in high resolution mode during polishing.

Consider reversed phase chromatography (RPC) for a polishing step, provided that the target protein can withstand the run conditions. RPC separates proteins and peptides on the basis of hydrophobicity. RPC is a high selectivity (high resolution) technique, requiring the use of organic solvents. The technique is widely used for purity check analyses when recovery of activity and tertiary structure are not essential. Since many proteins are denatured by organic solvents, the technique is not generally recommended for protein purification because recovery of activity and return to a correct tertiary structure may be compromised. However, in the polishing phase, when the majority of protein impurities have been removed, RPC can be excellent, particularly for small target proteins that are not often denatured by organic solvents.

CIPP does not mean that there must always be three purification steps. For example, capture and intermediate purification may be achievable in a single step, as may intermediate purification and polishing. Similarly, purity demands may be so low that a rapid capture step is sufficient to achieve the desired result. For purification of therapeutic proteins, a fourth or fifth purification step may be required to fulfil the highest purity and safety demands. The number of steps used will always depend upon the purity requirements and intended use for the protein.

Selection of media for multi-step purification

Having decided upon the most suitable purification techniques, the most suitable medium should be selected for each technique. Recommended prepacked columns to follow a standard purification protocol combining IEX, HIC and GF at laboratory scale are shown in Table 20. These recommendations are based on the assumption that the possibility of using affinity purification has been excluded.

Step	Starting scale	Increasing scale
Sample Preparation	HiTrap Desalting	HiPrep 26/10 Desalting
Capture IEX	HiTrap IEX Selection Kit (screen 7 different IEX media packed in 1 ml HiTrap columns to select optimal medium)	
	HiTrap Q FF, 1 ml or 5 ml	HiPrep 16/10 Q FF
	HiTrap SP FF, 1 ml or 5 ml	HiPrep 16/10 SP FF
	HiTrap DEAE FF, 1 ml or 5 ml	HiPrep 16/10 DEAE FF
	HiTrap CM FF, 1 ml or 5 ml	HiPrep 16/10 CM FF
	HiTrap Q XL, 1 ml or 5 ml	HiPrep 16/10 Q XL
	HiTrap SP XL, 1 ml or 5 ml	HiPrep 16/10 SP XL
	HiTrap ANX FF(high sub), 1 ml or 5 ml	HiPrep 16/10 ANX FF (high sub)
Capture HIC	HiTrap HIC Selection Kit (screen 5 different HIC media packed in 1 ml HiTrap columns to select optimal medium)	
	HiTrap Phenyl FF (high sub), 1 ml or 5 ml	HiPrep 16/10 Phenyl FF (high sub)
	HiTrap Phenyl FF (low sub), 1 ml or 5 ml	HiPrep 16/10 Phenyl FF (low sub)
	HiTrap Octyl FF, 1 ml or 5 ml	HiPrep 16/10 Octyl FF
	HiTrap Butyl FF, 1 ml or 5 ml	HiPrep 16/10 Butyl FF
	HiTrap Phenyl HP, 1 ml or 5 ml	HiLoad 16/10 Phenyl Sepharose HP
		HiLoad 26/10 Phenyl Sepharose HP
Intermediate Purification IEX (listed in order of bead size: use smaller bead sizes as sample purity increases to maximize resolution)	HiTrap Q HP, 1 ml or 5 ml	HiLoad 16/10 Q Sepharose HP
	HiTrap SP HP, 1 ml or 5 ml	HiLoad 26/10 Q Sepharose HP
	RESOURCE™ Q, 1 ml or 6 ml	HiLoad 16/10 SP Sepharose HP
	RESOURCE S, 1 ml or 6 ml	HiLoad 26/10 SP Sepharose HP
	Mono Q™ 5/50 GL	SOURCE™ 15Q (loose medium)
	Mono S™ 5/50 GL	SOURCE 15S (loose medium)
Intermediate Purification HIC	HiTrap Phenyl HP, 1 ml or 5 ml	HiLoad 16/10 Phenyl Sepharose HP
	RESOURCE 15ISO	HiLoad 26/10 Phenyl Sepharose HP
	RESOURCE 15PHE	SOURCE 15ISO (loose medium)
	RESOURCE 15ETH	SOURCE 15PHE (loose medium)
		SOURCE 15ETH (loose medium)
Polishing GF (select medium according to size of target molecules and contaminants)	Superdex 200 10/300 GL	HiLoad 16/60 Superdex 200 prep grade
		HiLoad 26/60 Superdex 200 prep grade
	Superdex 75 10/300 GL	HiLoad 16/60 Superdex 75 prep grade
		HiLoad 26/60 Superdex 75 prep grade
	Superdex Peptide 10/300 GL	HiLoad 16/60 Superdex 30 prep grade
		HiLoad 26/60 Superdex 30 prep grade

Table 20.

To increase the binding capacity and for larger scale purification, HiTrap columns can easily be linked in series.

Most media are available for packing in larger columns. Always check specific availability if the intention is to scale up. Custom-designed media and custom-packed columns can be produced.

If a purification is not intended for scale up (i.e. milligram quantities of product are needed), use high performance, prepacked media, such as Sepharose High Performance (IEX and HIC), SOURCE (IEX, HIC), Monobeads™ (IEX), or Superdex (GF) for all steps.

Selection of pH for purification by ion exchange

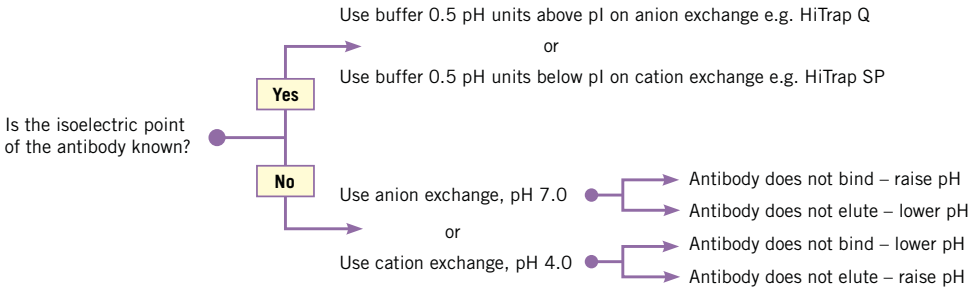
Knowledge of the characteristics of antibodies and fragments helps considerably in the selection of the correct purification conditions, particularly with regard to the elimination of known contaminants. Table 21 gives examples of the isoelectric point values for antibodies and some common contaminants.

Component	pI
Antibodies	4–9
Albumin	4.9
Transferrin	5.2–6.1
α_2-macroglobulin	4.1–4.9

Table 21.

The selection of anion or cation exchange and the correct pH is crucial for a successful purification. The principles of ion exchange chromatography are outlined briefly in Appendix 9, together with practical advice on the development and optimization of a purification method.

As shown below, the ion exchanger and buffer pH can be chosen according to the information available on the isoelectric point of the antibody.



If the pI of the antibody is sufficiently different from the contaminants, it may be possible to minimize contamination by using a cation exchange medium (negatively charged) 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 and the impurities (which could include negatively charged nucleic acids and endotoxins) pass through.

Examples of multi-step purification

The following examples demonstrate the successful application of the CIPP strategy to the purification of antibodies and antibody fragments. In many cases, with knowledge of the characteristics of the target protein and known contaminants techniques and elution conditions can be selected to yield a highly pure product in as few as two purification steps. The availability of a suitable affinity medium will often lead to a two-step purification process, combining affinity and gel filtration, as shown below, and as demonstrated by examples in Chapter 3.

Example 1: Two-step purification of mouse monoclonal IgG_1

This example demonstrates the effectiveness of using a high selectivity, affinity purification for initial capture. In common with most antibody preparations, there is a possibility that IgG aggregates and/or dimers are present. 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*, available from Amersham Biosciences.

Target molecule

Mouse monoclonal IgG_1.

Source material

Cell culture supernatant.

Extraction and clarification

Cell culture supernatant is filtered through a 0.45 µm filter.

Capture

The target protein is captured on a HiTrap rProtein A FF column. This step removes contaminating proteins, low molecular substances and significantly reduces the sample volume.

Scouting for optimal elution conditions

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 25 shows the results of a scouting experiment 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, results not shown).

Using ÄKTA design 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.

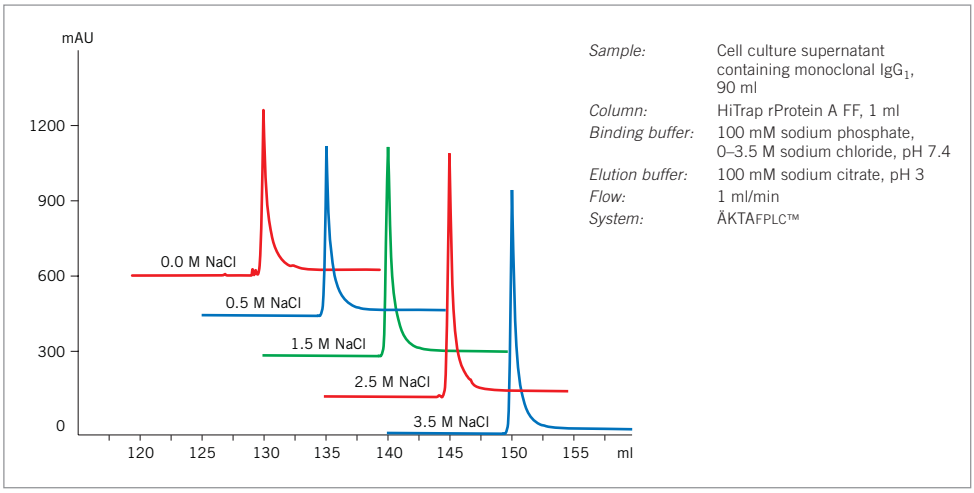


Fig. 25. Automatic scouting of optimal salt concentration in the binding buffer on HiTrap rProtein A FF.

Optimization of binding and elution conditions gives a well-resolved peak containing IgG_1, as shown in Figure 26.

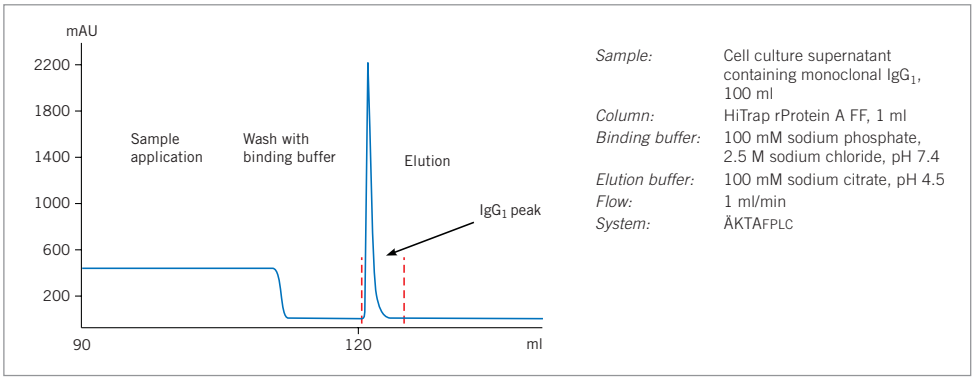


Fig. 26. Optimized capture step on HiTrap rProtein A FF.

Intermediate purification

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

Polishing

The polishing step shown in Figure 27 removes low or trace levels of contaminants, in this case IgG aggregates and/or dimers, by gel filtration on a HiLoad 16/60 Superdex 200 prep grade column.

