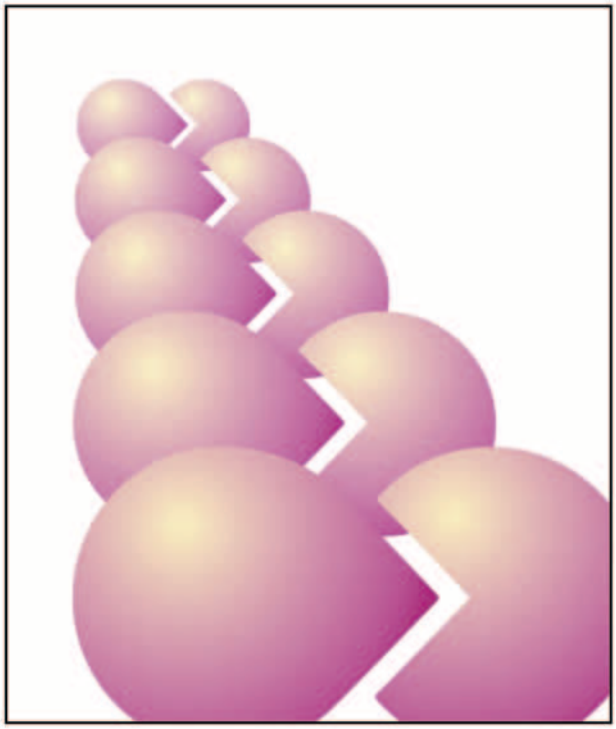
Affinity Chromatography

Principles and Methods



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Affinity Chromatography

Principles and Methods

Contents

Introduction

Biomolecules are purified using purification techniques that separate according to differences in specific properties, as shown in Figure 1.

Property	Technique
Biorecognition (ligand specificity)	Affinity chromatography
Charge	Ion exchange chromatography
Size	Gel filtration (sometimes called size exclusion)
Hydrophobicity	Hydrophobic interaction chromatography Reversed phase chromatography

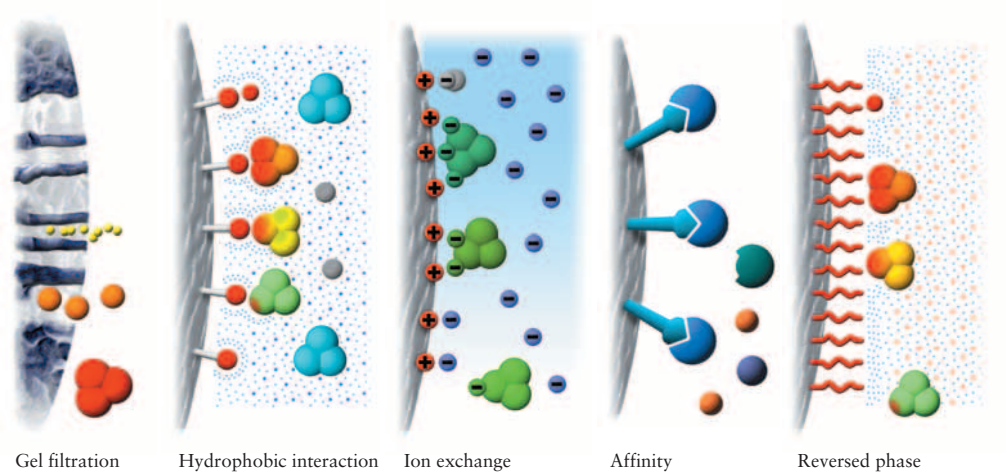


Fig. 1. Separation principles in chromatographic purification.

Affinity chromatography 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 offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest. Purification can be in the order of several thousand-fold and recoveries of active material are generally very high.

Affinity chromatography is unique in purification technology since it is the only technique that enables the purification of a biomolecule on the basis of its biological function or individual chemical structure. Purification that would otherwise be time-consuming, difficult or even impossible using other techniques can often be easily achieved with affinity chromatography. The technique can be used to separate active biomolecules from denatured or functionally different forms, to isolate pure substances present at low concentration in large volumes of crude sample and also to remove specific contaminants.

Amersham Biosciences offers a wide variety of prepacked columns, ready to use media, and pre-activated media for ligand coupling.

This handbook describes the role of affinity chromatography in the purification of biomolecules, the principle of the technique, the media available and how to select them, application examples and detailed instructions for the most commonly performed procedures. Practical information is given as a guide towards obtaining the best results.

The illustration on the inside cover shows the range of handbooks that have been produced by Amersham Biosciences to ensure that purification with any chromatographic technique becomes a simple and efficient procedure at any scale and in any laboratory.

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.

PBS phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4).

Chapter 1
Affinity chromatography in brief

Affinity chromatography 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 in a purification protocol and can be used whenever a suitable ligand is available for the protein(s) of interest. With high selectivity, hence high resolution, and high capacity for the protein(s) of interest, purification levels in the order of several thousand-fold with high recovery of active material are achievable. Target protein(s) is collected in a purified, concentrated form.

Biological interactions between ligand and target molecule can be a result of electrostatic or hydrophobic interactions, van der Waals' forces and/or hydrogen bonding. To elute the target molecule from the affinity medium the interaction can be reversed, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity.

In a single step, affinity purification can offer immense time-saving over less selective multi-step procedures. The concentrating effect enables large volumes to be processed. Target molecules can be purified from complex biological mixtures, native forms can be separated from denatured forms of the same substance and small amounts of biological material can be purified from high levels of contaminating substances.

For an even higher degree of purity, or when there is no suitable ligand for affinity purification, an efficient multi-step process must be developed using the purification strategy of Capture, Intermediate Purification and Polishing (CIPP). When applying this strategy affinity chromatography offers an ideal capture or intermediate step in any purification protocol and can be used whenever a suitable ligand is available for the protein of interest.

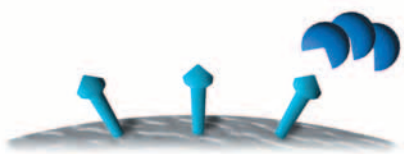
Successful affinity purification requires a biospecific ligand that can be covalently attached to a chromatography matrix. The coupled ligand must retain its specific binding affinity for the target molecules and, after washing away unbound material, the binding between the ligand and target molecule must be reversible to allow the target molecules to be removed in an active form. Any component can be used as a ligand to purify its respective binding partner. Some typical biological interactions, frequently used in affinity chromatography, are listed below:

- Enzyme ⇔ substrate analogue, inhibitor, cofactor.
- Antibody ⇔ antigen, virus, cell.
- Lectin ⇔ polysaccharide, glycoprotein, cell surface receptor, cell.
- Nucleic acid ⇔ complementary base sequence, histones, nucleic acid polymerase, nucleic acid binding protein.
- Hormone, vitamin ⇔ receptor, carrier protein.
- Glutathione ⇔ glutathione-S-transferase or GST fusion proteins.
- Metal ions ⇔ Poly (His) fusion proteins, native proteins with histidine, cysteine and/or tryptophan residues on their surfaces.

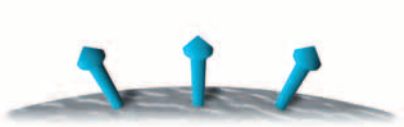
Affinity chromatography is also used to remove specific contaminants, for example Benzamidine Sepharose™ 6 Fast Flow can remove serine proteases, such as thrombin and Factor Xa. Figure 2 shows the key stages in an affinity purification.

1. Affinity medium is equilibrated in binding buffer.

2. Sample is applied under conditions that favor specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column.



3. Target protein is recovered by changing conditions to favor elution of the bound molecules. Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form.



4. Affinity medium is re-equilibrated with binding buffer.

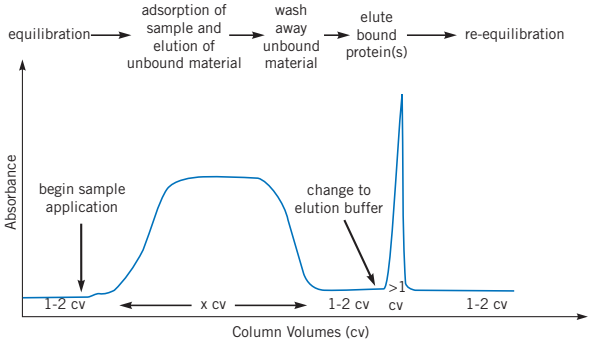


Fig. 2. Typical affinity purification.

The high selectivity of affinity chromatography enables many separations to be achieved in one simple step, including, for example, common operations such as the purification of monoclonal antibodies or fusion proteins. A wide variety of prepacked columns, ready to use media, and pre-activated media for ligand coupling through different functional groups, makes affinity chromatography readily available for a broad range of applications.

To save time, the HiTrap™ column range (Table 1) is excellent for routine laboratory scale applications in which the risk of cross-contamination between samples must be eliminated, for purification from crude samples or for fast method development before scaling up purification. HiTrap columns can be operated with a syringe, a peristaltic pump or any ÄKTA™design chromatography system. Several HiTrap columns can be connected in series to increase purification capacity and all columns are supplied with detailed protocols for use.

Table 1. HiTrap and HiPrep™ affinity columns for laboratory scale purification.

Application	HiTrap and HiPrep columns
Isolation of human immunoglobulins	
IgG, fragments and subclasses	HiTrap rProtein A FF, 1 ml and 5 ml
IgG, fragments and subclasses	HiTrap Protein A HP, 1 ml and 5 ml
IgG, fragments and subclasses including human IgG_3 strong affinity for monoclonal mouse IgG_1 and rat IgG	HiTrap Protein G HP, 1 ml and 5 ml MAbTrap™ Kit
Avian IgY from egg yolk	HiTrap IgY Purification HP, 5 ml
Mouse and human IgM	HiTrap IgM Purification HP, 1 ml
Purification of fusion proteins	
$(His)_6$ fusion proteins	HisTrap™ Kit HiTrap Chelating HP, 1 ml and 5 ml
GST fusion proteins	GSTrap™ FF, 1 ml and 5 ml GSTPrep™ FF 16/10, 20 ml
Other Group Specific Media	
Albumin and nucleotide-requiring enzymes	HiTrap Blue HP, 1 ml and 5 ml
Proteins and peptides with exposed His, Cys or Trp	HiTrap Chelating HP, 1 ml and 5 ml
Biotinylated substances	HiTrap Streptavidin HP, 1 ml
DNA binding proteins and coagulation factors	HiTrap Heparin HP, 1 ml and 5 ml HiPrep 16/10 Heparin FF, 20 ml
Trypsin-like serine proteases including Factor Xa, thrombin and trypsin	HiTrap Benzamidine FF (high sub), 1 ml and 5 ml
Matrix for preparation of affinity media. Coupling via primary amines	HiTrap NHS-activated HP, 1 ml and 5 ml

BioProcess Media for large-scale 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 BioProcess 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.

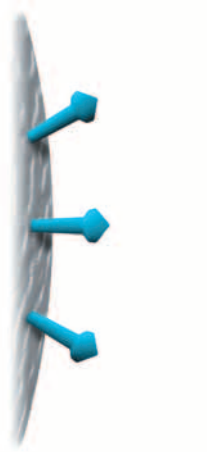
Please refer to the latest *BioProcess Products Catalog* from Amersham Biosciences for further details of our products and services for large-scale production.

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.

Common terms in affinity chromatography



Matrix: for ligand attachment. Matrix should be chemically and physically inert.

Spacer arm: used to improve binding between ligand and target molecule by overcoming any effects of steric hindrance.

Ligand: molecule that binds reversibly to a specific target molecule or group of target molecules.

Binding: buffer conditions are optimized to ensure that the target molecules interact effectively with the ligand and are retained by the affinity medium as all other molecules wash through the column.

Elution: buffer conditions are changed to reverse (weaken) the interaction between the target molecules and the ligand so that the target molecules can be eluted from the column.

Wash: buffer conditions that wash unbound substances from the column without eluting the target molecules or that re-equilibrate the column back to the starting conditions (in most cases the binding buffer is used as a wash buffer).

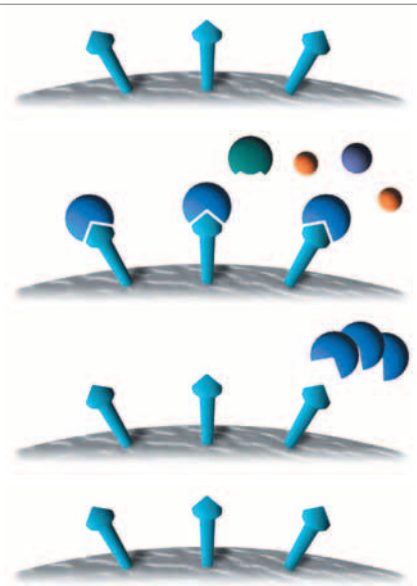
Ligand coupling: covalent attachment of a ligand to a suitable pre-activated matrix to create an affinity medium.

Pre-activated matrices: matrices which have been chemically modified to facilitate the coupling of specific types of ligand.

Chapter 2
Affinity chromatography in practice

This chapter provides guidance and advice that is generally applicable to any affinity purification. The first step towards a successful purification is to determine the availability of a suitable ligand that interacts reversibly with the target molecule or group of molecules. Ready to use affinity media, often supplied with complete separation protocols, already exist for many applications. The contents section of this handbook lists the full range of affinity media from Amersham Biosciences according to the specific molecule or group of molecules to be purified. Application- and product-specific information and advice for these media are supplied in other sections of this handbook. Practical information specific to the use of pre-activated matrices for the preparation of affinity medium is covered in Chapter 5.