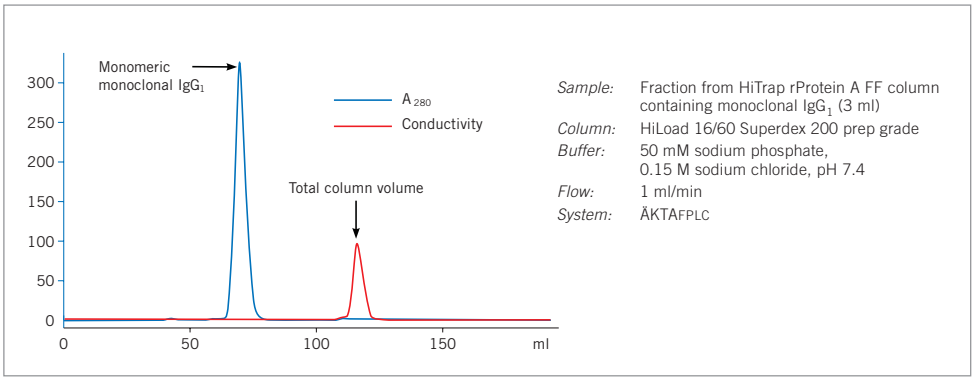


Fig. 27. Polishing on HiLoad 16/60 Superdex 200 prep grade.

An affinity purification reduces sample volume and concentrates the sample. Gel filtration is the slowest of all the chromatography techniques and the size of the column determines the volume of sample that can be applied. 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 50 ml cell culture supernatant. The recovery from the capture and polishing steps was above 95%. Figure 28 shows the SDS PAGE analysis of selected fractions.

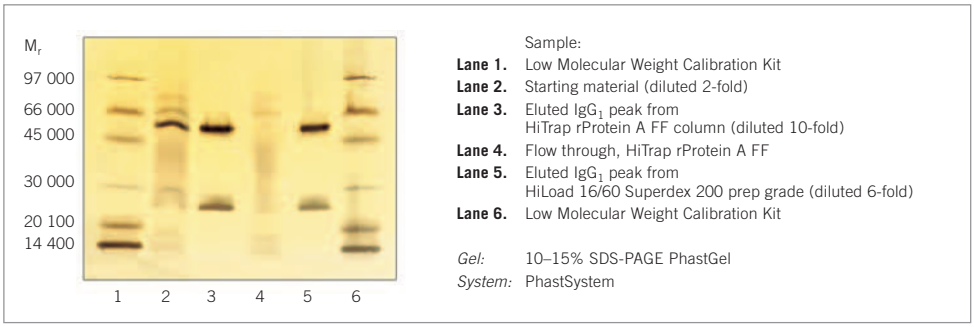


Fig. 28. Purity analysis using SDS-PAGE.

Example 2: Two-step purification of a mouse monoclonal IgG_1 for diagnostic use

The goal of this purification is production of a monoclonal antibody to achieve a level of purity sufficient for *in vitro* diagnostic use.

Target molecule

Mouse monoclonal IgG_1 anti-IgE.

Source material

Hybridoma cell culture.

Clarification

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

Capture

As shown in Figure 29 a HIC purification is chosen for the capture step because the antibody binds very strongly to the medium (Phenyl Sepharose High Performance) and most foetal calf serum proteins pass through the column. The sample is concentrated into a smaller volume for polishing.

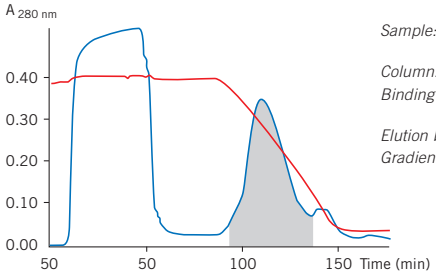
Screening of HIC media, using a HiTrap HIC Selection Kit or RESOURCE HIC Test Kit, is recommended to select the medium that gives the best results.

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

Binding buffer:	20 mM potassium phosphate, 0.05 M ammonium sulphate, pH 7.0.
Elution buffer:	20 mM potassium phosphate, pH 7.0.

Performing the separation

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 0.5–0 M ammonium sulphate.
5. Wash with 2–3 column volumes of 100% elution buffer.
6. Re-equilibrate with 2–3 column volumes of binding buffer.



Sample:	Hybridoma cell culture supernatant, mouse IgG_1, anti-IgE. Ammonium sulphate added to 0.5 M
Column:	Phenyl Sepharose High Performance, 10 cm bed height
Binding buffer:	20 mM potassium phosphate, 0.5 M ammonium sulphate, pH 7.0
Elution buffer:	20 mM potassium phosphate, pH 7.0
Gradient:	0–100% elution buffer, 10 column volumes

Fig. 29. Capture 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

Gel filtration is a suitable polishing step (Figure 30). Using Superdex 200 prep grade a final purity of > 99% is achieved.

Performing the separation

1. Equilibrate column in phosphate buffered saline, pH 7.5 at 15 ml/min.
2. Apply sample (maximum sample volume 1–2% of total column volume).
3. Elute sample in one column volume of buffer. Collect fractions.
4. Wash with 2–3 column volumes of buffer.

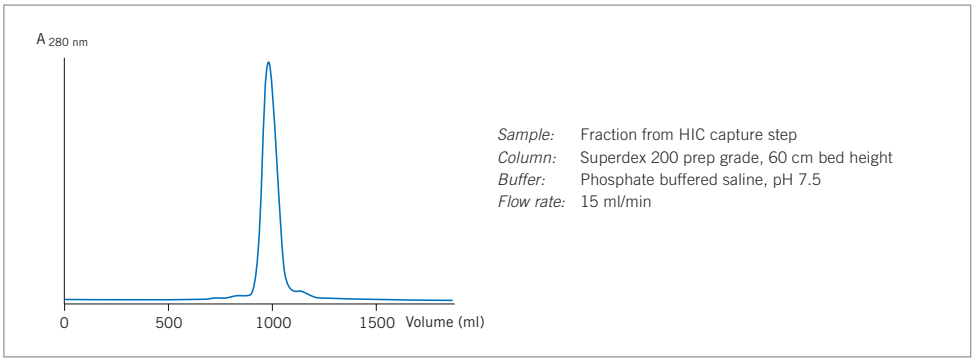


Fig. 30. Polishing on Superdex 200 prep grade.

Example 3: Three-step purification of a recombinant Fab fragment

This example demonstrates a three-step purification strategy in which the same purification principle is used in two different modes in the capture and polishing step: IEX for capture, HIC for intermediate purification and IEX for the polishing step. The objective of this work was to develop a strategy that could be scaled up for use as a routine procedure. A more detailed description of this work can be found in *Application Note Code No. 18-1111-23* from Amersham Biosciences.

Target Molecule

Recombinant antigen binding fragment (Fab) directed against HIV gp-120.

Source

The anti-gp 120 Fab was expressed in the periplasm of the *E. coli* strain BM170 MCT61. *E. coli* pellets were stored frozen after being harvested and washed once.

Sample extraction, clarification and capture

Thawed cells were lysed with sucrose. The lysate was treated with DNase I in the presence of 2 mM $MgCl_2$ at pH 7.5 before the capture step. The Fab fragment was captured from non-clarified homogenate by expanded bed adsorption with STREAMLINE™ SP (cation exchanger).

Expanded bed adsorption was chosen because the target protein could be captured directly from the crude homogenate in a single step, without the need for centrifugation or other preparatory clean-up steps. The technique is well suited for large-scale purification.

At laboratory scale, a prepacked cation exchange column can be used together with suitable sample preparation before beginning purification.

The result of the capture step is shown in Figure 31. The Fab fragment is concentrated and rapidly transferred into a stable environment by a step elution.

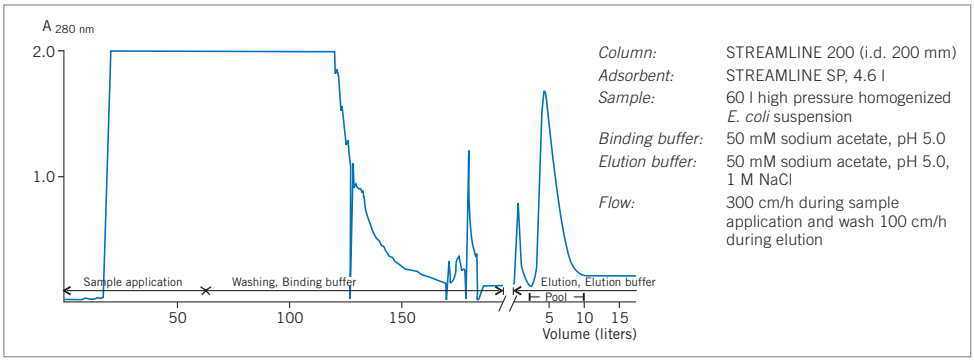


Fig. 31. Capture step using expanded bed adsorption.

Intermediate purification

HIC was selected because the separation principle is complementary to IEX and, since the sample was already in a high salt buffer after elution from STREAMLINE SP, a minimum amount of sample conditioning was required.

Hydrophobic properties are difficult to predict and it is always recommended to screen different media. A HiTrap HIC Selection Kit (containing five 1 ml columns prepacked with different HIC media suitable for use at production scale) was used to screen for the most appropriate medium. Buffer pH was kept at pH 5.0 to minimize the need for sample conditioning after capture. Results of the media screening are shown in Figure 32.

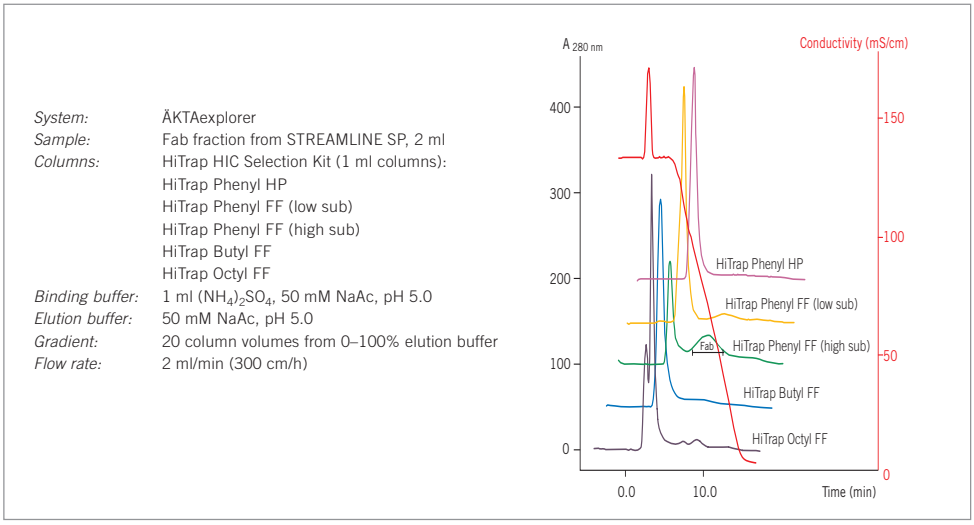
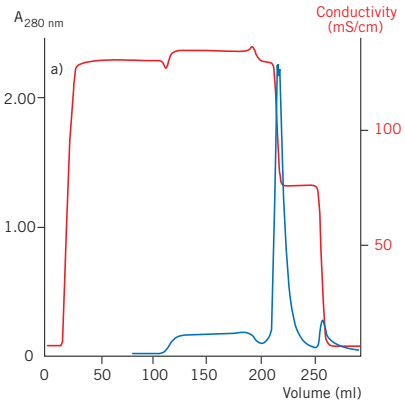


Fig. 32. HIC media scouting using HiTrap HIC Selection Kit.

Phenyl Sepharose 6 Fast Flow (high sub) was selected since the medium showed excellent selectivity for the target protein thereby removing the bulk contaminants. Optimization of elution conditions resulted in a step elution being used to maximize the throughput and the concentrating effect of the HIC technique. Figure 33 shows the optimized elution scheme and the subsequent scale up of the intermediate purification step.

System:	ÄKTAexplorer
Sample:	Fab fraction from STREAMLINE SP, 80 ml
Columns:	Phenyl Sepharose 6 Fast Flow (high sub) in XK 16/20 (10 cm bed height)
Binding buffer:	1 M $(NH_4)_2SO_4$, 50 mM NaAc, pH 5.0
Elution buffer:	50 mM NaAc, pH 5.0
Gradient:	Step gradient to 50% elution buffer
Flow rate:	5 ml/min
Column volume:	20 ml



System:	ÄKTAexplorer
Sample:	Fab fraction from STREAMLINE SP, 800 ml
Columns:	Phenyl Sepharose 6 Fast Flow (high sub) in XK 50/20
Binding buffer:	1 M $(NH_4)_2SO_4$, 50 mM NaAc, pH 5.0
Elution buffer:	50 mM NaAc, pH 5.0
Gradient:	Step gradient to 50% elution buffer
Flow rate:	Equilibration: 100 ml/min Loading and elution: 50 ml/min
Column volume:	200 ml

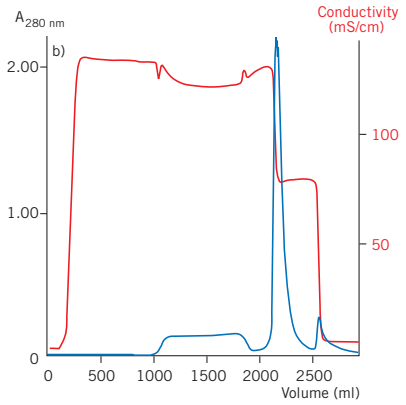


Fig. 33. Intermediate purification using HIC: optimization and scale-up.

Polishing

Gel filtration was investigated as the natural first choice for a final polishing step to remove trace contaminants and transfer the sample to suitable storage conditions. However, in this example, gel filtration could not resolve a M_r 52 000 contaminant from the M_r 50 000 Fab fragment (results not shown).

As an alternative another cation exchanger SOURCE 15S was used. In contrast to the cation exchange separation at the capture step, the polishing cation exchange separation was performed using a shallow gradient elution on a medium with a small, uniform bead size (SOURCE 15S) to give a high resolution result, as shown in Figure 34.

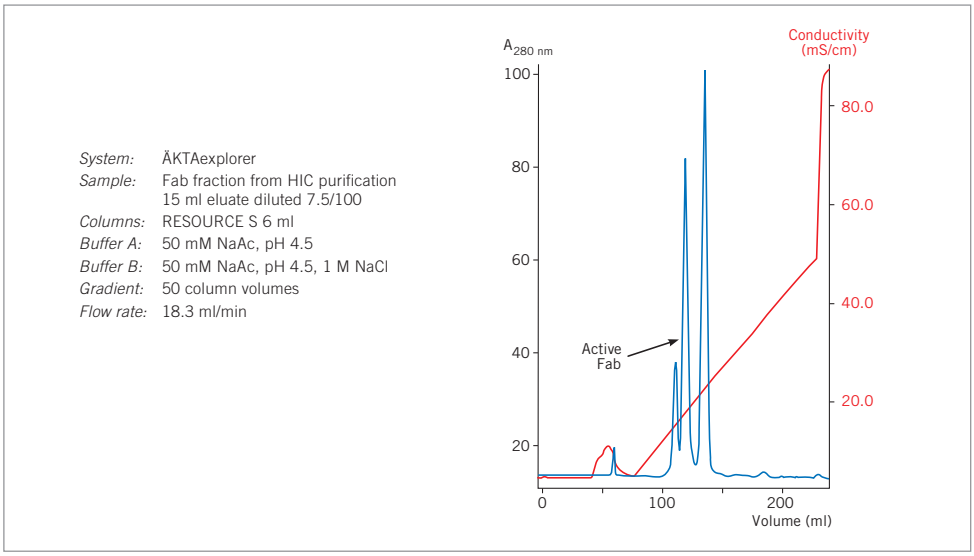


Fig. 34. Optimized Fab polishing step.

Analytical assays

Collected fractions were separated by SDS-PAGE and stained by Coomassie Blue using PhastSystem, following the protocols supplied with the instrument. Fab activity was measured by a goat-anti-human IgG Fab ELISA, an anti-gp120 ELISA and an *in vitro* assay which measured the inhibition of HIV-1 infection of T-cells. Nucleic acid was routinely monitored by measuring A_{260}/A_{280}. The correlation of a high A_{260}/A_{280} ratio (> 1) with the presence of DNA was verified for selected samples by agarose gel electrophoresis and EtBr staining. Endotoxin determination employed a kinetic chromogenic Limulus assay (COAMATIC Chromogenics AB, Mölndal, Sweden).

Chapter 6
Removal of specific contaminants after initial purification

For many applications at laboratory scale, contaminant molecules may not be a significant problem. Affinity chromatography 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 3 on page 13, 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 are albumin, transferrin and host or bovine immunoglobulins that originate in ascites, cell culture and serum. These three contaminants 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 of their properties to those of the target molecule.

For some cell culture preparations it is possible to decreased 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. The chromatographic technique best suited to separate specific contaminants from the target molecule will largely depend upon the physical characteristics of all the components.

Select the technique according to the characteristics that are significantly different: ion exchange (for separation by differences in charge), hydrophobic interaction (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.

If the pI value of the antibody is sufficiently different from the contaminants it may be, possible to minimize contamination by using a cation exchange medium (negatively charged) 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 and the impurities (including negatively charged nucleic acids) pass through.

When using ion exchange chromatography, apply the same principles for the removal of contaminants as those described on page 61.

Bovine immunoglobulins

Co-purification of host or bovine immunoglobulins is a problem associated with any affinity purification when using a native source or a source to which supplements such as calf serum or bovine serum albumin are added. This contamination problem is particularly significant for monoclonal antibodies intended for *in vivo* human applications. Difficulties have also been encountered when murine monoclonal antibodies are the target molecule. The similarities between the physical characteristics of the contaminant and the target molecule require careful selection of the most suitable chromatographic technique for purification. Both hydrophobic interaction and ion exchange can be used.

Hydrophobicity of proteins is difficult to predict. It is recommended to screen several chromatographic media with different hydrophobicities (e.g. using the HiTrap HIC Selection Kit) to enable selection of the medium that gives the best results In this example, SOURCE 15ISO, 15PHE and 15ETH were tested.

As shown in Figure 35, HiTrap HIC Selection Kit can be used to screen for the most suitable hydrophobic medium. A RESOURCE HIC Test Kit is also available.

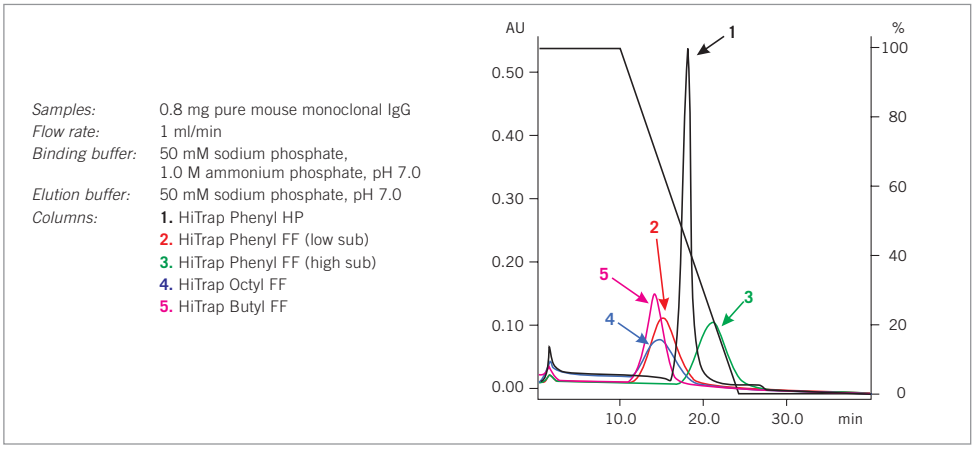


Fig. 35.

Albumin and transferrin

If albumin is present at very high levels in the original sample, it may occasionally be seen as a contaminant even after affinity purification, but other impurities will wash through the column.

IEX or HIC are usually the methods of choice for removing albumin and transferrin, separating the molecules on the basis of differences in their isoelectric points or differing 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 the target protein. In some cases, it may be possible to optimize the IEX step to improve the separation between the target protein and the contaminants by modifying the pH and elution conditions (see page 61).

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

Removal of albumin

Blue Sepharose media may be a useful alternative for removing albumin. This affinity technique can be used to remove albumin either before or after other purification steps. The albumin binds in a non-specific manner by electrostatic and/or hydrophobic interactions with the aromatic anionic ligand, Cibacron™ Blue F3G-A, coupled to Sepharose.

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 visualization of other protein peaks seen by UV absorption.

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

Purification options

	Capacity/ml medium*	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.

**Protein binding capacity will vary for different proteins.*
 # Higher binding capacity than Blue Sepharose CL-6B.

Purification

Sample: Human plasma, buffer exchanged to binding buffer with HiTrap Desalting
Column: HiTrap Blue HP 1 ml column
Binding buffer: 20 mM sodium phosphate, pH 7.0
Elution buffer: 20 mM sodium phosphate, 2.0 M sodium chloride, pH 7.0

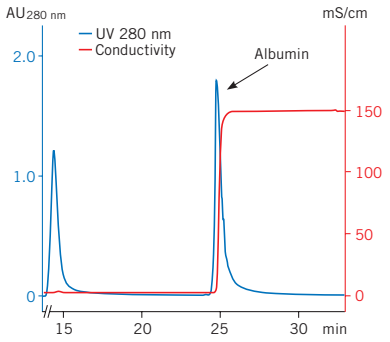


Fig. 36.

Performing a separation

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

Elution buffer: 20 mM sodium phosphate, 2.0 M NaCl, pH 7.0 or 50 mM KH_2PO_4, 1.5 M KCl, pH 7.0

1. Equilibrate the column with 5 column volumes of binding buffer.
2. Apply the sample, using a syringe or pump.
3. Wash with 10 column volumes of binding buffer or until no material appears in the eluent (monitored by absorption at A_{280nm}).
4. Elute with 5 column volumes of elution buffer. More may be required if the interaction is difficult to reverse.

Storage

Wash media and columns with 20% ethanol (use approximately 5 column volumes for packed media) and store at +4 to +8 °C.

α_2-macroglobulin and haptoglobulin

These 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 column.

In general, careful selection of the correct ion exchange medium and pH for purification can ensure that these contaminants are removed during an ion exchange purification. Blue Sepharose and Chelating Sepharose can also be considered for removing of α_2-macroglobulin.

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. When high salt concentrations are involved, dimers or polymers can be formed during freezing and thawing. These aggregates can lower the biological activity of the sample.

Gel filtration is the technique of choice for remving aggregates, and a medium such as Superdex 200 will give the best possible separation between monomer and dimer. It is used as the final polishing step in many purification strategies. The principles of gel filtration are outlined in Appendix 9.

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 the sample loading is limited to 1–3% of the total column volume in most cases.

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

Figure 37 shows an example of the purification of human IgG monomers and dimers on Superdex 200.

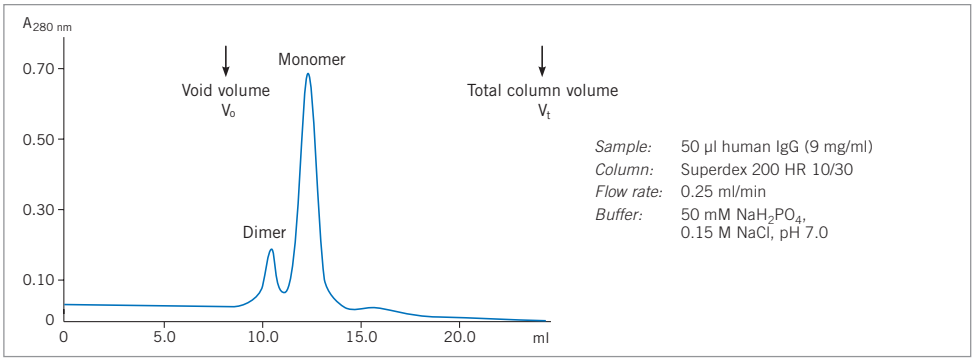


Fig. 37.