Purification steps



1. Affinity medium is equilibrated in binding buffer.

2. Sample is applied under conditions that favor specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column.

3. Target protein is recovered by changing conditions to favor elution of the bound molecules. Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form.

4. Affinity medium is re-equilibrated with binding buffer.

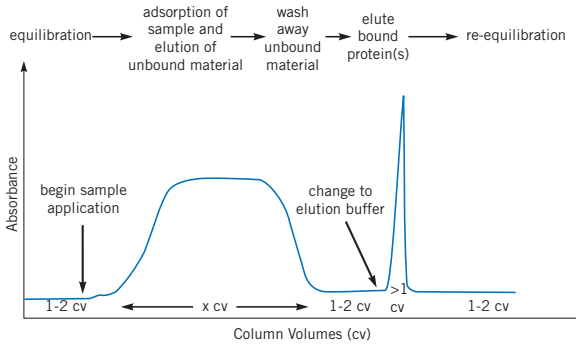


Fig. 3. Typical affinity purification.

Figure 4 shows the simple procedure used to perform affinity purification on prepacked HiTrap columns.

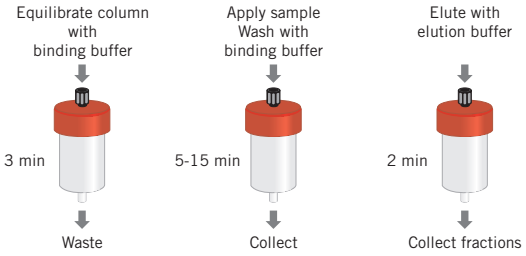


Fig. 4.

HiTrap columns may be used with a syringe, a peristaltic pump or a liquid chromatography system (see Selection of Purification Equipment, Appendix 2) and are supplied with a detailed protocol to ensure optimum results.

Media selection

A ligand already coupled to a matrix is the simplest solution. Selecting prepacked columns such as HiTrap or HiPrep will not only be more convenient, but will also save time in method optimization as these columns are supplied with detailed instructions for optimum performance.

If a ligand is available, but needs to be coupled to a pre-activated matrix, refer to Chapter 5.

If no suitable ligand is available, decide whether it is worth the time and effort involved to obtain a ligand and to develop a specific affinity medium. In many cases, it may be more convenient to use alternative purification techniques such as ion exchange or hydrophobic interaction chromatography.

Preparation of media and buffers

Storage solutions and preservatives should be washed away thoroughly before using any affinity medium. Re-swell affinity media supplied as freeze-dried powders in the correct buffer as recommended by the manufacturer.

Use high quality water and chemicals. Solutions should be filtered through 0.45 µm or 0.22 µm filters.

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

If an affinity medium is to be used routinely, care must be taken to ensure that any contaminants from the crude sample can be removed by procedures that do not damage the ligand.

Binding and elution buffers are specific for each affinity medium since it is their influence on the interaction between the target molecule and the ligand that facilitates the affinity-based separation. Some affinity media may also require a specific buffer in order to make the medium ready for use again.

Avoid using magnetic stirrers as they may damage the matrix. Use mild rotation or end-over-end stirring.

Sample preparation and application

Samples should be clear and free from particulate matter. Simple steps to clarify a sample before beginning purification will avoid clogging the column, may reduce the need for stringent washing procedures and can extend the life of the chromatographic medium. Appendix 1 contains an overview of sample preparation techniques.

If possible, test the affinity of the ligand: target molecule interaction. Too low affinity will result in poor yields since the target protein may wash through or leak from the column during sample application. Too high affinity will result in low yields since the target molecule may not dissociate from the ligand during elution.

Binding of the target protein may be made more efficient by adjusting the sample to the composition and pH of the binding buffer: perform a buffer exchange using a desalting column or dilute in binding buffer (see page 133). When working with very weak affinity interactions that are slow to reach equilibrium, it may be useful to stop the flow after applying sample in order to allow more time for the interaction to take place before continuing to wash the column. In some cases, applying the sample in aliquots may be beneficial.

Sample preparation techniques should ensure that components known to interfere with binding (the interaction between the target molecule and the ligand) are removed.

Since affinity chromatography is a binding technique, the sample volume does not affect the separation as long as conditions are chosen to ensure that the target protein binds strongly to the ligand.

It may be necessary to test for a flow rate that gives the most efficient binding during sample application since this parameter can vary according to the specific interaction between the target protein and the ligand and their concentrations.

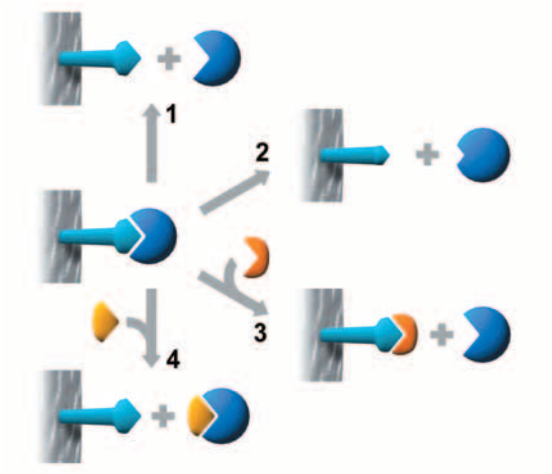
The column must be pre-equilibrated in binding buffer before beginning sample application. For interactions with strong affinity between the ligand and the target molecule that quickly reach equilibrium, samples can be applied at a high flow rate. However, for interactions with weak affinity and/or slow equilibrium, a lower flow rate should be used. The optimal flow rate to achieve efficient binding may vary according to the specific interaction and should be determined when necessary. Further details on the kinetics involved in binding and elution from affinity media are covered in Appendix 7.

When working with very weak affinity interactions that are slow to reach equilibrium, it may be useful to stop the flow after applying the sample to allow more time for the interaction to take place before continuing to wash the column. In some cases, applying the sample in aliquots may be beneficial.

Do not begin elution of target substances until all unbound material has been washed through the column by the binding buffer (determined by UV absorbance at 280 nm). This will improve the purity of the eluted target substance.

Elution

There is no generally applicable elution scheme for all affinity media. Reference to manufacturer's instructions, the scientific literature and a few simple rules should result in an effective elution method that elutes the target protein in a concentrated form. Elution methods may be either selective or non-selective, as shown in Figure 5.



Method 1
The simplest case. A change of buffer composition elutes the bound substance without harming either it or the ligand.

Method 2
Extremes of pH or high concentrations of chaotropic agents are required for elution, but these may cause permanent or temporary damage.

Methods 3 and 4
Specific elution by addition of a substance that competes for binding. These methods can enhance the specificity of media that use group-specific ligands.

Fig. 5. Elution methods.

When substances are very tightly bound to the affinity medium, it may be useful to stop the flow for some time after applying eluent (10 min. to 2 h is commonly used) before continuing elution. This gives more time for dissociation to take place and thus helps to improve recoveries of bound substances.

Selective elution methods are applied in combination with group-specific ligands whereas non-selective elution methods are used in combination with highly specific ligands. Forces that maintain the complex include electrostatic interactions, hydrophobic effects and hydrogen bonding. Agents that weaken these interactions may be expected to function as efficient eluting agents.

The optimal flow rate to achieve efficient elution may vary according to the specific interaction and should be determined when necessary. Further details on the kinetics involved in binding and elution of target molecules from affinity media are covered in Appendix 7.

A compromise may have to be made between the harshness of the eluent required for elution and the risk of denaturing the eluted material or damaging the ligand on the affinity medium.

Ready to use affinity media from Amersham Biosciences are supplied with recommendations for the most suitable elution buffer to reverse the interaction between the ligand and target protein of the specific interaction. Each of these recommendations will be based on one of the following elution methods:

pH elution

A change in pH alters the degree of ionization of charged groups on the ligand and/or the bound protein. This change may affect the binding sites directly, reducing their affinity, or cause indirect changes in affinity by alterations in conformation.

A step decrease in pH is the most common way to elute bound substances. The chemical stability of the matrix, ligand and target protein determines the limit of pH that may be used.

If low pH must be used, collect fractions into neutralization buffer such as 1 M Tris-HCl, pH 9 (60–200 µl per ml eluted fraction) to return the fraction to a neutral pH. The column should also be re-equilibrated to neutral pH immediately.

Ionic strength elution

The exact mechanism for elution by changes in ionic strength will depend upon the specific interaction between the ligand and target protein. This is a mild elution using a buffer with increased ionic strength (usually NaCl), applied as a linear gradient or in steps.

Enzymes usually elute at a concentration of 1 M NaCl or less.

Competitive elution

Selective eluents are often used to separate substances on a group specific medium or when the binding affinity of the ligand/target protein interaction is relatively high. The eluting agent competes either for binding to the target protein or for binding to the ligand. Substances may be eluted either by a concentration gradient of a single eluent or by pulse elution, see page 22.

When working with competitive elution the concentration of competing compound should be similar to the concentration of the coupled ligand. However, if the free competing compound binds more weakly than the ligand to the target molecule, use a concentration ten-fold higher than that of the ligand.

Reduced polarity of eluent

Conditions are used to lower the polarity of the eluent promote elution without inactivating the eluted substances. Dioxane (up to 10%) or ethylene glycol (up to 50%) are typical of this type of eluent.

Chaotropic eluents

If other elution methods fail, deforming buffers, which alter the structure of proteins, can be used, e.g. chaotropic agents such as guanidine hydrochloride or urea. Chaotropes should be avoided whenever possible since they are likely to denature the eluted protein.

Gradient and step elution

Figure 6 shows examples of step and gradient elution conditions. For prepacked affinity HiTrap columns, supplied with predefined elution conditions, a step elution using a simple syringe can be used. HiTrap columns can also be used with a chromatography system such as ÄKTAprime. The use of a chromatography system is essential when gradient elution is required.

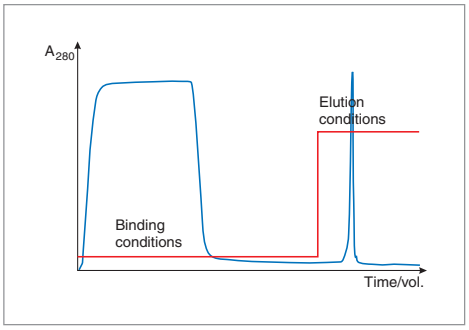


Fig. 6a. Step elution.

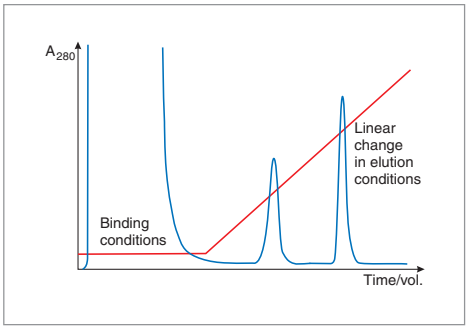


Fig. 6b. Gradient elution.

During development and optimization of affinity purification, use a gradient elution to scan for the optimal binding or elution conditions, as shown in Figure 7 and Figure 8.

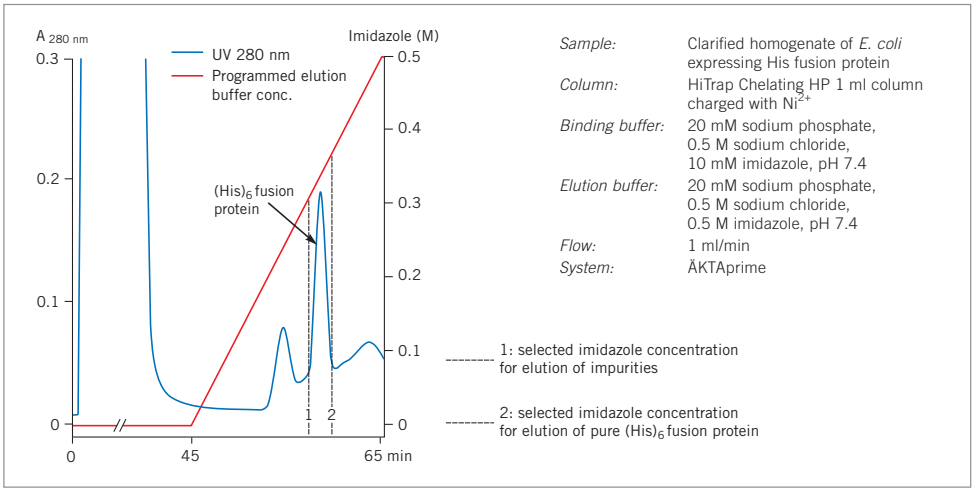
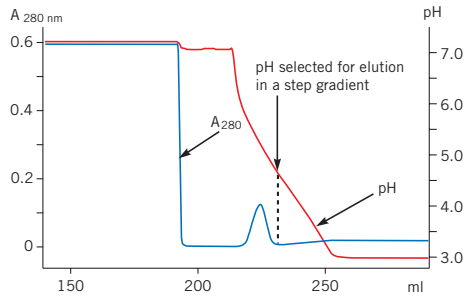


Fig. 7. Gradient elution of a $(His)_6$ fusion protein.



Sample:	Cell culture supernatant containing monoclonal IgG_1, 90 ml
Column:	HiTrap rProtein A FF, 1 ml
Binding buffer:	100 mM sodium phosphate, 100 mM sodium citrate, 2.5 M sodium chloride, pH 7.4
Elution buffer:	100 mM sodium phosphate, 100 mM sodium citrate, pH-gradient from 7.4 to 3.0
Flow:	1 ml/min
System:	ÄKTAFPLC™

Fig. 8. Scouting for optimal elution pH of a monoclonal IgG_1 from HiTrap rProtein A FF, using a pH gradient.