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 bio-assays to indicate if impurities are interfering with results.

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 target molecule will bind the target and allow the negatively charged molecules to wash through the column. Consequently, if cation exchange is used as the initial capture step these contaminants will be removed at an early stage in purification.

If endotoxins need to be removed from a purified product, anion exchange chromatography at a pH value slightly below the isoelectric point of the product will bind the endotoxins and the target molecule will wash through the column. Alternatively use a pH which binds both molecular species, but allows them to be clearly separated during gradient elution from the column.

Affinity ligands

With any affinity chromatography medium there is a risk of ligand leakage from the matrix, particularly if harsh conditions are required to elute the target molecule. In many cases this leakage is negligible and a satisfactory purity is achieved. At laboratory scale, leakage of ligands is not a significant problem. However, in pharmaceutical production processes it must be shown that even trace amounts of ligand have been removed from the final product.

Figure 38 shows an example of the removal of protein A from mouse IgG_{2b} on a 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, 1.0 M NaCl, pH 5.2
Flow rate:	4 ml/min
Gradient:	0–45% elution buffer in 15 column volumes

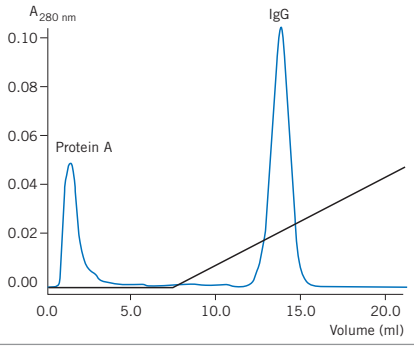


Fig. 38. Removal of protein A from mouse IgG_{2b} by cation exchange chromatography on HiTrap SP HP. Recombinant protein A was spiked into mouse IgG_{2b} previously purified on rProtein A Sepharose Fast Flow.

Chapter 7
Large-scale purification

Large-scale purification requires careful planning and close collaboration with process specialists. This chapter gives only a brief review of some of the issues involved in large-scale purification.

A downstream production process must achieve the required purity and recovery with complete safety and reliability, at a given scale and within a given economic framework. Economy is a very complex issue. In commercial production, time to market can override extensive optimization in favour of recovery, capacity or speed. Robustness and reliability are always of great concern since a batch failure can have major consequences. Special safety issues may be involved in the purification of biopharmaceuticals, such as detection or removal of infectious agents, pyrogens, immunogenic contaminants and tumorigenic hazards. It may be necessary to use analytical techniques targeted towards specific contaminants in order to demonstrate that they have been removed to acceptable levels.

High safety demands mean that considerable attention is paid not only to the purification strategy, but also to the source of the product. At production scale, considerable emphasis is placed on minimizing the contaminants that can enter the process from the very beginning, as well as focusing on the removal of impurities downstream. Currently, therapeutic antibodies are being developed in Chinese Hamster Ovary (CHO) cells and *E. coli*, as well as hybridomas. Expression levels are often higher than those used in research applications (up to several grams per liter of culture supernatant) and impurities are minimized by, for example, the use of protein-free or highly characterized culture media. With well established characteristics for both target molecule and contaminants and with detection assays and sample preparation procedures in place, the CIPP (Capture, Intermediate Purification, Polishing) strategy, as described in Chapter 5, is frequently used in industry. This strategy ensures faster method development and a short process to pure product and good economy. Each step has clearly defined goals in terms of concentration, stabilization, removal of critical contaminants and conditioning.

Since a production process has goals and constraints that differ from those in the laboratory, CIPP can lead to a different combination of techniques as compared to traditional laboratory scale approaches. Process development is performed in a down-scaled model of the final production process. Scalability is therefore a key issue and media or conditions that are unproven or inappropriate at large-scale are not worth testing in the laboratory. In large-scale capture, throughput will often be the focus during method development. It is important to consider all aspects: sample extraction and clarification, sample loading capacity, flow rate during equilibration, binding, washing, elution and cleaning. The need for cleaning-in-place procedures may even exclude media that were suitable at laboratory scale. In principle, a capture step is optimized to maximize capacity and/or speed at the expense of some resolution. However, there is usually considerable resolution and purification from molecules that have significant physicochemical differences compared to the target protein. Recovery will be of concern in any preparative situation, especially for production of a high value product, and it is important to assay for recovery during optimization of the capture step.

Considerations for monoclonal antibody purification

CIPP can be applied to any protein purification, including any antibody class or fragment, whether from native or genetically engineered sources. In the case of native monoclonal antibodies, certain combinations of techniques can be prioritized for production of large quantities of material, since a great deal is known about their characteristics and the characteristics of their most common contaminants. These are illustrated in Table 22.

Purification step	Technique/Products	Comments
Capture		
AC	Protein A Sepharose 4 Fast Flow rProtein A Sepharose Fast Flow rmpProtein A Sepharose Fast Flow* Protein G Sepharose 4 Fast Flow	High purity.
	MabSelect	High purity. Optimized for rapid processing of large volumes.
EBA.AC	STREAMLINE rProtein A	As above, but offers simultaneous clarification and purification.
IEX	SP Sepharose Fast Flow	Cation exchange. Since pI of MAb is usually higher than that of main contaminants: major serum proteins (e.g. albumin, transferrin) are washed through the column. MAb often more stable in acidic conditions than many other proteins. High resolution and capacity.
EBA.IEX	STREAMLINE SP XL	As above, but offers simultaneous clarification and purification.
HIC	Phenyl Sepharose Fast Flow (high sub) Phenyl Sepharose Fast Flow (low sub)	Major serum contaminants (e.g. albumin, transferrin) washed through the column.
Intermediate Purification		
IEX	Q or SP Sepharose Fast Flow Q or SP Sepharose High Performance	Cation or anion exchange according to the charge properties of components remaining after capture step. Use cation exchange when pI of MAb is higher than that of main contaminants. Sepharose matrix selected according to the performance required (scale, speed etc.)
HIC	Phenyl Sepharose Fast Flow (high sub) Phenyl Sepharose Fast Flow (low sub) Phenyl Sepharose High Performance SOURCE 15ISO or 15PHE	Follows after EBA and/or IEX as samples are already in salt. Screen to determine the medium that gives the best purification.
Polishing		
IEX	Q Sepharose Fast Flow or SP Sepharose Fast Flow Q Sepharose High Performance or SP Sepharose High Performance	Cation or anion exchange, depending on previous step, remaining impurities and other purposes. Sepharose matrix selected according to the performance required (scale, speed etc.).
HIC	SOURCE 15ISO or 15PHE	Follows after AC or IEX. Separates contaminants such as bovine IgG.
GF	Superdex 200 Superdex 200 prep grade	Follows after IEX, HIC or AC. Highest resolution for separation of dimers and aggregates.

** rmpProtein A Sepharose Fast Flow may help to minimize regulatory concerns at process scale since no mammalian components are involved in production or purification.*

Table 22.

Combining sample preparation and capture for Fc-containing antibodies

There are two approaches to the purification of Fc-containing antibodies. Conventionally, filtration is used to remove debris before the capture step on a packed bed of protein A coupled to an affinity matrix. A modern alternative is expanded bed adsorption (EBA), a technique that clarifies, concentrates and captures the target molecules from cell culture supernatant in a single step. Apart from offering better yields, because of fewer steps and quicker removal of degrading agents, EBA has been found to be more gentle for the removal of cells, which consequently reduces the load of intracellular contaminants on subsequent steps. The principles of this technique are described in Appendix 9.

EBA is particularly suitable for large-scale recombinant protein and monoclonal antibody purification. Crude sample containing particles can be applied to the expanded bed without filtration or centrifugation. STREAMLINE adsorbents are specially designed for use in STREAMLINE columns. Together they enable the high flow rates needed for high productivity in industrial applications. The technique combines sample preparation and capture in a single step. STREAMLINE adsorbents are designed to handle feed directly from both fermentation homogenate and crude feedstock from cell culture/fermentation. In Figure 39 IgG_1 is captured from a crude sample as it is applied to an expanded bed of STREAMLINE rProtein A, while cell debris, cells, particulate matter, whole cells, and contaminants pass through. Flow is reversed and the IgG_1 is desorbed in the elution buffer.

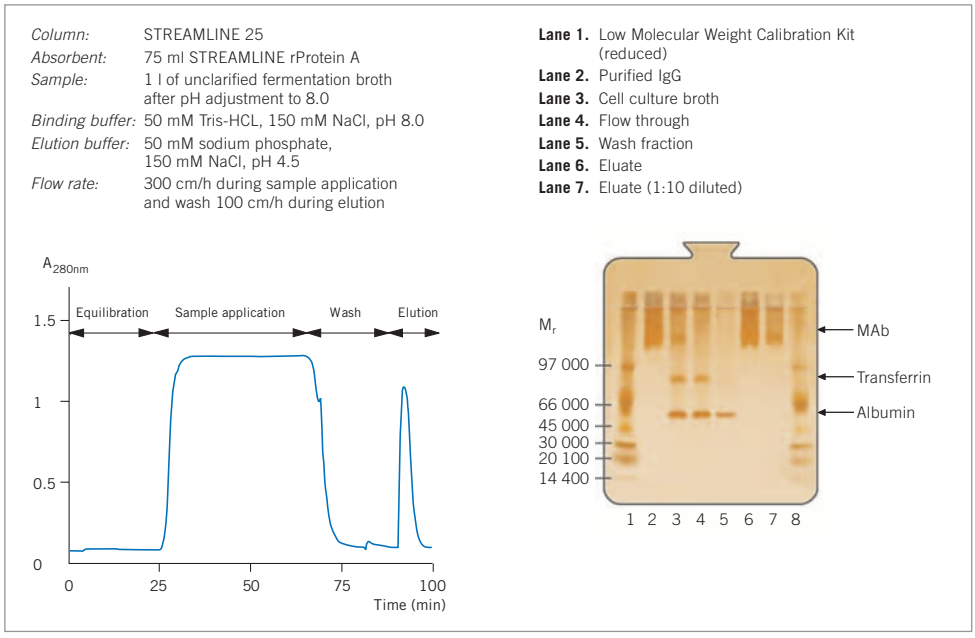


Fig. 39. Capture of IgG_1 by EBA on STREAMLINE rProtein A and analysis by SDS PAGE using silver staining under non-reduced conditions.

BioProcess Media for production

Specific BioProcess™ Media have been designed for each chromatographic stage in a process from Capture to Polishing. Large capacity production integrated with clear ordering and delivery routines ensure that these media are available in the right quantity, at the right place, at the right time. Amersham Biosciences can assure future supplies of BioProcess Media, making them a safe investment for long-term production. The media are produced following validated methods and tested under strict control to fulfil high performance specifications. A certificate of analysis is available with each order.

Regulatory Support Files contain details of performance, stability, extractable compounds and analytical methods. The essential information in these files gives an invaluable starting point for process validation, as well as providing support for submissions to regulatory authorities. Using BioProcess Media for every stage results in an easily validated process. High flow rate, high capacity and high recovery contribute to the overall economy of an industrial process.

All BioProcess Media have chemical stability to allow efficient cleaning and sanitization procedures. Packing methods are established for a wide range of scales and compatible large-scale columns and equipment are available.

Please refer to the latest *BioProcess Products Catalog* from Amersham Pharmacia Biotech for further details of our products and services for large-scale production.

Custom Designed Media and Columns

Prepacked columns, made according to the client's choice from the Amersham BioSciences range of columns and media, can be supplied by the Custom Products Group.

Custom Designed Media (CDM) can be produced for specific industrial process separations when suitable media are not available from the standard range. The CDM group at Amersham BioSciences works in close collaboration with the user to design, manufacture, test and deliver media for specialized separation 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. Amersham Biosciences provides a wide range of columns that ensures the highest performance from all our purification media and meets the demands of modern pharmaceutical manufacturing. Please ask your local representative for further details of CDM products and services.