Flow rates

It is not possible to specify a single optimal flow rate in affinity chromatography because dissociation rates of ligand/target molecule interactions vary widely.

For ready to use affinity media follow the manufacturer's instructions and optimize further if required:

-determine the optimal flow rate to achieve efficient binding
-determine the optimal flow rate for elution to maximize recovery
-determine the maximum flow rate for column re-equilibration to minimize total run times

To obtain sharp elution curves and maximum recovery with minimum dilution of separated molecules, use the lowest acceptable flow rate.

Analysis of results and further steps

The analysis of results from the first separation can indicate if the purification needs to be improved to increase the yield, achieve higher purity, speed up the separation or increase the amount of sample that can be processed in a single run. Commonly used assays are outlined in Appendix 8.

It is generally recommended to follow any affinity step with a second technique, such as a high resolution gel filtration to remove any aggregates, or ligands that may have leached from the medium. For example, Superdex™ can be used to separate molecules, according to differences in size, and to transfer the sample into storage buffer, removing excess salt and other small molecules. The chromatogram will also give an indication of the homogeneity of the purified sample.

Alternatively, a desalting column that gives low resolution, but high sample capacity, can be used to quickly transfer the sample into storage buffer and remove excess salt (see page 133).

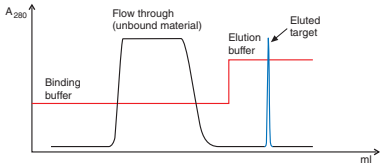
Equipment selection

Appendix 2 provides a guide to the selection of purification systems.

Troubleshooting

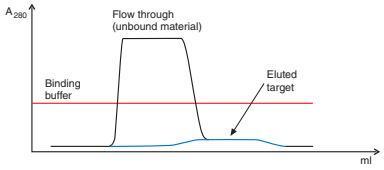
This section focuses on practical problems that may occur when running a chromatography column. The diagrams below give an indication of how a chromatogram may deviate from the ideal during affinity purification and what measures can be taken to improve the results.

Target elutes as a sharp peak. Satisfactory result



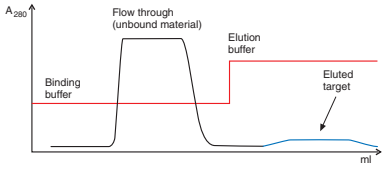
- If it is difficult or impossible to retain biological activity when achieving this result, either new elution conditions or a new ligand must be found.
- If using low pH for elution, collect the fractions in neutralization buffer (60–200 µl 1 M Tris-HCl, pH 9.0 per ml eluted fraction).

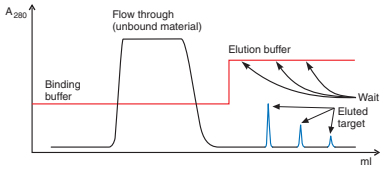
Target is a broad, low peak that elutes while binding buffer is being applied



- Find better binding conditions.

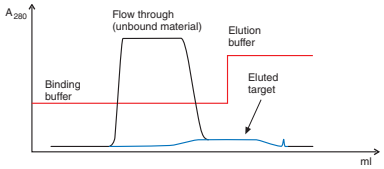
Target elutes in a broad, low peak

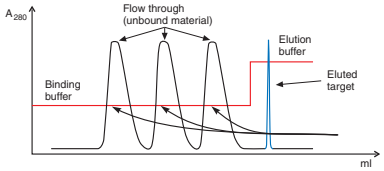




- Try different elution conditions.
- If using competitive elution, increase the concentration of the competitor in the elution buffer.
- Stop flow intermittently during elution to allow time for the target molecule to elute and so collect the target protein in pulses (see second figure beneath).
  Note: This result may also be seen if the target protein has denatured and aggregated on the column or if there is non-specific binding.

Some of the target molecule elutes as a broad, low peak while still under binding conditions





- Allow time for the sample to bind and/or apply sample in aliquots, stopping the flow for a few minutes between each sample application (see second figure beneath).

Situation	Cause	Remedy
Protein does not bind or elute as expected.	Sample has not been filtered properly.	Clean the column, filter the sample and repeat.
	Sample has altered during storage.	Prepare fresh samples.
	Sample has wrong pH or buffer conditions are incorrect.	Use a desalting column to transfer sample into the correct buffer (see page 133).
	Solutions have wrong pH.	Calibrate pH meter, prepare new solutions and try again.
	The column is not equilibrated sufficiently in the buffer.	Repeat or prolong the equilibration step.
	Proteins or lipids have precipitated on the column.	Clean and regenerate the column or use a new column.
	Column is overloaded with sample.	Decrease the sample load.
	Microbial growth has occurred in the column.	Microbial growth rarely occurs in columns during use, but, to prevent infection of packed columns, store in 20% ethanol when possible.
	Precipitation of protein in the column filter and/ or at the top of the bed.	Clean the column, exchange or clean the filter or use a new column.
Low recovery of activity, but normal recovery of protein.	Protein may be unstable or inactive in the elution buffer.	Determine the pH and salt stability of the protein.
		Collect fractions into neutralization buffer such as 1 M Tris-HCl, pH 9 (60–200 µl per fraction).
	Enzyme separated from co-factor or similar.	Test by pooling aliquots from the fractions and repeating the assay.
Lower yield than expected.	Protein may have been degraded by proteases.	Add protease inhibitors to the sample and buffers to prevent proteolytic digestion. Run sample through a medium such as Benzamidine 4 Fast Flow (high sub) to remove serine proteases.
	Adsorption to filter during sample preparation.	Use another type of filter.
	Sample precipitates.	May be caused by removal of salts or unsuitable buffer conditions.
	Hydrophobic proteins. Protein is still attached to ligand.	Use chaotropic agents, polarity reducing agents or detergents.
More activity is recovered than was applied to the column.	Different assay conditions have been used before and after the chromatographic step.	Use the same assay conditions for all the assays in the purification scheme.
	Removal of inhibitors during separation.	
Reduced or poor flow through the column.	Presence of lipoproteins or protein aggregates.	Remove lipoproteins and aggregrates during sample preparation (see Appendix 1).
	Protein precipitation in the column caused by removal of stabilizing agents during fractionation.	Modify the eluent to maintain stability.
	Clogged column filter.	Replace the filter or use a new column. Always filter samples and buffer before use.
	Clogged end-piece or adaptor or tubing.	Remove and clean or use a new column.
	Precipitated proteins.	Clean the column using recommended methods or use a new column.
	Bed compressed.	Repack the column, if possible, or use a new column.
	Microbial growth.	Microbial growth rarely occurs in columns during use, but, to prevent infection of packed columns, store in 20% ethanol when possible.

Situation	Cause	Remedy
Back pressure increases during a run or during successive runs.	Turbid sample.	Improve sample preparation (see Appendix 1). Improve sample solubility by the addition of ethylene glycol, detergents or organic solvents.
	Precipitation of protein in the column filter and/or at the top of the bed.	Clean using recommended methods. Exchange or clean filter or use a new column.
		Include any additives that were used for initial sample solubilization in the solutions used for chromatography.
Bubbles in the bed.	Column packed or stored at cool temperature and then warmed up.	Remove small bubbles by passing de-gassed buffer upwards through the column. Take special care if buffers are used after storage in a fridge or cold-room. Do not allow column to warm up due to sunshine or heating system. Repack column, if possible, (see Appendix 3).
	Buffers not properly de-gassed.	De-gas buffers thoroughly.
Cracks in the bed.	Large air leak in column.	Check all connections for leaks. Repack the column if possible (see Appendix 3).
Distorted bands as sample runs into the bed.	Air bubble at the top of the column or in the inlet adaptor.	Re-install the adaptor taking care to avoid air bubbles.
	Particles in buffer or sample.	Filter or centrifuge the sample. Protect buffers from dust.
	Clogged or damaged net in upper adaptor.	Dismantle the adaptor, clean or replace the net. Keep particles out of samples and eluents.
Distorted bands as sample passes down the bed.	Column poorly packed.	Suspension too thick or too thin. Bed packed at a temperature different from run. Bed insufficiently packed (too low packing pressure, too short equilibration). Column packed at too high pressure.

Chapter 3
Purification of specific groups of molecules

A group specific medium has an affinity for a group of related substances rather than for a single type of molecule. The same general ligand can be used to purify several substances (for example members of a class of enzymes) without the need to prepare a new medium for each different substance in the group. Within each group there is either structural or functional similarity. The specificity of the affinity medium derives from the selectivity of the ligand and the use of selective elution conditions.

Immunoglobulins

The diversity of antibody-antigen interactions has created many uses for antibodies and antibody fragments. They are used for therapeutic and diagnostic applications as well as for immunochemical techniques within general research. The use of recombinant technology has greatly expanded our ability to manipulate the characteristics of these molecules to our advantage. The potential exists to create an infinite number of combinations between immunoglobulins and immunoglobulin fragments with tags and other selected proteins.

A significant advantage for the purification of antibodies and their fragments is that a great deal of information is available about the properties of the target molecule and the major contaminants, no matter whether the molecule is in its a native state or has been genetically engineered and no matter what the source material.

The *Antibody Purification Handbook* from Amersham Biosciences presents the most effective and frequently used strategies for sample preparation and purification of the many different forms of antibodies and antibody fragments used in the laboratory.

The handbook also includes more detailed information on antibody structure and classification, illustrated briefly here in Figures 9 and 10.

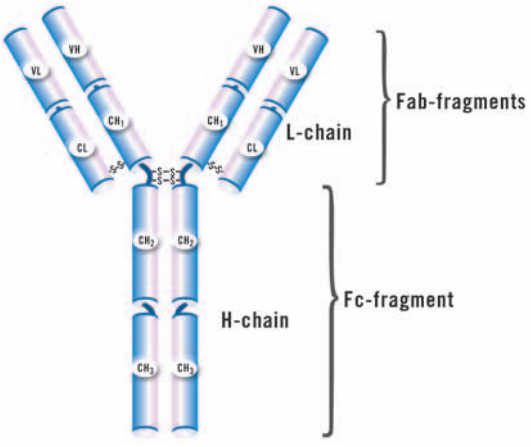


Fig. 9. H2L2 structure of a typical immunoglobulin.

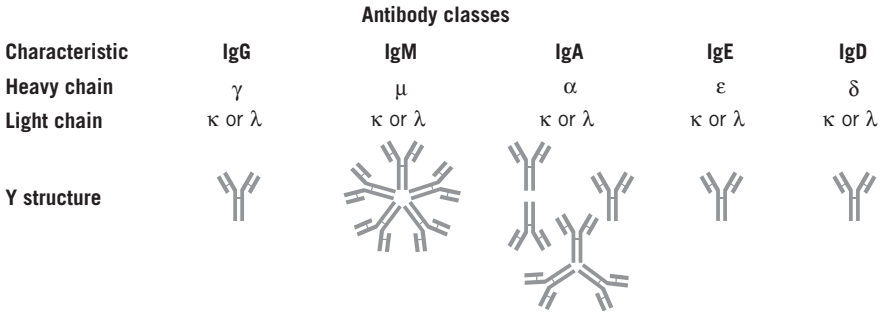


Fig. 10. Antibody classes.

IgG, IgG fragments and subclasses

The basis for purification of IgG, IgG fragments and subclasses is the high affinity of protein A and protein G for the Fc region of polyclonal and monoclonal IgG-type antibodies, see Figure 9.

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 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 2 shows a comparison of the relative binding strengths of protein A and protein G to different immunoglobulins compiled from various publications.

A useful reference on this subject is also: Structure of the IgG-binding regions of *streptococcal* Protein G, *EMBO J.*, **5**, 1567–1575 (1986).

Binding strengths are tested with free protein A or protein G and can be used as a guide 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.

Table 2. Relative binding strengths of protein A and protein G to various immunoglobulins. No binding: –, relative strength of binding: +, ++, +++, ++++.

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.

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.

Genetically engineered antibodies and antibody fragments can have altered biological properties and also altered properties to facilitate their purification. For example, tags can be introduced into target molecules for which no affinity media were previously available thus creating a fusion protein that can be effectively purified by affinity chromatography. Details for the purification of tagged proteins are covered in the section Recombinant Fusion Proteins on page 42 of this handbook. For information on the purification of recombinant proteins in general, refer to *The Recombinant Protein Handbook*: *Protein Amplification and Simple Purification* and the *GST Fusion System Handbook* from Amersham Biosciences.

HiTrap Protein G HP, Protein G Sepharose 4 Fast Flow, MAbTrap Kit

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 2) 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 deleted genetically, 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 results in very low leakage levels over a wide range of elution conditions.

Purification options

	Binding capacity	Maximum operating flow	Comments
HiTrap Protein G HP	Human IgG, > 25 mg/column Human IgG, >125 mg/column	4 ml/min (1 ml column) 20 ml/min (5 ml column)	Purification of IgG, fragments and subclasses, including human IgG_3. Strong affinity for monoclonal mouse IgG_1 and rat IgG. Prepacked columns.
MAbTrap Kit	Human IgG, > 25 mg/column	4 ml/min	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 x 1 ml), accessories, pre-made buffers for 10 purifications and detailed experimental protocols.
Protein G Sepharose 4 Fast Flow	Human IgG, > 20 mg/ml medium Cow IgG, 23 mg/ml medium Goat IgG, 19 mg/ml medium Guinea pig IgG, 17 mg/ml medium Mouse IgG, 10 mg/ml medium Rat IgG, 7 mg/ml medium	400 cm/h*	Supplied as a suspension ready for column packing.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Purification examples

Figure 11 shows the purification of mouse monoclonal IgG_1 on HiTrap Protein G HP 1 ml. The monoclonal antibody was purified from a hybridoma cell culture supernatant.