Appendix 1

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 to help during 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 used most frequently to determine the total protein content. 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

6X 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 6X SDS loading buffer to 5–10 µl of supernatant from crude extracts, cell lysates or purified fractions as appropriate.
2. Vortex briefly and heat for 5 minutes at +90 to +100 °C.
3. Load the samples onto an SDS-polyacrylamide gel.
4. Run the gel 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 23).

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

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

Table 23.

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

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, such as Hybond™ ECL™ (for subsequent ECL detection) or Hybond P (for subsequent ECL Plus™ detection).
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 and Hybond ECL instruction manual*, both from Amersham Biosciences.

- 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 reaction kinetics.

Detection and assay of tagged proteins

SDS-PAGE, Western blotting and ELISAs can also be applied to the detection and assay of genetically engineered molecules to which a specific tag has been attached. In some cases, an assay based on the properties associated with the tag itself can be developed, e.g. the GST Detection Module for enzymatic detection and quantification of GST tagged proteins. Further details on the detection and quantification of GST and $(His)_6$ tagged proteins are available in *The Recombinant Protein Handbook: Protein Amplification and Simple Purification* and the *GST Gene Fusion System Handbook* from Amersham Biosciences.

Appendix 2

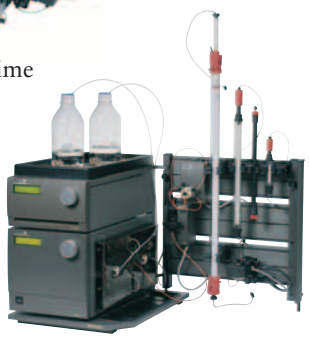
Selection of purification equipment

Amersham Biosciences offers solutions from the simplest purification through to large-scale protein production. Using this guide will assist in the selection of the most appropriate solution to suit the immediate purification task and possible needs in the future.

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

ÄKTAexplorer

ÄKTAprime



ÄKTApurifier

ÄKTAFPLC

Appendix 3

General instructions for affinity purification with HiTrap columns

Alternative 1. Manual purification with a syringe

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. Allow the column and buffers to reach room temperature.
2. Remove the top cap of the column.
3. Fill the syringe with binding buffer.
4. Connect the column to the syringe using the adapter supplied ('drop to drop' to avoid introducing air into the column).
5. Remove the twist-off end.
6. Equilibrate the column with 5 column volumes of binding buffer.
7. Apply the sample using the syringe. For best results, maintain a flow rate of 0.2–1 ml/min (1 ml column) and 1–5 ml/min (5 ml column) as the sample is applied.
8. Wash with 5–10 column volumes of binding buffer. Maintain flow rates of 1–2 ml/min (1 ml column) and 5–10 ml/min (5 ml column) during the wash.
9. Elute with 5–10 column volumes of elution buffer. Maintain flow rates of 1–2 ml/min (1 ml column) and 5–10 ml/min (5 ml column) during elution.
10. Immediately re-equilibrate the column with 5 ml binding buffer.

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

Alternative 2. Simple purification with ÄKTAprime

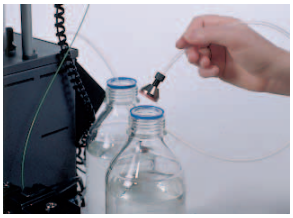
ÄKTAprime contains pre-programmed templates for purification of IgG, IgM and IgY using the appropriate HiTrap columns.

Prepare at least 500 ml of each buffer.

1. Follow instructions supplied on the ÄKTAprime 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.

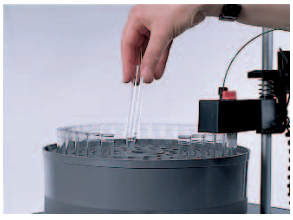
Typical procedures using ÄKTAprime

A. Preparing the buffers

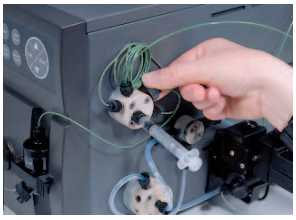


B. Connecting the column

C. Preparing the fraction collector



D. Loading the sample



Appendix 4

Column packing and preparation

Prepacked columns from Amersham Biosciences will ensure reproducible results and the highest performance. However, if column packing is required, the following guidelines will apply at any scale of operation:

- With a high binding capacity medium, short, wide columns can be used for rapid purification, even with low linear flow rates.
- Ready to use affinity media are supplied with details of the binding capacity per ml of medium. Unless otherwise stated, estimate the amount of medium required to bind the target molecules and use two- to five times this amount to pack the column. Refer to the product instructions for more specific information regarding buffers, flow rates etc.
- For affinity media made from pre-activated matrices, determine the binding capacity of the medium. Estimate the amount of medium required to bind the target molecules and use two- to five times this amount to pack the column.

Affinity media can be packed in either Tricorn or XK columns available from Amersham Biosciences. A step-by-step demonstration of column packing can be seen in "The Movie", available in CD format (see Ordering Information).

1. Equilibrate all materials to the temperature at which the separation will be performed.
2. Eliminate air by flushing column end pieces with the recommended buffer. Ensure no air is trapped under the column net. Close column outlet leaving 1–2 cm of buffer in the column.
3. Gently resuspend the medium.

For media not supplied in suspension, use a medium: buffer ratio of approximately 1:2 to produce a suspension for mixing during rehydration.

Avoid using magnetic stirrers since they may damage the matrix.

4. Estimate the amount of slurry (resuspended medium) required on the basis of the recommendations supplied.
5. Pour the required volume of slurry into the column. Pouring down a glass rod held against the wall of the column will minimize the introduction of air bubbles.
6. Immediately fill the column with buffer.
7. Mount the column top piece and connect to a pump.
8. Open the column outlet and set the pump to the desired flow rate.

If the recommended flow rate cannot be obtained, use the maximum flow rate the pump can deliver.

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

9. Maintain the packing flow rate for at least 3 column volumes after a constant bed height is obtained. Mark the bed height on the column.

Do not exceed 75% of the packing flow rate during any purification.

10. Stop the pump and close the column outlet. Remove the top piece and carefully fill the rest of the column with buffer to form an upward meniscus at the top.
11. Insert the adaptor into the column at an angle, ensuring that no air is trapped under the net.
12. Slide the adaptor slowly down the column (the outlet of the adaptor should be open) until the mark is reached. Lock the adaptor in position.
13. Connect the column to the pump and begin equilibration. Re-position the adaptor if necessary.

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

Many media equilibrated with sterile phosphate-buffered saline containing an antimicrobial agent may be stored at +4 °C for up to 1 month, but always follow the specific storage instructions supplied with the product.

Column selection

Tricorn and XK columns are fully compatible with the high flow rates achievable with modern media and a broad range of column dimensions are available. Columns most suitable for packing affinity media are listed below. In most cases the capacity of the affinity medium and the amount of sample to be purified will determine the column size required. For a complete listing refer to the Amersham Biosciences BioDirectory or web catalog (*www.chromatography.amershambiosciences.com*).

Columns	Volume (ml)	Code no
Tricorn 5/20	0.31–0.55	18-1163-08
Tricorn 5/50	0.90–1.14	18-1163-09
Tricorn 10/20	1.26–2.20	18-1163-13
Tricorn 10/50	3.61–4.56	18-1163-14
XK 16/20	2–34	18-8773-01
XK 26/20	0–80	18-1000-72
XK 50/20	0–275	18-1000-71

Appendix 5

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, if essential. Stabilizing agents are often required for storage of purified proteins.
- Serum, culture supernatants and ascitic fluid should be kept frozen at -20 °C or -70 °C, in small aliquots.
- Avoid repeated freeze/thawing or freeze drying/re-dissolving that may reduce biological activity.
- Avoid conditions close to stability limits 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. Above 24 hours at +4 °C, add a preserving agent if possible (e.g. merthiolate 0.01%).

Sodium azide can interfere with many coupling methods and some biological assays and can be a health hazard. It can be removed by using a desalting column (see page 21).

General recommendations for purified proteins

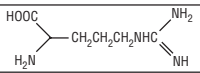
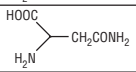
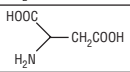
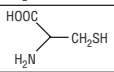
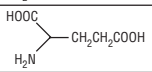
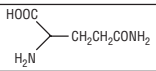
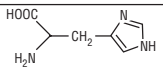
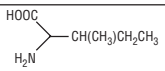
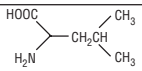
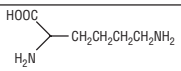
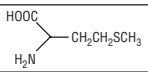
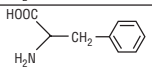
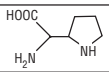
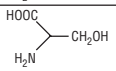
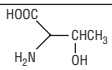
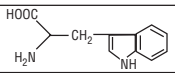
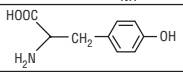
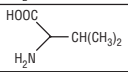
- Store as a precipitate in high concentration of ammonium sulphate, for example 4.0 M.
- Freeze in 50% glycerol, especially suitable for enzymes.
- Avoid the use of preserving agents if the product is to be used for a biological assay. Preserving agents should not be added if *in vivo* experiments are to be performed. Instead store samples in small aliquots and keep frozen.
- Sterile filter to prolong storage time.
- Add stabilizing agents, e.g. glycerol (5–20%), serum albumin (10 mg/ml), ligand (concentration is selected based on concentration of active protein) to help 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/re-dissolving that may reduce biological activity.

Sodium azide can interfere with many coupling methods and some biological assays. It can be removed by using a desalting column (see page 21).

Cryoproteins are a group of proteins, including some mouse antibodies of the IgG_3 subclass, that should not be stored at +4 °C as they precipitate at this temperature. Keep at room temperature in the presence of a preserving agent.

Appendix 6

Table of amino acids

Amino acid	Three-letter code	Single-letter code	Structure
Alanine	Ala	A	
Arginine	Arg	R	
Asparagine	Asn	N	
Aspartic Acid	Asp	D	
Cysteine	Cys	C	
Glutamic Acid	Glu	E	
Glutamine	Gln	Q	
Glycine	Gly	G	
Histidine	His	H	
Isoleucine	Ile	I	
Leucine	Leu	L	
Lysine	Lys	K	
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	
Serine	Ser	S	
Threonine	Thr	T	
Tryptophan	Trp	W	
Tyrosine	Tyr	Y	
Valine	Val	V	

Formula	M_r	Middle unit residue ($-H_2O$) Formula	M_r	Charge at pH 6.0-7.0	Hydrophobic (non-polar)	Uncharged (polar)	Hydrophilic (polar)
$C_3H_7NO_2$	89.1	C_3H_5NO	71.1	Neutral	■		
$C_6H_{14}N_4O_2$	174.2	$C_6H_{12}N_4O$	156.2	Basic (+ve)			■
$C_4H_8N_2O_3$	132.1	$C_4H_6N_2O_2$	114.1	Neutral		■	
$C_4H_7NO_4$	133.1	$C_4H_5NO_3$	115.1	Acidic(-ve)			■
$C_3H_7NO_2S$	121.2	C_3H_5NOS	103.2	Neutral		■	
$C_5H_9NO_4$	147.1	$C_5H_7NO_3$	129.1	Acidic (-ve)			■
$C_5H_{10}N_2O_3$	146.1	$C_5H_8N_2O_2$	128.1	Neutral		■	
$C_2H_5NO_2$	75.1	C_2H_3NO	57.1	Neutral		■	
$C_6H_9N_3O_2$	155.2	$C6H_7N_3O$	137.2	Basic (+ve)			■
$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	■		
$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	■		
$C_6H_{14}N_2O_2$	146.2	$C_6H_{12}N_2O$	128.2	Basic(+ve)			■
$C_5H_{11}NO_2S$	149.2	C_5H_9NOS	131.2	Neutral	■		
$C_9H_{11}NO_2$	165.2	C_9H_9NO	147.2	Neutral	■		
$C_5H_9NO_2$	115.1	C_5H_7NO	97.1	Neutral	■		
$C_3H_7NO_3$	105.1	$C_3H_5NO_2$	87.1	Neutral		■	
$C_4H_9NO_3$	119.1	$C_4H_7NO_2$	101.1	Neutral		■	
$C_{11}H_{12}N_2O_2$	204.2	$C_{11}H_{10}N_2O$	186.2	Neutral	■		
$C_9H_{11}NO_3$	181.2	$C_9H_9NO_2$	163.2	Neutral		■	
$C_5H_{11}NO_2$	117.1	C_5H_9NO	99.1	Neutral	■		

Appendix 7

Converting from linear flow (cm/hour) to volumetric flow rates (ml/min) and *vice versa*

It is convenient when comparing results for columns of different sizes to express flow as linear flow (cm/hour). However, flow is usually measured in volumetric flow rate (ml/min). To convert between linear flow and volumetric flow rate use one of the formulae below.