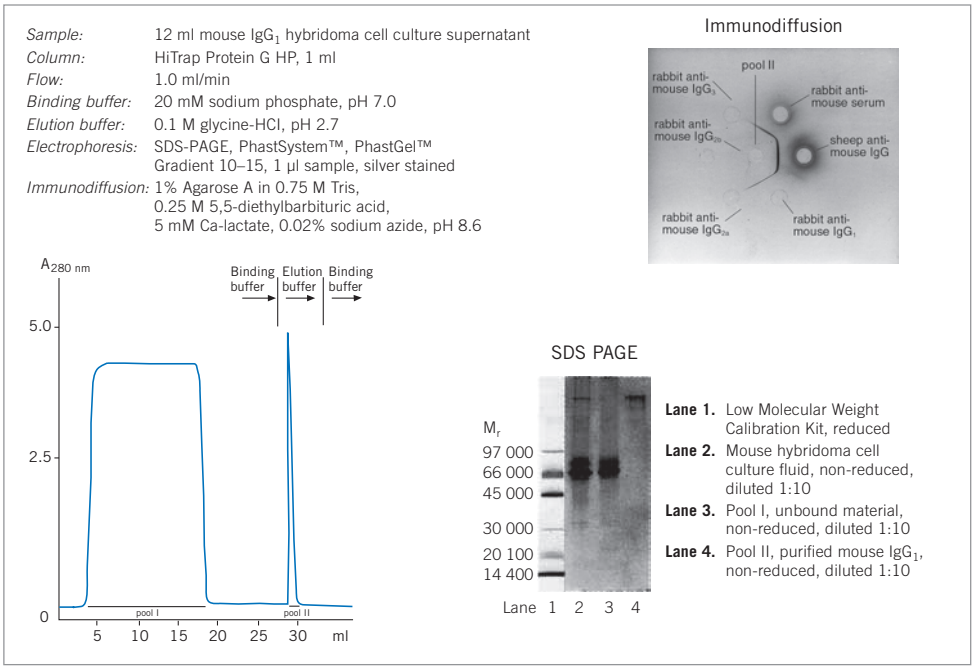


Fig. 11. Purification of monoclonal mouse IgG_1 on HiTrap Protein G HP, 1 ml.

Figure 12 shows the purification of recombinant mouse Fab fragments, expressed in *E. coli*, using Protein G Sepharose 4 Fast Flow. 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.

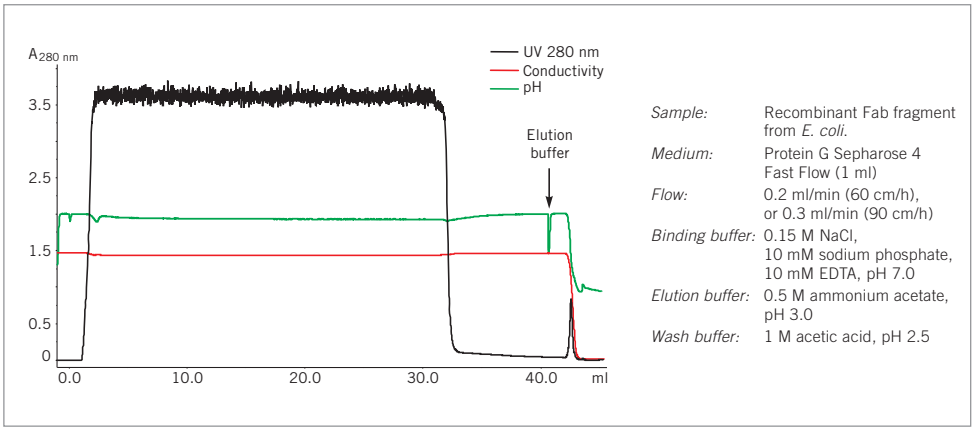


Fig. 12. Purification of recombinant Fab fragments directed to the envelope protein gp120 of HIV-1 (anti-gp120 Fab), expressed in *E. coli*.

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 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 either by buffer exchange on a desalting column or by dilution and pH adjustment (see page 133).

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 neutralization buffer (60 µl – 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 133).

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.

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 (see Appendix 3).

MAbTrap Kit

Fig. 13. MAbTrap Kit, ready for use.

MAbTrap Kit contains a HiTrap Protein G HP 1 ml column, stock solutions of binding, elution and neutralization buffers, a syringe with fittings and an optimized purification protocol, as shown in Figure 13. The kit contains sufficient material for up to 20 purifications of monoclonal or polyclonal IgG from serum, cell culture supernatant or ascitic fluid, using a syringe. The column can also be connected to a peristaltic pump, if preferred. Figure 14 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 15.

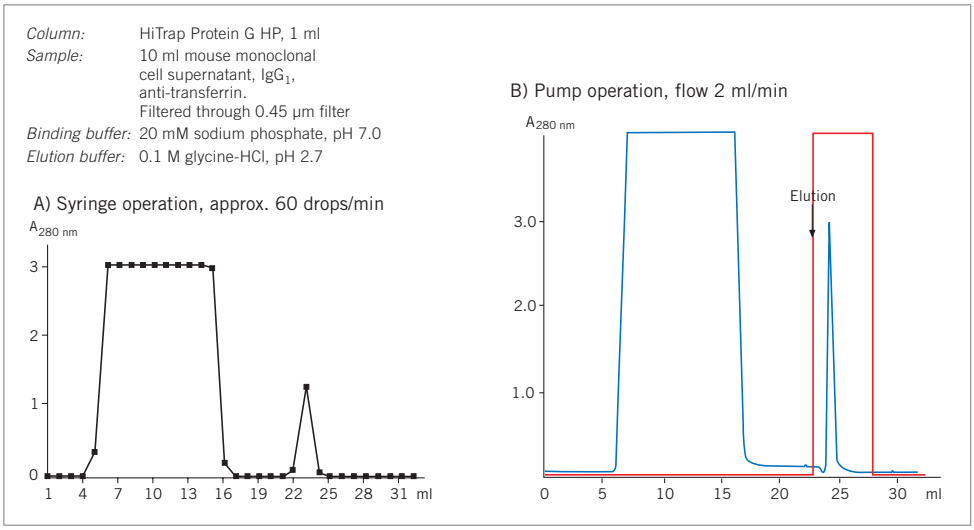


Fig. 14. Purification of mouse monoclonal IgG_1 from cell culture supernatant. A. with syringe operation. B. with pump operation.

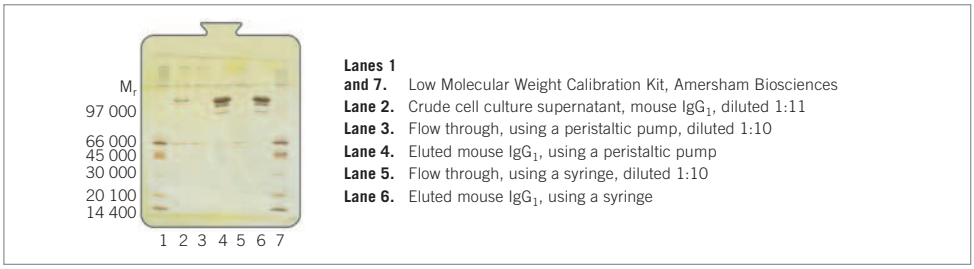


Fig. 15. SDS-PAGE on PhastSystem using PhastGel 10–15, non-reduced, and silver staining.

Performing a separation

Column:	HiTrap Protein G HP, 1 ml
Recommended flow rate:	1 ml/min
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 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 either by buffer exchange on a desalting column (see page 133) or by dilution and pH adjustment.

Fig. 16. Using HiTrap Protein G HP with a syringe. A: Dilute buffers and prepare 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 warm to room temperature.
2. Dilute the binding and elution 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.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
HiTrap Protein G HP (MAbTrap Kit)	2 mg/ml	Ligand coupled to Sepharose HP by N-hydroxysuccinimide activation (gives stable attachment through alkylamine and ether links).	Long term 3–9 Short term 2–9	34 µm
Protein G Sepharose 4 Fast Flow	2 mg/ml	Ligand coupled to Sepharose 4 Fast Flow by cyanogen bromide activation.	Long term 3–9 Short term 2–10	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

Stable in all common aqueous buffers.

Storage

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

HiTrap Protein A HP, Protein A Sepharose 4 Fast Flow, HiTrap rProtein A FF, rProtein A Sepharose 4 Fast Flow, MabSelect

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 IgG. One molecule of protein A can bind at least two molecules of IgG.

Both protein A and a recombinant protein A are available from Amersham Biosciences. These molecules share similar specificities 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 to Sepharose. Single point coupling often results in an enhanced binding capacity.

The binding strength of protein A for IgG depends on the source species of the immunoglobulin as well as the subclass of IgG (see Table 2). The dynamic binding capacity depends on the binding strength and also on several other factors, such as flow rate during sample application.

Although IgG is the major reactive human immunoglobulin, some other types have also been demonstrated to bind to protein A. Interaction takes place with human colostral IgA as well as human myeloma IgA_2 but not IgA_1. Some human monoclonal IgMs and some IgMs from normal and macroglobulinaemic sera can bind 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 A to Sepharose results in very low leakage levels over a wide range of elution conditions.

Purification options

	Binding capacity	Maximum operating flow	Comments
HiTrap Protein A HP	Human IgG, > 20 mg/column Human IgG, > 100 mg/column	4 ml/min (1 ml column) 20 ml/min (5 ml column)	Purification of IgG, fragments and sub-classes. Prepacked columns.
Protein A Sepharose 4 Fast Flow*	Human IgG, > 35 mg/ml medium Mouse IgG, 3–10 mg/ml medium	400 cm/h**	Supplied as a suspension ready for column packing.
HiTrap rProtein A FF	Human IgG, > 50 mg/column Human IgG, > 250 mg/column	4 ml/min (1 ml column) 20 ml/min (5 ml column)	Purification of IgG, fragments and sub-classes. Enhanced binding capacity. Prepacked columns.
rProtein A Sepharose 4 Fast Flow*	Human IgG, > 50 mg/ml medium Mouse IgG, 8–20 mg/ml medium	300 cm/h**	Enhanced binding capacity. Supplied as a suspension ready for column packing.
MabSelect™ (recombinant protein A ligand)	Human IgG, approx. 30 mg/ml medium	500 cm/h**	For 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 before column packing.*
***See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Purification examples

Figure 17 shows the purification of mouse IgG_{2b} from ascites fluid on 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%.

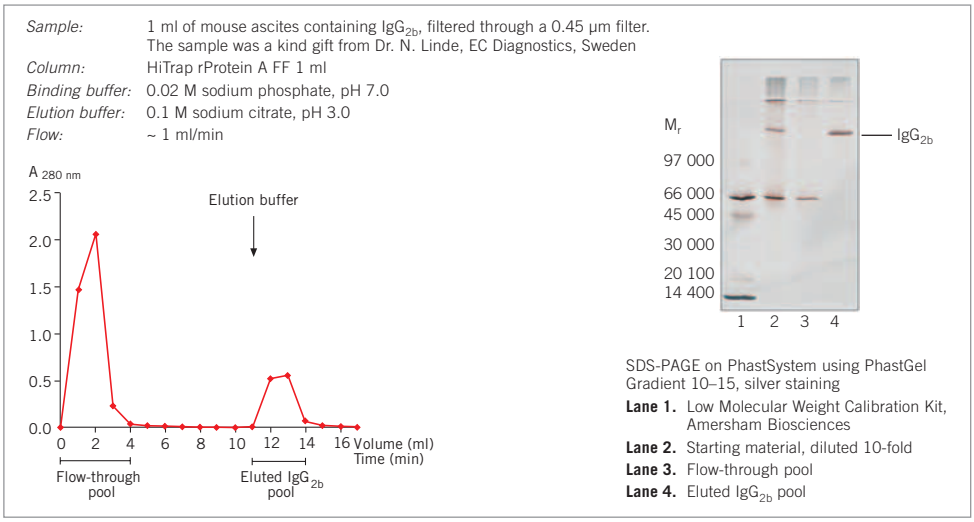


Fig. 17. Purification of mouse IgG_{2b} from ascites on HiTrap rProtein A FF 1 ml column using a syringe.

Figure 18 shows a larger scale purification of monoclonal mouse IgG_{2a} from a clarified hybridoma cell culture on rProtein A Sepharose Fast Flow. Sample loading was over 9 mg IgG/ml of medium, with a 95% recovery of highly purified antibody.

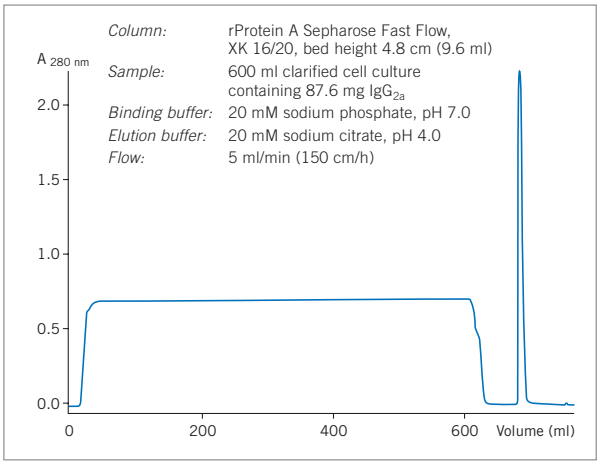


Fig. 18a. Purification of a monoclonal IgG_{2a} from clarified cell culture on rProtein A Sepharose 4 Fast Flow.