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

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

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

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/hour?

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

Z = Volumetric flow rate = 1 ml/min

d = column inner diameter = 0.5 cm

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

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

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 x 10^{13} molecules	10 µg
50 000	20 pmol; 1.2 x 10^{13} molecules	50 µg
100 000	10 pmol; 6.0 x 10^{12} molecules	100 µg
150 000	6.7 pmol; 4.0 x 10^{12} molecules	150 µg

Protein	A_{280} for 1 mg/ml
IgG	1.35
IgM	1.20
IgA	1.30
Protein A	0.17
Avidin	1.50
Streptavidin	3.40
Bovine Serum Albumin	0.70

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

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

Column pressures

The maximum operating back pressure refers to the pressure above which the column contents may begin to compress.

Pressure units may be expressed in megaPascals, bar or pounds per square inch and can be converted as follows: 1MPa = 10 bar = 145 psi

Appendix 9

Principles and standard conditions for purification techniques

Affinity Chromatography (AC)

AC separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix. The technique is ideal for a capture or intermediate step and can be used whenever a suitable ligand is available for the protein(s) of interest. AC offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest.

The target protein(s) is specifically and reversibly bound by a complementary binding substance (ligand). The sample is applied under conditions that favour specific binding to the ligand. Unbound material is washed away, and the bound target protein is recovered by changing conditions to those favouring desorption. Desorption is performed specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Samples are concentrated during binding and protein is collected in a purified, concentrated form. The key stages in a purification are shown in Figure 40. Affinity chromatography is also used to remove specific contaminants, for example Benzamidine Sepharose 6B can remove serine proteases.

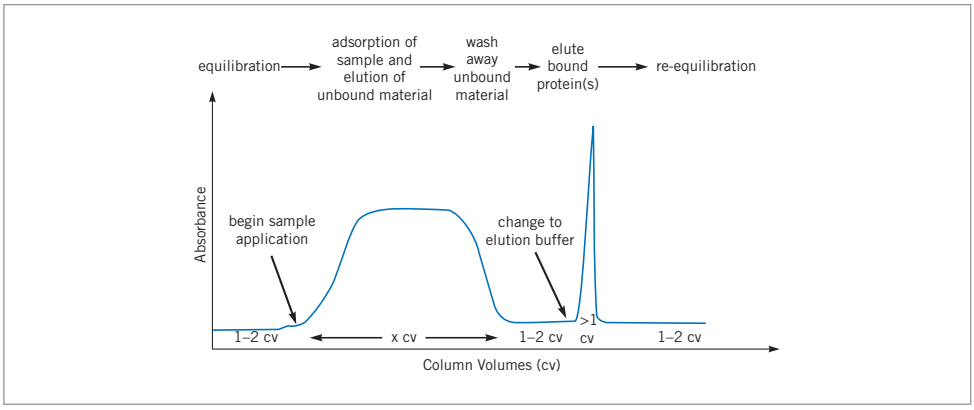


Fig. 40. Typical affinity purification.

General troubleshooting

Column has clogged

Cell debris in the sample may clog the column. Clean the column and ensure that samples have been filtered or centrifuged.

No binding to the purification column

Decrease the flow rate to improve binding.

Check pH and buffer composition.

If re-using a prepacked column, check that the column has been regenerated correctly. Replace with fresh medium or a new column if binding capacity does not return after regeneration.

Column capacity may have been exceeded. If using HiTrap columns (1 ml or 5 ml), link 2 or 3 columns in series to increase capacity or pack a larger column.

Poor elution from the column

Decrease the flow rate to improve elution.

Check pH and buffer composition.

Change to a different eluent.

Further information

Protein Purification Handbook
Affinity Chromatography Handbook: Principles and Methods

Ion exchange (IEX)

IEX separates proteins on the basis of differences in charge to give a very high resolution separation with high sample loading capacity. The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. Proteins bind when 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 by changing pH. Changes are made stepwise or with a continuous gradient. Most commonly, samples are eluted with salt (NaCl), using gradient elution (Figure 41). Target proteins are concentrated during binding and collected in a purified, concentrated form.

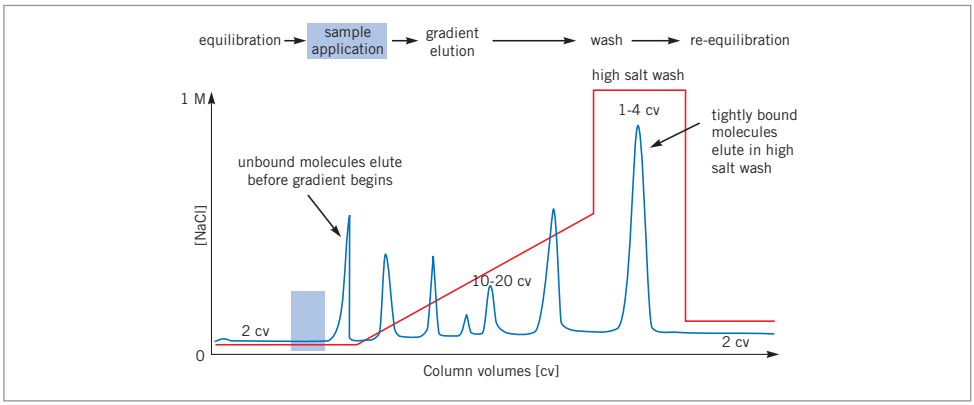


Fig. 41. Typical IEX gradient elution.

The net surface charge of proteins varies according to the surrounding pH. When above its isoelectric point (pI) a protein will bind to an anion exchanger; when below its pI a protein will behind to a cation exchanger. Typically IEX is used to bind the target molecule, but it can also be used to bind impurities if required. IEX can be repeated at different pH values to separate several proteins that have distinctly different charge properties, as shown in Figure 42.

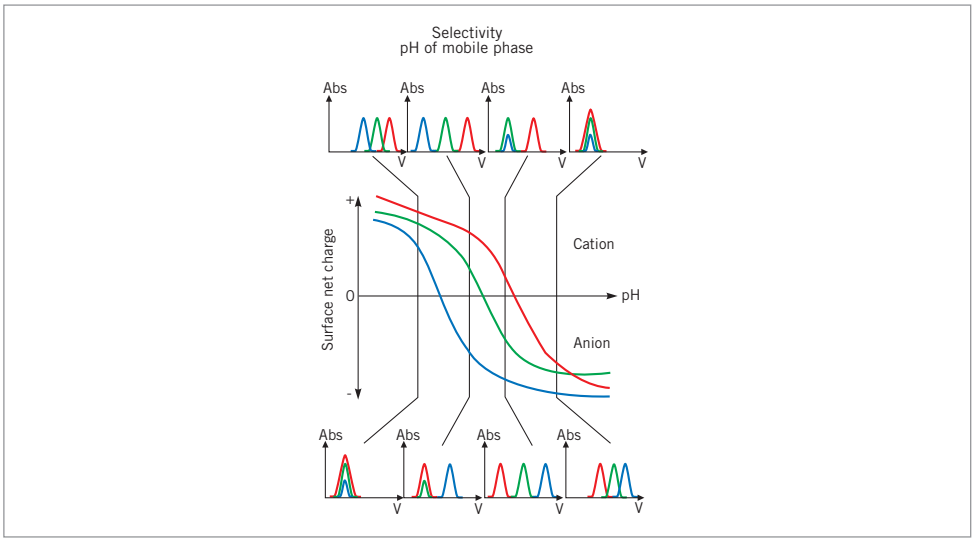


Fig. 42. Effect of pH on protein elution patterns.

Method development (in priority order)

1. Select the optimal ion exchanger using small columns as in the HiTrap IEX Selection Kit to save time and sample.
2. Scout for the optimal pH. Begin 0.5–1 pH unit away from the isoelectric point of the target protein if known.
3. Select the steepest gradient to give acceptable resolution at the selected pH.
4. Select the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.

To reduce separation times and buffer consumption, design and optimize a method for step elution as shown in Figure 43. It is often possible to increase sample loading when using step elution.

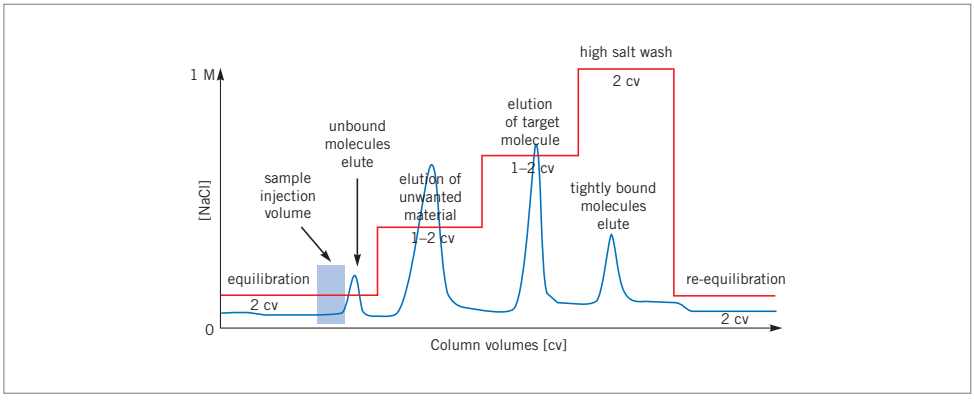


Fig. 43. Step elution.

Further information

Protein Purification Handbook
Ion Exchange Chromatography Handbook: Principles and Methods

Hydrophobic interaction chromatography (HIC)

HIC separates proteins on the basis of differences in hydrophobicity. The technique is ideal for the capture or intermediate steps in a purification. Separation is based on the reversible interaction between a protein and the hydrophobic surface of a chromatographic medium. This interaction is enhanced by high ionic strength buffer which makes HIC an ideal 'next step' after precipitation with ammonium sulphate or elution in high salt during IEX. Samples in high ionic strength solution (e.g. 1.5 M ammonium sulphate) bind as they are loaded onto a column. Conditions are then altered so that the bound substances are eluted differentially.

Elution is usually performed by decreasing in salt concentration (Figure 44). Changes are made stepwise or with a continuous decreasing salt gradient. Most commonly, samples are eluted with a decreasing gradient of ammonium sulphate. Target proteins are concentrated during binding and collected in a purified, concentrated form. Other elution procedures include reducing eluent polarity (ethylene glycol gradient up to 50%), adding chaotropic species (urea, guanidine hydrochloride) or detergents, changing pH or temperature.