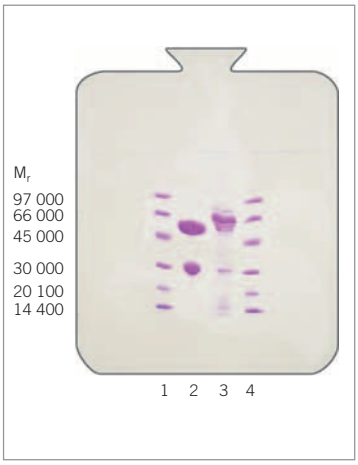


Fig. 18b. SDS-PAGE of starting material (lane 2) and eluate (lane 3). The samples were concentrated 10 times and reduced. Lane 1 and 4 are LMW markers. PhastSystem, PhastGel Gradient 10–15.

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 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 either by buffer exchange on a desalting column (see page 133) or by dilution and pH adjustment.

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

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. Immediately re-equilibrate with 5–10 column volumes of binding buffer.

**Since elution conditions are quite harsh, collect fractions into neutralization buffer (60 µl – 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 3 gives examples of some typical binding and elution conditions that have been used with Protein A Sepharose.

Table 3.

Species	Subclass	Binding to free protein A	Protein A Sepharose binding pH	Protein A Sepharose 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)

Binding strengths are tested with free protein A. They can be used as a guide to predict the binding behaviour to a protein A affinity 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.

IgGs from most species and subclasses bind protein A near 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.

With some antibodies, such as mouse IgG_1, it might be necessary to add sodium chloride up to 3 M in the binding buffer to achieve efficient binding when using protein A, for example 1.5 M glycine, 3 M NaCl, pH 8.9.

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.

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.

To increase capacity, connect several HiTrap Protein A HP or HiTrap rProtein A FF columns (1 ml or 5 ml) in series or pack a larger column with Protein A Sepharose 4 Fast Flow or rProtein A Sepharose 4 Fast Flow (see Appendix 3).

Desalt and/or transfer purified IgG fractions into a suitable buffer using a desalting column (see page 133).

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

Media characteristics

Product	Ligand density	Composition	pH stability*	Mean particle size
HiTrap Protein A HP	3 mg/ml	Ligand coupled to Sepharose HP by N-hydroxysuccinimide activation (stable attachment through alkylamine and ether links).	Short term 2–10 Long term 3–9	34 µm
Protein A Sepharose 4 Fast Flow**	6 mg/ml	Ligand coupled to Sepharose 4 Fast Flow by cyanogen bromide activation.	Short term 2–10 Long term 3–9	90 µm
HiTrap rProtein A FF	6 mg/ml	Ligand coupled to Sepharose 4 Fast Flow by epoxy activation, thioether coupling.	Short term 2–11 Long term 3–10	90 µm
rProtein A Sepharose 4 Fast Flow**	6 mg/ml	Ligand coupled to Sepharose 4 Fast Flow by epoxy activation, thioether coupling.	Short term 2–11 Long term 3–10	90 µm
MabSelect Contact your local specialist for details.				

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*
***Protein A Sepharose 4 Fast Flow and rProtein A Sepharose 4 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 before column packing.*

Chemical stability

These media and columns tolerate high concentrations of urea, guanidine HCl and chaotropic agents.

Storage

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

Monoclonal IgM from hybridoma cell culture

HiTrap IgM Purification HP

The technique described here 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 option

	Binding capacity	Maximum operating flow	Comments
HiTrap IgM Purification HP	Human IgM, 5 mg/column	4 ml/min	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 example

Figure 19 shows results from the purification of monoclonal α-Shigella IgM from hybridoma cell culture supernatant. SDS-PAGE analysis demonstrates a purity level of over 80%. Results from an ELISA (not shown) indicated a high activity of the antibody in the purified fraction.

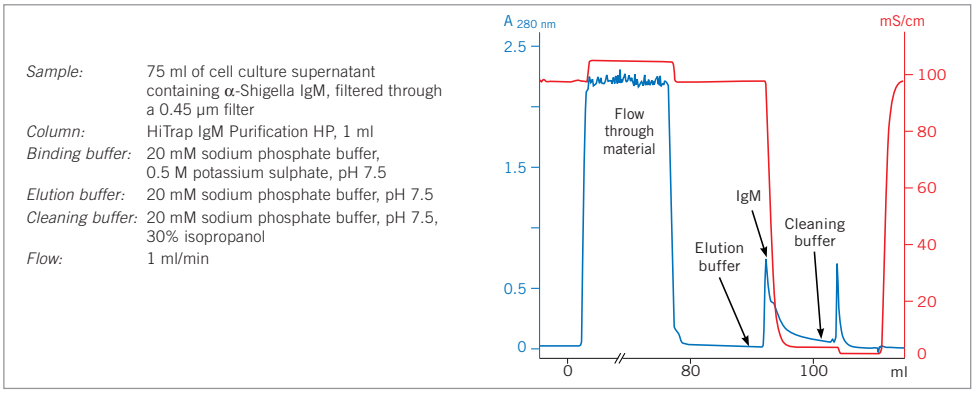


Fig. 19a. Purification of α-Shigella IgM on HiTrap IgM Purification HP.

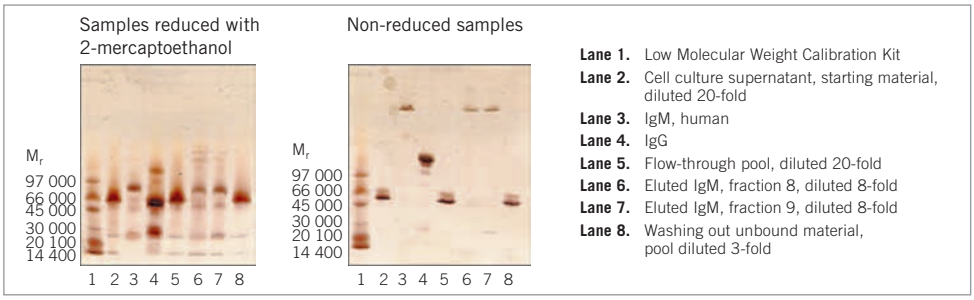


Fig. 19b. SDS-PAGE on PhastSystem, using PhastGel 4–15 with silver staining.

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 of hybridoma cell culture supernatant 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 sequentially with at least 5 column volumes of binding, elution and wash 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 wash buffer.
7. Immediately re-equilibrate the column with 5 column volumes of binding buffer.

Potassium sulphate (0.5 M) can be used instead of ammonium sulphate. Most monoclonal IgMs bind 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 IgMs may bind too tightly to the column for complete elution in binding buffer. The remaining IgM will be eluted with wash buffer, but the high content of isopropanol will cause precipitation of IgM. Perform an immediate buffer exchange (see page 133) or dilute the sample to preserve the IgM. Lower concentrations of isopropanol may elute the IgM and decrease the risk of precipitation.

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.

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.

Media characteristics

	Ligand and density	pH stability*	Mean particle size
HiTrap IgM Purification HP	2-mercaptopyridine 2 mg/ml	Long term 3–11 Short term 2–13	34 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Storage

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

Avian IgY from egg yolk

HiTrap IgY Purification HP

Purification option

	Binding capacity	Maximum operating flow	Comments
HiTrap IgY Purification HP	100 mg pure IgY/column (1/4 egg yolk)	20 ml/min	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 example

Figure 20 shows the purification of α-Hb IgY from 45 ml of egg yolk extract (corresponding to one quarter of a yolk) and Figure 21 shows the SDS-PAGE analysis indicating a purity level of over 70%.

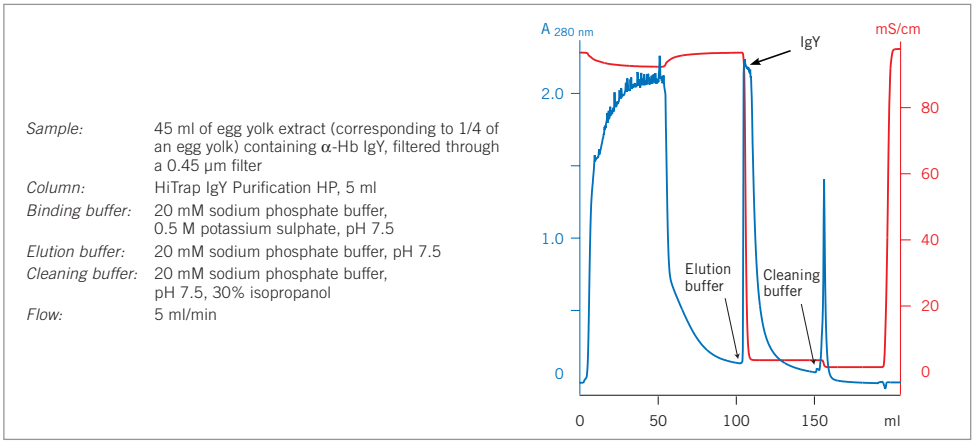
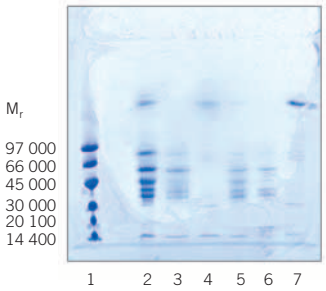


Fig. 20. Purification of avian IgY on HiTrap IgY Purification HP.



Lane 1. Low Molecular Weight Calibration Kit
Lane 2. Egg yolk extract
Lane 3. Flow-through pool
Lane 4. Eluted IgY
Lane 5. Egg yolk extract, diluted 4-fold
Lane 6. Flow-through pool, diluted 4-fold
Lane 7. Eluted IgY, diluted 4-fold

Fig. 21. 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 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 wash buffer.
7. Immediately 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. The sample should have the same concentration of Na_2SO_4 as the binding buffer.

An increase in salt concentration 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 elution with 100% elution buffer for a few column volumes.

To increase 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, columns should only be reused with identical samples.

Media characteristics

	Ligand and density	pH stability*	Mean particle size
HiTrap IgY Purification HP	2-mercaptopyridine 3 mg/ml	Long term 3–11 Short term 2–13	34 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Storage

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

Recombinant fusion proteins

The purification of recombinant proteins can often be simplified by incorporating a tag of known size into the protein. As well as providing a marker for expression and facilitating detection of the recombinant protein, an important role for the tag is to enable a simple purification by affinity chromatography. The two most commonly used tags are glutathione-S-transferase (GST) and 6 x histidine residues $(His)_6$. Protein A fusion proteins have also been produced to take advantage of the affinity between IgG and protein A for affinity purification.

GST fusion proteins

GST MicroSpin Purification Module, GSTrap FF, GSTPrep FF 16/10, Glutathione Sepharose 4 Fast Flow, Glutathione Sepharose 4B

Glutathione S-transferase (GST) is one of the most common tags used to facilitate the purification and detection of recombinant proteins and a range of products for simple, one step purification of GST fusion proteins are available (see Purification options).

Purification and detection of GST-tagged proteins, together with information on how to handle fusion proteins when they are expressed as inclusion bodies, are dealt with in depth in the *GST Gene Fusion System Handbook* and *The Recombinant Protein Handbook: Protein Amplication and Simple Purification*, available from Amersham Biosciences.

Purification options

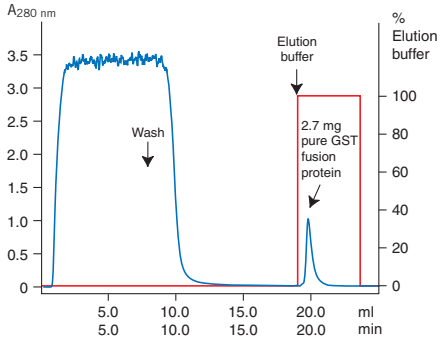
	Binding capacity	Maximum operating flow	Comments
GST MicroSpin™ Purification Module	400 µg/column	n.a.	Ready to use, prepacked columns, buffers and chemicals. High throughput when used with MicroPlex™ 24 Vacuum (up to 48 samples simultaneously).
GSTrap FF 1 ml	10–12 mg recombinant GST/column	4 ml/min	Prepacked column, ready to use.
GSTrap FF 5 ml	50–60 mg recombinant GST/column	15 ml/min	Prepacked column, ready to use.
GSTPrep FF 16/10	>200 mg recombinant GST/column	>10 ml/min	Prepacked column, ready to use.
Glutathione Sepharose 4 Fast Flow	10–12 mg recombinant GST/ml medium	450 cm/h*	For packing high performance columns for use with purification systems and scaling up.
Glutathione Sepharose 4B	8 mg horse liver GST/ml medium	75 cm/h*	For packing small columns and other formats.

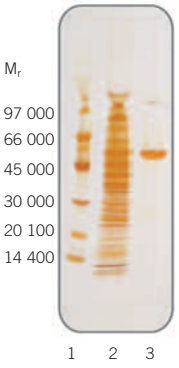
**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Purification examples

Figure 22 shows a typical purification of GST fusion protein on GSTrap FF 1 ml with an SDS-PAGE analysis of the purified protein.

Column: GSTrap FF, 1 ml
Sample: 8 ml cytoplasmic extract from *E. coli* expressing a GST fusion protein
Binding buffer: PBS, pH 7.3
Elution buffer: 50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow: 1 ml/min
Chromatographic procedure: 4 ml binding buffer, 8 ml sample, 10 ml binding buffer, 5 ml elution buffer, 5 ml binding buffer
System: ÄKTAexplorer





SDS-PAGE on ExcelGel™ SDS Gradient 8–18% using Multiphor™ II followed by silver staining.

Lane 1. Low Molecular Weight (LMW) Calibration kit, reduced, Amersham Pharmacia Biotech

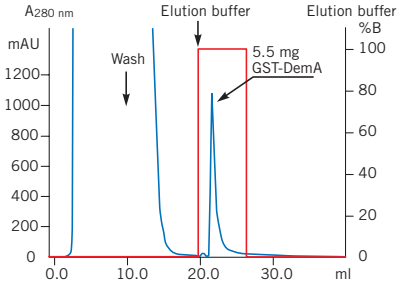
Lane 2. Cytoplasmic extract of *E. coli* expressing GST fusion protein, 1 g cell paste/10 ml

Lane 3. GST fusion protein eluted from GSTrap FF 1 ml

Fig. 22. Purification of a GST fusion protein.

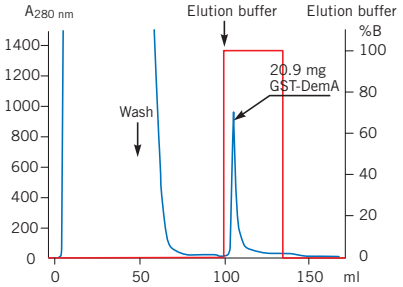
Figure 23 shows how the purification of a GST fusion protein can be scaled up 20-fold from a GSTrap FF 1 ml column to a GSTPrep FF 16/10 column.

a) GSTrap FF 1 ml



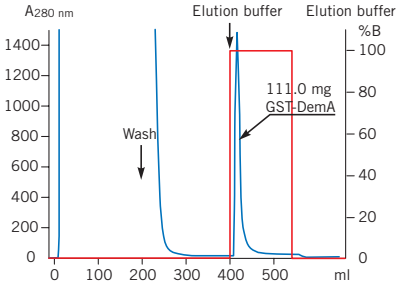
Column:	GSTrap FF 1 ml
Sample:	10 ml extract from *E. coli* expressing GST-DemA
Binding buffer:	PBS, pH 7.4
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow:	Sample loading: 0.5 ml/min Wash and elution: 1 ml/min
Chromatographic procedure:	5 ml binding buffer, 10 ml sample, 10 ml binding buffer, 7 ml elution buffer, 5 ml binding buffer
System:	ÄKTAexplorer

b) GSTrap FF 5 ml



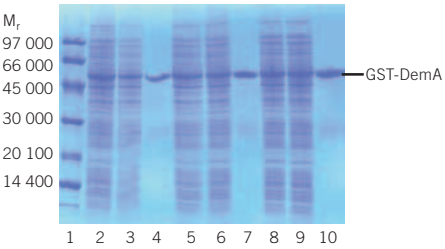
Column:	GSTrap FF 5 ml
Sample:	50 ml extract from *E. coli* expressing GST-DemA
Binding buffer:	PBS, pH 7.4
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow:	Sample loading: 2.5 ml/min Wash and elution: 5 ml/min
Chromatographic procedure:	25 ml binding buffer, 50 ml sample, 50 ml binding buffer, 35 ml elution buffer, 25 ml binding buffer
System:	ÄKTAexplorer

c) GSTPrep FF 16/10



Column:	GSTPrep FF 16/10
Sample:	200 ml extract from *E. coli* expressing GST-DemA
Binding buffer:	PBS, pH 7.4
Elution buffer:	50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione
Flow:	Sample loading: 5 ml/min Wash and elution: 10 ml/min
Chromatographic procedure:	100 ml binding buffer, 200 ml sample, 200 ml binding buffer, 140 ml elution buffer, 100 ml binding buffer
System:	ÄKTAexplorer

Electrophoresis: SDS-PAGE, 12.5% gel, Coomassie Blue staining



Lane 1: Low Molecular Weight (LMW) calibration kit, reduced
Lane 2: Extract of *E. coli* expressing GST-DemA, 1 g cell paste/5 ml
Lane 3: Flow-through from GSTrap FF 1 ml
Lane 4: GST-DemA eluted from GSTrap FF 1 ml
Lane 5: Extract of *E. coli* expressing GST-DemA, 1 g cell paste/5 ml
Lane 6: Flow-through from GSTrap FF 5 ml
Lane 7: GST-DemA eluted from GSTrap FF 5 ml
Lane 8: Extract of *E. coli* expressing GST-DemA, 1 g cell paste/5 ml
Lane 9: Flow-through from GSTPrep FF 16/10
Lane 10: GST-DemA eluted from GSTPrep FF 16/10

Fig. 23. Scaling up purification of a GST fusion protein.

Performing a separation

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

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

Fig. 24. Using GSTrap FF with a syringe. **A:** Prepare buffers and sample. Remove the column's top cap and twist off the end. **B:** Equilibrate column, load the sample and begin collecting fractions. **C:** Wash and elute, continuing to collect fractions.

1. Equilibrate the column with 5 column volumes of binding buffer.
2. Apply the sample.
3. Wash with 5–10 column volumes of binding buffer.
4. Elute with 5–10 column volumes of elution buffer.
5. Wash with 5–10 column volumes of binding buffer.

It is important to keep a low flow rate during sample loading and elution as the kinetics of the binding interaction between GST and glutathione are relatively slow. The binding capacity is protein dependent and therefore yield will vary according to the type of protein. Yield may be improved by using a slower flow rate or passing the sample through the column several times.

For a single purification of a small quantity of product or for high throughput screening, GST MicroSpin columns are convenient and simple to use with either centrifugation or MicroPlex 24 Vacuum.

To increase capacity, connect several GSTrap FF columns (1 ml or 5 ml) in series. For greater capacity, use GSTPrep 16/10 FF columns or pack Glutathione Sepharose 4 Fast Flow into a suitable column (see Appendix 3). GSTrap FF columns can be used with a syringe, a peristaltic pump or a chromatography system.

Enzyme-specific recognition sites are often included to allow the removal of the GST tag by enzymatic cleavage when required. Thrombin is commonly used for enzymatic cleavage, and must, subsequently, be removed from the recombinant product. HiTrap Benzamidine FF (high sub) 1 ml or 5 ml columns provide simple, ready-made solutions for this process (see page 54).

Reuse of GSTrap FF depends on the nature of the sample. To prevent cross-contamination, columns should only be reused with identical samples.

Cleaning

These procedures are applicable to Glutathione Sepharose 4 Fast Flow and Glutathione Sepharose 4B.

1. Wash with 2–3 column volumes of alternating high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5) buffers.
2. Repeat the cycle 3 times.
3. Re-equilibrate immediately with 3–5 column volumes of binding buffer.

If the medium is losing binding capacity, this may be due to an accumulation of precipitated, denatured or non-specifically bound proteins.

To remove precipitated or denatured substances:

1. Wash with 2 column volumes of 6 M guanidine hydrochloride.
2. Wash immediately with 5 column volumes of binding buffer.

To remove hydrophobically bound substances:

1. Wash with 3–4 column volumes of 70% ethanol (or 2 column volumes of a non-ionic detergent (Triton™ X-100 1%)).
2. Wash immediately with 5 column volumes of binding buffer.

Media characteristics

	Spacer arm	Ligand and density	pH stability*	Mean particle size
Glutathione Sepharose 4 Fast Flow (GSTrap FF, GSTPrep FF 16/10)	10 carbon linker	Glutathione 120–320 µmoles/ml	Short term 3–12 Long term 3–12	90 µm
Glutathione Sepharose 4B	10 carbon linker	Glutathione 7–15 µmoles/ml	Short term 4–13 Long term 4–13	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

No significant loss of binding capacity when exposed to 0.1 M citrate (pH 4.0), 0.1 M NaOH, 70% ethanol or 6 M guanidine hydrochloride for 2 hours at room temperature. No significant loss of binding capacity after exposure to 1% SDS for 14 days.

Storage

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

Poly (His) fusion proteins

His MicroSpin Purification Module, HisTrap Kit, HiTrap Chelating HP, Chelating Sepharose Fast Flow

The $(His)_6$ tag is one of the most common tags used to facilitate the purification and detection of recombinant proteins and a range of products for simple, one step purification of $(His)_6$ fusion proteins are available (see Purification options). Polyhistidine tags such as $(His)_4$ or $(His)_{10}$ are also used. They may provide useful alternatives to $(His)_6$ for improving purification results. For example, since $(His)_{10}$ binds more strongly to the affinity medium, a higher concentration of eluent (imidazole) can be used during the washing step before elution. This can facilitate the removal of contaminants which may otherwise be co-purified with a $(His)_6$ fusion protein.

Chelating Sepharose, when charged with Ni^{2+} ions, selectively binds proteins if complex-forming amino acid residues, in particular histidine, are exposed on the protein surface. $(His)_6$ fusion proteins can be easily bound and then eluted with buffers containing imidazole.

Purification and detection of His-tagged proteins, together with information on how to handle fusion proteins when they are expressed as inclusion bodies, are dealt with in depth in *The Recombinant Protein Handbook: Protein Amplication and Simple Purification*, available from Amersham Biosciences.

Purification options

	Binding capacity	Maximum operating flow	Comments
His MicroSpin Purification Module	100 µg/column	n.a.	Ready to use, prepacked columns, buffers and chemicals. High throughput when used with MicroPlex 24 Vacuum (up to 48 samples simultaneously).
HisTrap Kit	12 mg*/column	4 ml/min	As above, but includes buffers for up to 12 purifications using a syringe.
HiTrap Chelating HP 1 ml	12 mg*/column	4 ml/min	Prepacked column, ready to use.
HiTrap Chelating HP 5 ml	60 mg*/column	20 ml/min	Prepacked column, ready to use.
Chelating Sepharose Fast Flow	12 mg*/ml medium	400 cm/h**	Supplied as suspension for packing columns and scale up.

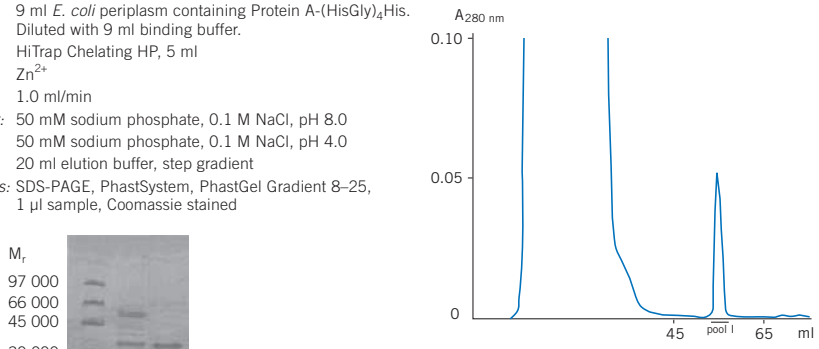
**Estimate for a $(His)_6$ fusion protein of M_r 27 600, binding capacity varies according to specific protein.*
***See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

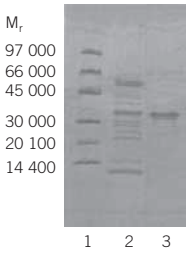
Purification examples

Figures 25 and 26 show the purification of recombinant proteins expressed in soluble form or as inclusion bodies and Figure 27 gives an example of simultaneous on-column purification and refolding of a recombinant protein expressed as an inclusion body.

Soluble recombinant proteins

Sample:	9 ml *E. coli* periplasm containing Protein A-$(HisGly)_4$His. Diluted with 9 ml binding buffer.
Column:	HiTrap Chelating HP, 5 ml
Metal ion:	Zn^{2+}
Flow:	1.0 ml/min
Binding buffer:	50 mM sodium phosphate, 0.1 M NaCl, pH 8.0
Elution buffer:	50 mM sodium phosphate, 0.1 M NaCl, pH 4.0
Gradient:	20 ml elution buffer, step gradient
Electrophoresis:	SDS-PAGE, PhastSystem, PhastGel Gradient 8–25, 1 µl sample, Coomassie stained





Lane 1. Low Molecular Weight Calibration Kit (LMW), reduced
Lane 2. Crude periplasmic fraction, reduced
Lane 3. Pool I, purified Protein A-$(HisGly)_4$His, reduced

Fig. 25. Purification of recombinant proteins on HiTrap Chelating HP, 5 ml, charged with Zn^{2+}.

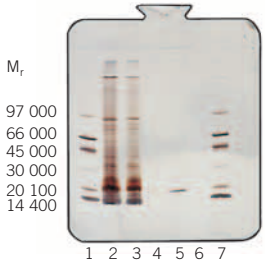
Recombinant protein expressed in inclusion bodies

Sample:	8 ml cell extract containing $(His)_{10}$-tagged protein. The clone was a kind gift from Dr. C. Fuller and S. Brasher, Department of Biochemistry, University of Cambridge, UK.
Column:	HiTrap Chelating HP, 1 ml
Metal ion:	Ni^{2+}
Binding buffers:	20 mM sodium phosphate, 0.5 M NaCl, 100 mM imidazole, 8 M urea or 6 M guanidine hydrochloride, pH 7.4
Elution buffers:	20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, 8 M urea or 6 M guanidine hydrochloride, pH 7.4
Flow:	Approx. 4 ml/min
Equipment:	Syringe
Electrophoresis:	SDS-PAGE, PhastSystem, PhastGel 10–15, 1 µl sample, silver staining

Purification in 8 M urea

Lane 1. Low Molecular Weight Calibration Kit (LMW)
Lane 2. Starting material, cell extract, diluted 20-fold
Lane 3. Flow-through, diluted 10-fold
Lane 4. Wash
Lane 5. Elution (first two ml)
Lane 6. Elution (last two ml)
Lane 7. LMW

Purification in 6 M guanidine hydrochloride



Lane 1. Low Molecular Weight Calibration Kit (LMW)
Lane 2. Starting material, cell extract, diluted 10-fold
Lane 3. Flow-through
Lane 4. Wash
Lane 5. Elution (first two ml)
Lane 6. Elution (last two ml)
Lane 7. LMW

Fig. 26. Purification of $(His)_{10}$-tagged protein from inclusion bodies on HiTrap Chelating HP, 1 ml, charged with Ni^{2+}.