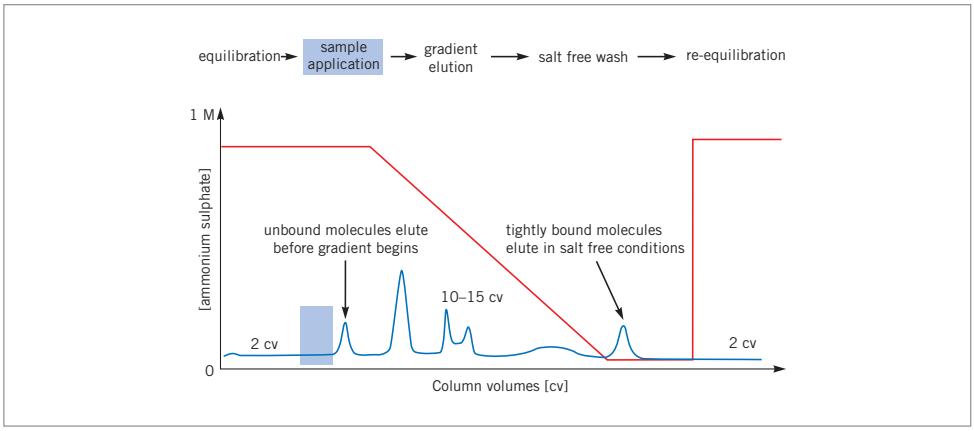


Fig. 44. Typical HIC gradient elution.

Method development (in priority order)

1. The hydrophobic behaviour of a protein is difficult to predict and binding conditions must be studied carefully. Use HiTrap HIC Selection Kit or RESOURCE HIC Test Kit to select the medium that gives the optimal binding and elution over the required range of salt concentration. For proteins with unknown hydrophobic properties begin with 0–100%B (0%B = 1 M ammonium sulphate).
2. Select the gradient that gives acceptable resolution.
3. Select the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.
4. If samples adsorb strongly to a medium, then conditions that cause conformational changes, such as pH, temperature, chaotropic ions or organic solvents can be altered. Conformational changes caused by these agents are specific to each protein. Use screening procedures to investigate the effects of these agents. Alternatively, change to a less hydrophobic medium.

To reduce separation times and buffer consumption, transfer to a step elution after method optimization, as shown in Figure 45. It is often possible to increase sample loading when using step elution, an additional benefit for larger scale purification.

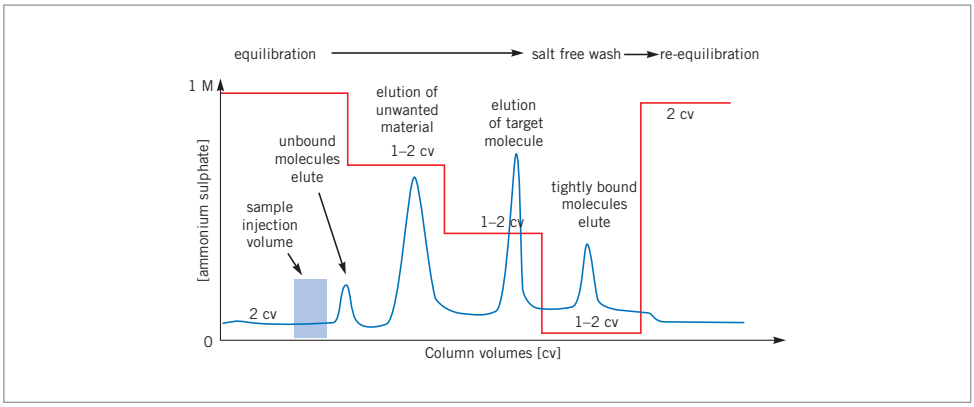


Fig. 45. Step elution.

Further information

Protein Purification Handbook
Hydrophobic Interaction Chromatography Handbook: Principles and Methods

Gel filtration (GF)

GF separates proteins on the basis of differences in molecular size. The technique is ideal for the final polishing steps in purification when sample volumes have been reduced (sample volume significantly influences speed and resolution in gel filtration). Samples are eluted isocratically (single buffer, no gradient Figure 46). Buffer conditions are varied to suit the sample type or the requirements for further purification, analysis or storage step, since buffer composition does not directly affect resolution. Proteins are collected in a purified form in the chosen buffer.

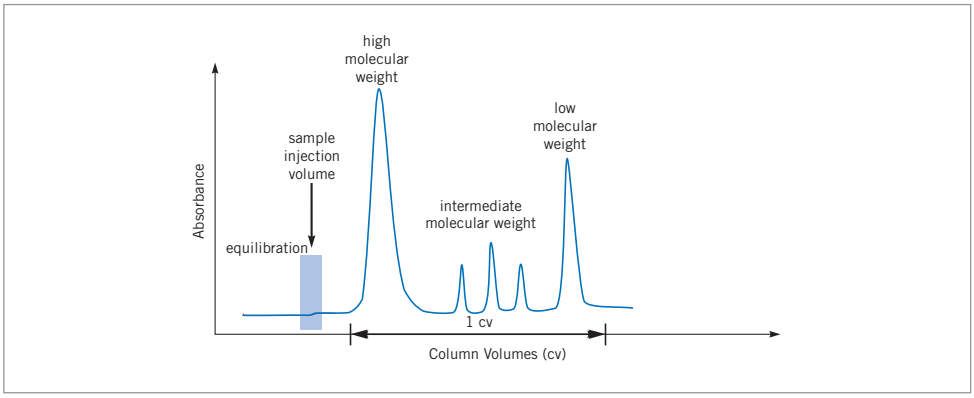


Fig. 46. Typical GF elution.

Further information

Protein Purification Handbook
Gel Filtration Handbook: Principles and Methods

Reversed phase chromatography (RPC)

RPC separates proteins and peptides with differing hydrophobicity based on their reversible interaction with the hydrophobic surface of a chromatographic medium. Samples bind when they are loaded onto a column. Conditions are then altered so that the bound substances are eluted differentially. Due to the nature of the reversed phase matrices, the binding is usually very strong and requires the use of organic solvents and other additives (ion pairing agents). Elution is usually performed by increases in organic solvent concentration, most commonly acetonitrile. Samples, which are concentrated during the binding and separation process, are collected in a purified, concentrated form. The key stages in a separation are shown in Figure 47.

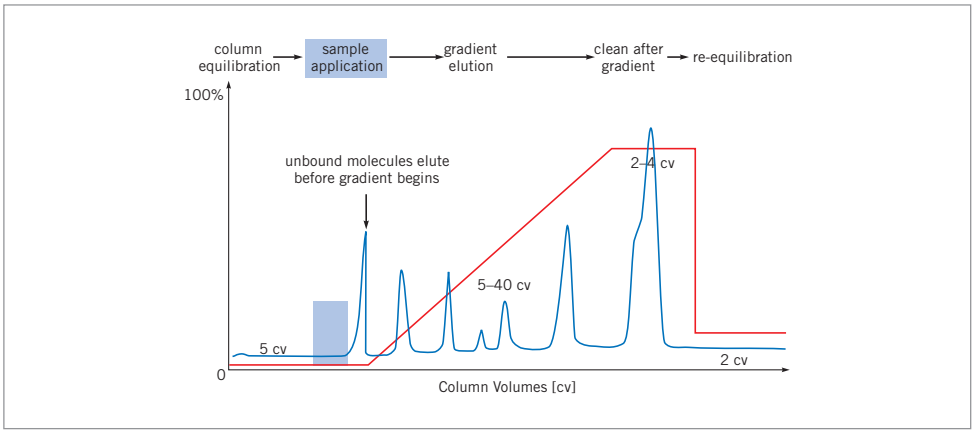


Fig. 47. Typical RPC gradient elution.

RPC is often used in the final polishing of oligonucleotides and peptides and is ideal for analytical separations, such as peptide mapping.

RPC is not recommended for protein purification if recovery of activity and return to a correct tertiary structure are required, since many proteins are denatured in the presence of organic solvents.

Method Development

1. Select the medium from screening results.
2. Select the optimal gradient to give acceptable resolution. For unknown samples begin with 0–100% elution buffer.
3. Select the 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 from a less hydrophobic medium.

Further information

Protein Purification Handbook
Reversed Phase Chromatography Handbook: Principles and Methods

Expanded bed adsorption (EBA)

EBA is a single pass operation in which target proteins are purified from crude sample, without the need for separate clarification, concentration and initial purification to remove particulate matter. Crude sample is applied to an expanded bed of STREAMLINE adsorbent particles within a specifically designed STREAMLINE column. Target proteins are captured on the adsorbent. Cell debris, cells, particulate matter, whole cells, and contaminants pass through and target proteins are then eluted. Figure 48 shows a representation of the steps involved in an EBA purification and Figure 49 shows a typical EBA elution.

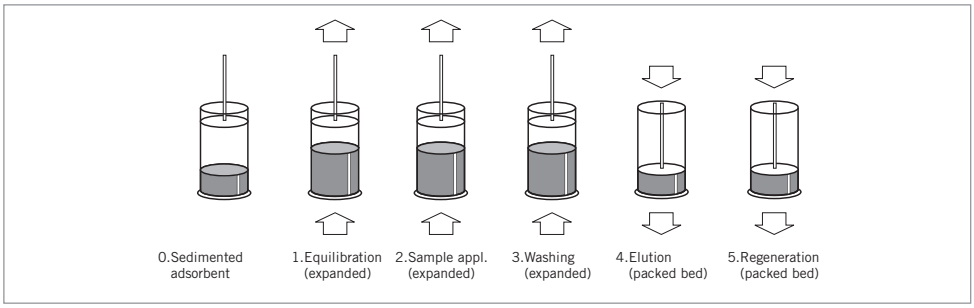


Fig. 48. Steps in an EBA purification process.

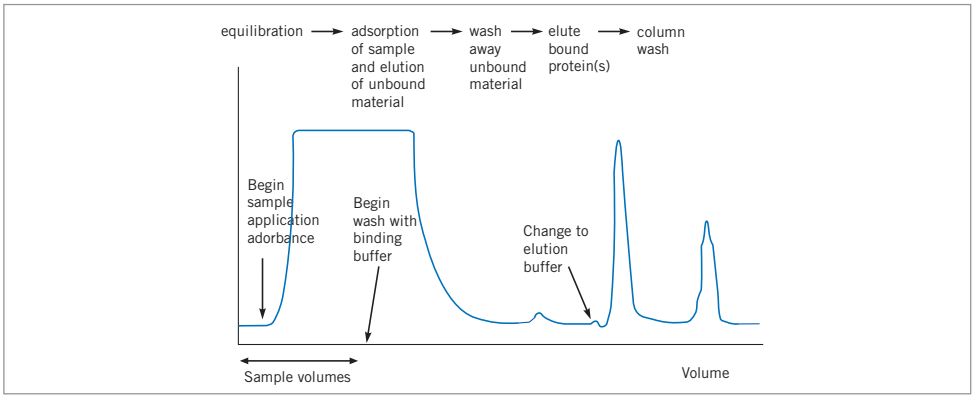


Fig. 49. Typical EBA elution.

Method development

1. Select suitable ligand to bind the target protein.
2. Scout for the optimal binding and elution conditions using clarified material in a packed column (0.02–0.15 liters bed volume of media). Gradient elution may be used during scouting, but the goal is to develop a step elution.
3. Optimize binding, elution, wash and cleaning-in-place procedures using unclarified sample in expanded mode at small-scale (0.02–0.15 liters bed volume of media).
4. Begin scale up process at pilot scale (0.2–0.9 liters bed volume of media).
5. Full scale production (up to several hundred liters bed volume of media).

Further information

Protein Purification Handbook
Expanded Bed Adsorption Handbook: Principles and Methods

Product index

Additional reading

	Code No.
Purification	
Protein Purification Handbook	18-1132-29
Recombinant Protein Handbook: Protein Amplification and Simple Purification	18-1142-75
GST Gene Fusion System Handbook	18-1157-58
Gel Filtration Handbook: Principles and Methods	18-1022-18
Ion Exchange Chromatography Handbook: Principles and Methods	18-1114-21
Hydrophobic Interaction Chromatography Handbook: Principles and Methods	18-1020-90
Affinity Chromatography Handbook: Principles and Methods	18-1022-29
Reversed Phase Chromatography Handbook: Principles and Methods	18-1134-16
Expanded Bed Adsorption Handbook: Principles and Methods	18-1124-26
Protein and Peptide Purification Technique Selection Guide	18-1128-63
Fast Desalting and Buffer Exchange of Proteins and Peptides	18-1128-62
Gel Filtration Columns and Media Selection Guide	18-1124-19
Ion Exchange Columns and Media Selection Guide	18-1127-31
HIC Columns and Media Product Profile	18-1100-98
Affinity Columns and Media Product Profile	18-1121-86
Convenient Protein Purification, HiTrap Column Guide	18-1129-81
HiTrap Protein A HP and HiTrap Protein G HP Data File	18-1134-76
HiTrap IgM Purification HP Data File	18-1127-43
HiTrap IgY Purification HP Data File	18-1127-42
HiTrap NHS-activated HP Data File	18-1134-80
MAbTrap Kit Data File	18-1034-14
MabSelect Data File	18-1149-94
ÄKTA design Brochure	18-1158-77
GST Gene Fusion System Brochure	18-1159-30
Column Packing CD, "The Movie"	18-1165-33
Rapid optimization and development of an automated two-step purification procedure for monoclonal IgG antibodies	18-1128-93
Rapid development of a purification process for a recombinant antigen binding fragment expressed in *E. coli*	18-1111-23
Analysis	
Gel Media Guide (electrophoresis)	18-1129-79
2D Electrophoresis Handbook	80-6429-60
Protein Electrophoresis Technical Manual	80-6013-88
ECL Western and ECL Plus Western Blotting Application Note	18-1139-13

Many of these items can be downloaded from ***www.chromatography.amershambiosciences.com***

References

Reference lists are available from ***www.chromatography.amershambiosciences.com***

	Code No.
Reference list MAbTrap Kit	18-1156-71
Reference list HiTrap Protein G HP	18-1156-72
Reference list HiTrap Protein A HP	18-1156-73
Reference list HiTrap rProtein A HP	18-1156-74
Reference list HiTrap Desalting	18-1156-70
Reference list HiPrep 26/10 Desalting	18-1156-89
Reference list HiLoad Superdex 200 pg	18-1156-96
Reference list HiLoad Superdex 75 pg	18-1156-95
Reference list HiLoad Superdex 30 pg	18-1156-94

General reading

Chamow, S. and Ashkenazi, A. (eds.) *Antibody Fusion Proteins*, John Wiley and Sons Inc. Publisher, New York (1999)

Harlow, E. and Lane, D. (eds.) *Using Antibodies: a laboratory manual*, Cold Spring Harbor Laboratory Press, New York (1998)

Gagnon, P., *Purification Tools for Monoclonal Antibodies*, Validated Biosystems, Inc.,Tucson (1996)

Ordering information

Product	Quantity	Code No.
Affinity: prepacked columns		
HiTrap rProtein A FF	5 × 1 ml	17-5079-01
	1 × 5 ml	17-5080-01
	2 × 1 ml	17-5079-02
HiTrap Protein A HP	5 × 1 ml	17-0402-01
	2 × 1 ml	17-0402-03
	1 × 5 ml	17-0403-01
HiTrap Protein G HP	5 × 1 ml	17-0404-01
	2 × 1 ml	17-0404-03
	1 × 5 ml	17-0405-01
MAbTrap Kit	HiTrap Protein G HP (1 × 1 ml), accessories, pre-made buffers for 10 purifications	17-1128-01
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	17-0716-01
	1 × 5 ml	17-0717-01
GSTrap FF	2 × 1 ml	17-5130-02
	5 × 1 ml	17-5130-01
	1 × 5 ml	17-5131-01
GSTPrep FF 16/10	1 × 20 ml	17-5234-01
HisTrap Kit	3 × 1 ml HiTrap Chelating HP columns, pre-made buffers and accessories for up to 12 purifications	17-1880-01
HiTrap Chelating HP	5 × 1 ml	17-0408-01
	1 × 5 ml	17-0409-01
HiTrap Blue HP	5 × 1 ml	17-0412-01
	1 × 5 ml	17-0413-01
Affinity: loose media (larger quantities available on request)		
Immunoprecipitation Starter Pack Protein A Sepharose 4 Fast Flow Protein G Sepharose 4 Fast Flow	2 × 2 ml	17-6002-35
Protein A Sepharose 4 Fast Flow	5 ml	17-0974-01
	25 ml	17-0974-04
rProtein A Sepharose 4 Fast Flow	5 ml	17-1279-01
	25 ml	17-1279-02
MabSelect	25 ml	17-5199-01
	200 ml	17-5199-02
rmpProtein A Sepharose Fast Flow	5ml	17-5138-01
	25 ml	17-5138-02
STREAMLINE rProtein A	75 ml	17-1281-01
Protein A Sepharose 6MB	10 ml	17-0469-01
Protein G Sepharose 4 Fast Flow	5 ml	17-0618-01
	25 ml	17-0618-02
NHS-activated Sepharose Fast Flow	25 ml	17-0906-01
CNBr-activated Sepharose 4 Fast Flow	10 g	17-0981-01
Glutathione Sepharose 4 Fast Flow	25 ml	17-5132-01
	100 ml	17-5132-02
	500 ml	17-5132-03
Chelating Sepharose Fast Flow	50 ml	17-0575-01
IgG Sepharose 6 Fast Flow	10 ml	17-0969-01
Blue Sepharose 6 Fast Flow	50 ml	17-0948-01

Product	Quantity	Code No.
IEX: 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	17-1153-01
	5 × 5 ml	17-1154-01
HiTrap SP HP	5 × 1 ml	17-1151-01
	5 × 5 ml	17-1152-01
HiTrap Q XL	5 × 1 ml	17-5158-01
	5 × 5 ml	17-5159-01
HiTrap SP XL	5 × 1 ml	17-5160-01
	5 × 5 ml	17-5161-01
HiTrap ANX FF (high sub)	5 × 1 ml	17-5162-01
	5 × 5 ml	17-5163-01
HiTrap DEAE FF	5 × 1 ml	17-5055-01
	5 × 5 ml	17-5154-01
HiTrap CM FF	5 × 1 ml	17-5056-01
	5 × 5 ml	17-5155-01
HiTrap Q FF	5 × 1 ml	17-5053-01
	5 × 5 ml	17-5156-01
HiTrap SP FF	5 × 1 ml	17-5054-01
	5 × 5 ml	17-5157-01
HiPrep 16/10 Q FF	1 × 20 ml	17-5190-01
HiPrep 16/10 SP FF	1 × 20 ml	17-5192-01
HiPrep 16/10 ANX FF (high sub)	1 × 20 ml	17-5191-01
HiPrep 16/10 SP XL	1 × 20 ml	17-5093-01
HiPrep 16/10 Q XL	1 × 20 ml	17-5092-01
HiPrep 16/10 DEAE FF	1 × 20 ml	17-5090-01
HiPrep 16/10 CM FF	1 × 20 ml	17-5091-01
Mono Q 5/50 GL (Tricorn)	1 × 1 ml	17-5166-01
Mono S 5/50 GL (Tricorn)	1 × 1 ml	17-5168-01
IEX: loose media		
Q Sepharose Fast Flow	25 ml	17-0510-10
	300 ml	17-0510-01
SP Sepharose Fast Flow	25 ml	17-0729-10
	300 ml	17-0729-01
DEAE Sepharose Fast Flow	25 ml	17-0709-10
	500 ml	17-0709-01
CM Sepharose Fast Flow	25 ml	17-0719-10
	500 ml	17-0719-01
ANX Sepharose 4 Fast Flow (high sub)	25 ml	17-1287-10
	500 ml	17-1287-01
Q Sepharose High Performance	75 ml	17-1014-01
SP Sepharose High Performance	75 ml	17-1087-01
SOURCE 15Q	10 ml	17-0947-20
	50 ml	17-0974-01
SOURCE 15S	10 ml	17-0944-10
	50 ml	17-0944-01
STREAMLINE SP XL	100 ml	17-5076-05

Product	Quantity	Code No.
HIC: prepacked columns		
HiTrap HIC Selection Kit HiTrap Phenyl HP HiTrap Phenyl FF (low sub) HiTrap Phenyl FF (high sub) HiTrap Butyl FF HiTrap Octyl FF	5 x 1 ml	17-1349-01
HIC: prepacked columns		
HiTrap Phenyl FF (high sub)	5 × 1 ml	17-1355-01
	5 × 5 ml	17-5193-01
HiTrap Phenyl FF (low sub)	5 × 1 ml	17-1353-01
	5 × 5 ml	17-5194-01
HiTrap Phenyl HP	5 × 1 ml	17-1351-01
	5 × 5 ml	17-5195-01
HiTrap Butyl FF	5 × 1 ml	17-1357-01
	5 × 5 ml	17-5197-01
HiTrap Octyl FF	5 × 1 ml	17-1359-01
	5 × 5 ml	17-5196-01
HiPrep 16/10 Phenyl FF (high sub)	1 × 20 ml	17-5095-01
HiPrep 16/10 Phenyl FF (low sub)	1 × 20 ml	17-5094-01
HiPrep 16/10 Butyl FF	1 × 20 ml	17-5096-01
HiPrep 16/10 Octyl FF	1 × 20 ml	17-5097-01
HiLoad 16/10 Phenyl Sepharose HP	1 × 20 ml	17-1085-01
HiLoad 26/10 Phenyl Sepharose HP	1 × 53 ml	17-1086-01
HIC: loose media		
Phenyl Sepharose 6 Fast Flow (high sub)	25 ml	17-0973-10
	200 ml	17-0973-05
Phenyl Sepharose 6 Fast Flow (low sub)	25 ml	17-0965-10
	200 ml	17-0965-05
Butyl Sepharose 4 Fast Flow	25 ml	17-0980-10
	200 ml	17-0980-01
Octyl Sepharose 4 Fast Flow	25 ml	17-0946-10
	200 ml	17-0946-02
Phenyl Sepharose High Performance	75 ml	17-1082-01
SOURCE 15ETH	50 ml	17-0146-01
SOURCE 15ISO	50 ml	17-0148-01
SOURCE 15PHE	50 ml	17-0147-01
Gel Filtration: prepacked columns (desalting and buffer exchange)		
PD-10 Desalting	30 columns	17-0851-01
HiTrap Desalting	5 × 5 ml	17-1408-01
HiPrep 26/10 Desalting	1 × 53 ml	17-5097-01
(high resolution)		
Superdex 200 10/300 GL (Tricorn)	1 × 24 ml	17-5175-01
HiLoad 16/60 Superdex 200 pg	1 × 120 ml	17-1069-01
HiLoad 26/60 Superdex 200 pg	1 × 320 ml	17-1071-01
Superdex 75 10/300 GL (Tricorn)	1 × 24 ml	17-5174-01
HiLoad 16/60 Superdex 75 pg	1 × 120 ml	17-1068-01
HiLoad 26/60 Superdex 75 pg	1 × 320 ml	17-1070-01
Superdex Peptide 10/300 GL (Tricorn)	1 × 24 ml	17-5176-01
HiLoad 16/60 Superdex 30 pg	1 × 120 ml	17-1139-01
HiLoad 26/60 Superdex 30 pg	1 × 320 ml	17-1140-01

Product	Quantity	Code No.
Gel filtration: loose media		
Superdex 200 prep grade	25 ml	17-1043-10
	150 ml	17-1043-01
Superdex 75 prep grade	25 ml	17-1044-10
	150 ml	17-1044-01
Superdex 30 prep grade	25 ml	17-0905-10
	150 ml	17-0905-01
Western Blotting		
Hybond P	10 sheets	RPN2020F
Hybond ECL	10 sheets	RPN2020D
ECL Western Blotting Detection Reagents	for 1000 cm^2	RPN2109
ECL Plus Western Blotting Detection System	for 1000 cm^2	RPN2132

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