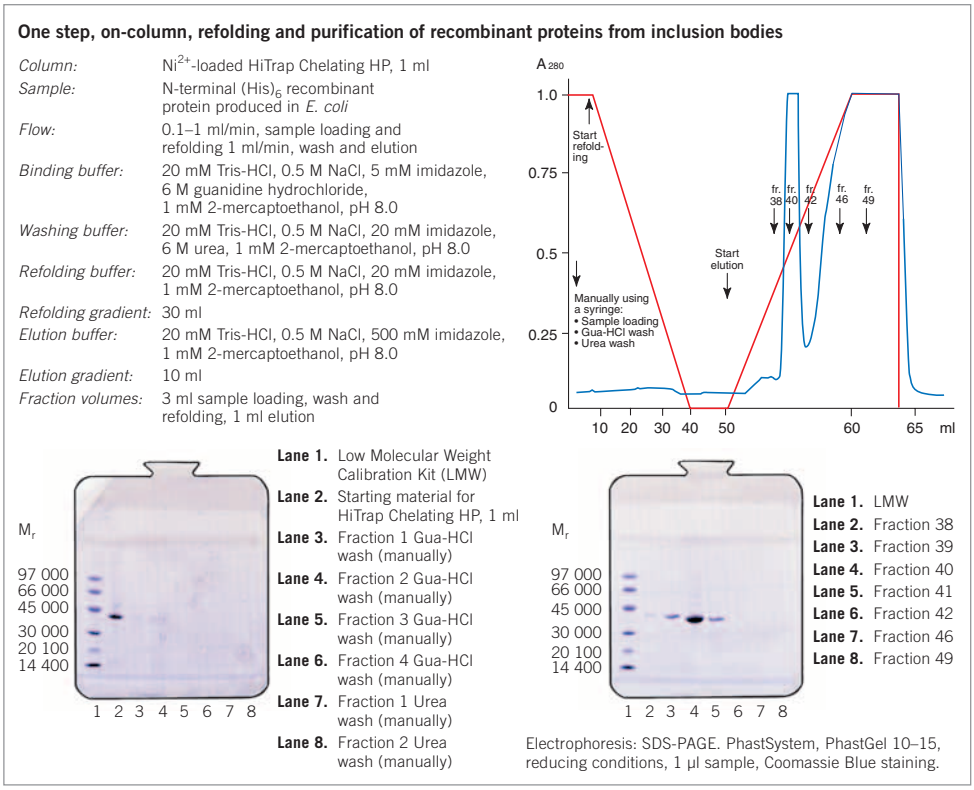


Fig. 27. One step refolding and purification of a $(His)_6$-tagged recombinant protein on HiTrap Chelating HP, 1 ml, charged with Ni^{2+}. The sample is bound to the column and all unbound material is washed through. Refolding of the bound protein is performed by running a linear 6–0 M urea gradient, starting with the wash buffer and finishing with the refolding buffer. A gradient volume of 30 ml or higher and a flow rate of 0.1–1 ml/min can be used. The optimal refolding rate should be determined experimentally for each protein. The refolded recombinant protein is eluted using a 10–20 ml linear gradient starting with refolding buffer and ending with the elution buffer.

Performing a separation

Figure 28 shows the simplicity of a poly (His) fusion protein purification when using a prepacked HiTrap Chelating HP column. The protocol described has been optimized for a high yield purification of $(His)_6$ fusion proteins and can be used as a base from which to scale up. An alternative optimization protocol designed to achieve high purity is supplied with the HisTrap Kit and is also described in *The Recombinant Protein Handbook: Protein Amplification and Simple Purificatio*n from Amersham Biosciences.

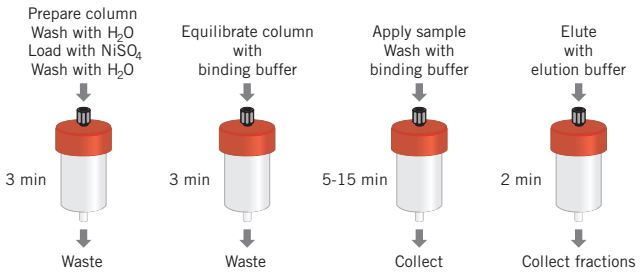


Fig. 28. HiTrap Chelating HP and a schematic overview of poly (His) fusion protein purification.

Nickel solution: 0.1 M $NiSO_4$

Binding buffer: 20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4

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

1. Wash the column with 5 column volumes of distilled water.

Use water, not buffer, to wash away the column storage solution which contains 20% ethanol. This avoids the risk of nickel salt precipitation in the next step. If air is trapped in the column, wash the column with distilled water until the air disappears.

2. Load 0.5 column volumes of the 0.1 M nickel solution onto the column.
3. Wash with 5 column volumes of distilled water.
4. Equilibrate the column with 10 column volumes of binding buffer.
5. Apply sample at a flow rate 1–4 ml/min (1 ml column) or 5 ml/min (5 ml column). Collect the flow-through fraction. A pump is more suitable for application of sample volumes greater than 15 ml.
6. Wash with 10 column volumes of binding buffer. Collect wash fraction.
7. Elute with 5 column volumes of elution buffer. Collect eluted fractions in small fractions such as 1 ml to avoid dilution of the eluate.
8. Wash with 10 column volumes of binding buffer. The column is now ready for a new purification and there is rarely a need to reload with metal if the same $(His)_6$ fusion protein is to be purified.

Imidazole absorbs at 280 nm. Use elution buffer as blank when monitoring absorbance. If imidazole needs to be removed, use a desalting column (see page 133).

For a single purification of a small quantity of product or for high throughput screening His MicroSpin columns are convenient and simple to use with either centrifugation or MicroPlex 24 Vacuum.

To increase capacity use several HiTrap Chelating HP columns (1 ml or 5 ml) in series. HiTrap Chelating HP columns (1 ml or 5 ml) can be used with a syringe, a peristaltic pump or a chromatography system. For even larger capacity, pack Chelating Sepharose Fast Flow into a suitable column (see Appendix 3).

The loss of metal ions is more pronounced at lower pH. The column does not have to be stripped (i.e. all metal ions removed) between each purification if the same protein is going to be purified. In this case, strip and re-charge (i.e. replace metal ions) the column after 5–10 purifications.

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

Purification using HisTrap Kit

HisTrap Kit includes everything needed for 12 purifications using a syringe. Three ready to use HiTrap Chelating HP 1 ml columns and ready-made buffer concentrates are supplied with easy-to-follow instructions.

Cleaning

Removal of nickel ions before re-charging or storage:

1. Wash with 5 column volumes of 20 mM sodium phosphate, 0.5 M NaCl, 0.05 M EDTA, pH 7.4.
2. Wash with 10 column volumes of distilled water.
3. For storage, wash with 5 column volumes of 20% ethanol.

Removal of precipitated proteins:

1. Fill column with 1 M NaOH and incubate for 2 hours.
2. Wash out dissolved proteins with 5 column volumes of water and a buffer at pH 7.0 until the pH of the flow-through reaches pH 7.0.

Media characteristics

	Composition	Metal ion capacity	pH stability*	Mean particle size
Chelating Sepharose High Performance (HiTrap Chelating HP)	Iminodiacetic acid coupled to Sepharose High Performance via an ether bond.	23 µmoles Cu^{2+}/ml	Short term 2–14 Long term 3–13	34 µm
Chelating Sepharose Fast Flow	Iminodiacetic acid coupled Sepharose Fast Flow via a spacer arm using epoxy coupling.	22–30 µmoles Zn^{2+}/ml	Short term 2–14 Long term 3–13	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

Stable in all commonly used aqueous buffers and denaturants such as 6 M guanidine hydrochloride and 8 M urea.

Storage

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

The column must be recharged with metal ions after long term storage to reactivate the medium.

Protein A fusion proteins

IgG Sepharose 6 Fast Flow

Recombinant fusion proteins containing a protein A tail and protein A can be purified on IgG Sepharose 6 Fast Flow.

Purification option

Product	Binding capacity/ml medium	Maximum operating flow
IgG Sepharose 6 Fast Flow	2 mg protein A at pH 7.5	400 cm/h*

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Purification example

Figure 29 shows automatic on-line monitoring of the production of a secreted fusion protein during fermentation. The fusion protein, ZZ-IGF-1 is insulin-like growth factor 1 fused with a derivative of protein A (designated ZZ), expressed in *E. coli*.

A) Sample: Bacterial suspension containing ZZ-IGF-1 fusion protein, automatically sampled from fermentation broth, 500 µl
Column: IgG Sepharose 6 Fast Flow (0.5 x 2.5 cm)
Binding buffer: 0.05 M Tris-HCl, 0.05% Tween™ 20, pH 7.6
Wash buffer: 10 mM ammonium acetate, pH 4.6
Elution buffer: 0.2 M acetic acid, pH 3.2

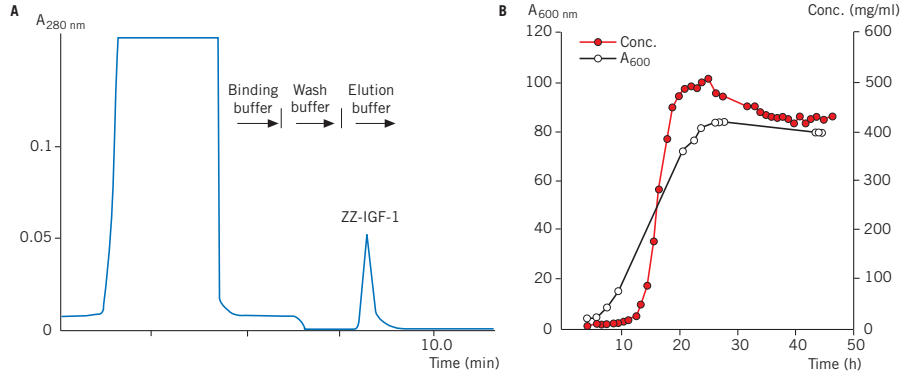


Fig. 29. A) Chromatogram of a sample taken at one time point during fermentation. B) Results from automatic monitoring of the product concentration during fermentation. Concentration of ZZ-IGF-1 is determined by integration of the ZZ-IGF-1 peak obtained during each chromatographic analysis. Bacterial density is measured manually at $A_{600\ nm}$.

Performing a separation

Binding buffer:	0.05 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.6
Wash buffer:	5 mM ammonium acetate, pH 5.0
Elution buffer:	0.5 M acetic acid, adjusted to pH 3.4 with ammonium acetate
Neutralization buffer:	1 M Tris-HCl, pH 9.0

1. Pack the column (see Appendix 3) and wash with at least 5 column volumes of binding buffer.
2. Equilibrate the column with approximately 5 column volumes of binding buffer.
3. Wash with 2–3 column volumes of acetic acid followed by 5 column volumes of binding buffer.
4. Apply the sample.
5. Wash with 10 column volumes binding buffer.
6. Wash with 2 column volumes of wash buffer or until no material appears in the eluent (determined by UV absorbance at $A_{280\ nm}$).
7. Elute with 2–5 column volumes of elution buffer.*
8. Immediately re-equilibrate the column with binding buffer until the eluent reaches pH 7.0 (the IgG may denature if left at a lower pH).

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

This method, while giving a concentrated eluate, can only be used if the fusion product is stable under the acid conditions.

An alternative eluent is 0.1 M glycine-HCl, pH 3.0. Chaotropic agents may also be used for elution.

Media characteristics

	Ligand	Composition	pH stability*	Particle size
IgG Sepharose 6 Fast Flow	Human polyclonal IgG	IgG coupled to Sepharose Fast Flow by the cyanogen bromide method.	Short term 3–10 Long term 3–10	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

Avoid reducing agents such as 2-mercaptoethanol or DTT since they may disrupt disulphide bonds within the IgG ligand.

Storage

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

Purification or removal of serine proteases, e.g. thrombin and trypsin, and zymogens

HiTrap Benzamidine FF (high sub), Benzamidine Sepharose 4 Fast Flow (high sub)

Sample extraction procedures often release proteases into solution, requiring the addition of protease inhibitors to prevent unwanted proteolysis. An alternative to the addition of inhibitors is to use a group specific affinity medium to remove the proteases from the sample. The same procedure can be used to either specifically remove these proteases or purify them.

The synthetic inhibitor para-aminobenzamidine is used as the affinity ligand for trypsin, trypsin-like serine proteases and zymogens. Benzamidine Sepharose 4 Fast Flow (high sub) is frequently used to remove molecules from cell culture supernatant, bacterial lysate or serum. During the production of recombinant proteins, tags such as GST are often used to facilitate purification and detection. Enzyme specific recognition sites are included in the recombinant protein to allow the removal of the tag by enzymatic cleavage when required. Thrombin is commonly used for enzymatic cleavage, and must often be removed from the recombinant product. HiTrap Benzamidine FF (high sub) provides a simple, ready to use solution for this process. Figure 30 shows the partial structure of Benzamidine Sepharose 4 Fast Flow (high sub) and Table 4 gives examples of different serine proteases.

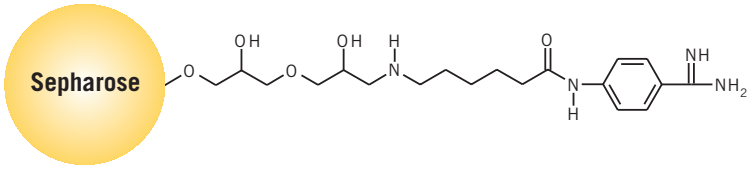


Fig. 30. Partial structure of Benzamidine Sepharose 4 Fast Flow (high sub).

Table 4. Examples of different serine proteases.

	Source	M_r	pI
Thrombin	Bovine pancreas	23 345	10.5
Trypsin	Human plasma chain A Human plasma chain B	5 700 31 000	 7.1
Urokinase	Human urine	54 000	8.9
Enterokinase	Porcine intestine heavy chain Porcine intestine light chain	134 000 62 000	4.2
Plasminogen	Human plasma	90 000	6.4–8.5
Prekallikrein	Human plasma	nd	nd
Kallikrein	Human plasma Human saliva	86 000 nd	nd (plasma) 4.0 (saliva)

Purification options

	Binding capacity	Maximum operating flow	Comments
HiTrap Benzamidine FF (high sub)	Trypsin, > 35 mg/column Trypsin, > 175 mg/column	4 ml/min (1 ml column) 15 ml/min (5 ml column)	Prepacked columns**.
Benzamidine Sepharose 4 Fast Flow (high sub)	Trypsin, > 35 mg/ml medium	300 cm/h*	Supplied as a suspension ready for column packing**.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*
***Supplied in 0.05 M acetate, pH 4 containing 20% ethanol.*

Purification examples

Figure 31 shows an example of the removal of trypsin-like proteases from human plasma to prevent proteolysis of the plasma components, using a low pH elution. The activity test demonstrated that almost all trypsin-like protease activity is removed from the sample and bound to the column.

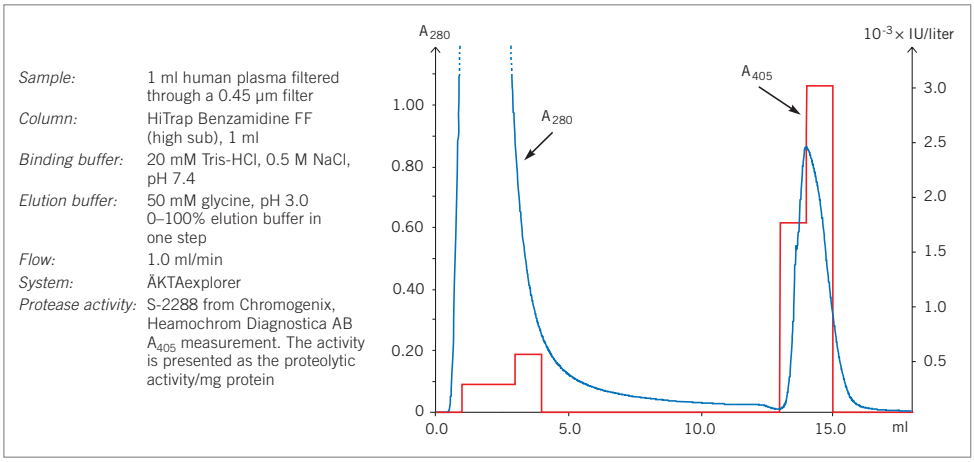
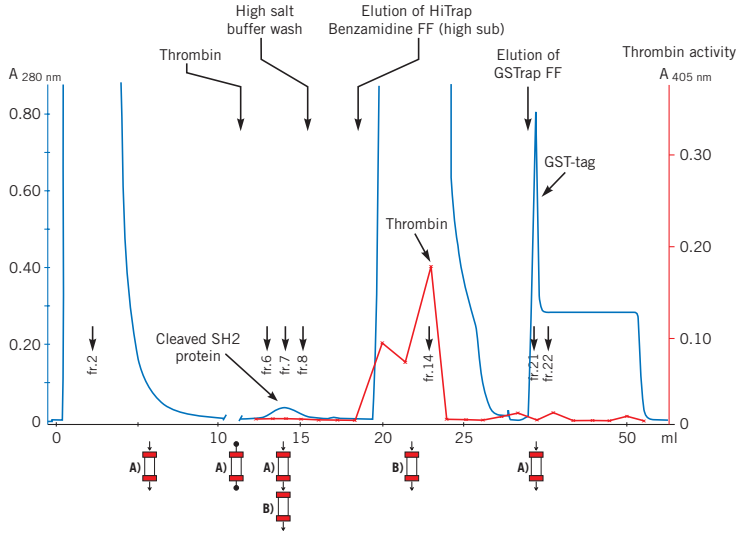


Fig. 31. Removal of trypin-like serine proteases from human plasma using HiTrap Benzamidine FF (high sub), 1 ml.

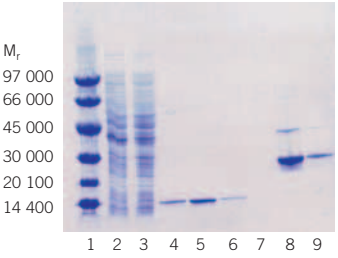
Figure 32 shows the effectiveness of using a GSTrap FF column with a HiTrap Benzamidine FF (high sub) for purification of a GST fusion protein, followed by cleavage of the GST tag via the thrombin cleavage site and subsequent removal of the thrombin enzyme. The GST fusion protein binds to the GSTrap FF column as other proteins wash through the column. Thrombin is applied to the column and incubated for 2 hours.

A HiTrap Benzamidine FF (high sub) column, pre-equilibrated in binding buffer, is attached after the GSTrap FF column and both columns are washed in binding buffer followed by a high salt buffer. The cleaved protein and thrombin wash through from the GSTrap FF column, thrombin binds to the HiTrap Benzamidine FF (high sub) column, and the eluted fractions contain pure cleaved protein.

Sample:	2 ml clarified *E. coli* homogenate expressing a M_r 37 000 SH2-GST fusion protein with a thrombin cleavage site
Columns:	GSTrap FF, 1 ml and HiTrap Benzamidine FF (high sub), 1 ml
Binding buffer:	20 mM sodium phosphate, 0.15 M NaCl, pH 7.5
High salt wash buffer:	20 mM sodium phosphate, 1.0 M NaCl, pH 7.5
Benzamidine elution buffer:	20 mM p-aminobenzamidine in binding buffer
GST elution buffer:	20 mM reduced glutathione, 50 mM Tris, pH 8.0
Flow:	0.5 ml/min
System:	ÄKTAprime
Protease treatment:	20 units/ml thrombin (Amersham Biosciences) for 2 hours at room temperature
Thrombin activity:	S-2238 (Chromogenix, Haemochrom Diagnostica AB) was used as a substrate and its absorbance at 405 nm was measured



A) GSTrap FF, 1 ml
B) HiTrap Benzamidine FF (high sub), 1 ml



Gel:	ExcelGel SDS Gradient 8–18%, Coomassie Blue staining
Lane 1.	Low Molecular Weight Calibration Kit (LMW)
Lane 2.	Clarified *E. coli* homogenate expressing SH2-GST fusion protein
Lane 3.	Flow-through from GSTrap FF (Fraction 2)
Lane 4.	SH2 GST-tag cleaved, washed off with binding buffer through both columns (Fraction 6)
Lane 5.	as above (Fraction 7)
Lane 6.	as above (Fraction 8)
Lane 7.	Elution of thrombin, HiTrap Benzamidine FF (high sub)
Lane 8.	Elution of GST-tag and some non-cleaved SH2-GST, GSTrap FF (Fraction 21)
Lane 9.	as above (Fraction 22)

Fig. 32. On-column cleavage of a GST fusion protein and removal of thrombin after on-column cleavage, using GSTrap FF and HiTrap Benzamidine FF (high sub).

Performing a separation

Binding buffer: 0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4

Elution buffer alternatives:

- pH elution: 0.05 M glycine-HCl, pH 3.0 or 10 mM HCl, 0.05 M NaCl, pH 2.0
- competitive elution: 20 mM p-aminobenzamidine in binding buffer
- denaturing eluents: 8 M urea or 6 M guanidine hydrochloride

1. Equilibrate the column with 5 column volumes of binding buffer.
2. Apply the sample.
3. Wash with 5–10 column volumes of binding buffer or until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
4. Elute with 5–10 column volumes of elution buffer. Collect fractions in neutralization buffer if low pH elution is used*. The purified fractions can be buffer exchanged using desalting columns (see page 133).

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

Since Benzamidine Sepharose 4 Fast Flow (high sub) has some ionic binding characteristics, the use of 0.5 M NaCl and pH elution between 7.4–8.0 is recommended. If lower salt concentrations are used, include a high salt wash step after sample application and before elution.

The elution buffer used for competitive elution has a high absorbance at 280 nm. The eluted protein must be detected by other methods, such as an activity assay, total protein or SDS-PAGE analysis. The advantage with competitive elution is that the pH is kept constant throughout the purification.

Cleaning

Wash with 3–5 column volumes of 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5 followed with 3–5 column volumes of 0.1 M sodium acetate, 0.5 M NaCl, pH 4.5 and re-equilibrate immediately with 3–5 column volumes of binding buffer.

Remove severe contamination by washing with non-ionic detergent such as 0.1% Triton X-100 at +37 °C for 1 minute.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
Benzamidine Sepharose 4 Fast Flow (high sub)	≥ 12 µmoles p-aminobenzamidine/ml	Amide coupling of ligand via a 14 atom spacer to highly cross-linked 4% agarose	Short term 1–9 Long term 2–8	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

All commonly used aqueous buffers.

Storage

Wash media and columns with 20% ethanol in 0.05 M sodium acetate, pH 4.0 (use approximately 5 column volumes for packed media) and store at +4 to +8 °C.

Serine proteases and zymogens with an affinity for arginine

Arginine Sepharose 4B

Arginine Sepharose 4B is an L-arginine derivative of Sepharose 4B that can be used for any biomolecule with a biospecific or charge dependent affinity for arginine, such as serine proteases and zymogens. Specific examples include prekallikrein, clostripain, prothrombin, plasminogen and plasminogen activator.

The L-arginine is coupled via its α-amino group, leaving the guanidino and α-carboxyl groups free to interact with samples. Electrostatic and stereospecific effects may contribute to the binding and elution process depending upon the specific sample involved. Figure 33 shows the partial structure of Arginine Sepharose 4B.

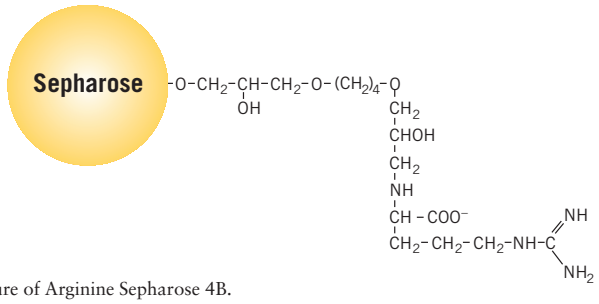


Fig. 33. Partial structure of Arginine Sepharose 4B.

Purification option

	Binding capacity/ml medium	Maximum operating flow	Comments
Arginine Sepharose 4B	No data available	75 cm/h*	Supplied as a suspension ready for column packing.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Performing a separation

Determine the capacity of the medium for the sample of interest over a range of different pH and flow rates. The sample must be at the same pH as the binding buffer for each experiment.

1. Pack the column (see Appendix 3) and wash with 5 column volumes of binding buffer.
2. Equilibrate the column with 10 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 (monitored by UV absorption at $A_{280\ nm}$).
5. Elute with 10–20 column volumes of elution buffer.

Biomolecules bound non-specifically can be eluted by:

- step or gradient elution with increasing ionic strength (up to 1 M NaCl)
- increasing concentration of urea or guanidine hydrochloride (up to 0.7 M)

Specifically bound biomolecules can be eluted by competitive elution with a buffer containing arginine or another competing agent for the target molecule.

Cleaning

Wash with 2–3 column volumes of alternate high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 5.0). Repeat 3 times. Re-equilibrate immediately with 5 column volumes of binding buffer.

Remove strongly bound proteins with 2–3 column volumns of 0.5 M NaOH or include 8 M urea or 6 M guanidine hydrochloride in the normal wash buffer to minimize adsorption.

Remove severe contamination by washing with non-ionic detergent, e.g. Triton X-100 (0.1%) at +37 °C for 1 min. Re-equilibrate immediately with binding buffer.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
Arginine Sepharose 4B	14–20 µmoles/ml	Arginine is coupled by an epoxy coupling method through a long hydrophilic spacer and stable ether and alkylamine bonds.	Short term 2–13 Long term 2–13	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

Stable to all commonly used aqueous buffers.

Storage

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

DNA binding proteins

HiTrap Heparin HP, HiPrep 16/10 Heparin FF, Heparin Sepharose 6 Fast Flow

DNA binding proteins form an extremely diverse class of proteins sharing a single characteristic, their ability to bind to DNA. Functionally the group can be divided into those responsible for the replication and orientation of the DNA such as histones, nucleosomes and replicases and those involved in transcription such as RNA/DNA polymerases, transcriptional activators and repressors and restriction enzymes. They can be produced as fusion proteins to enable more specific purification (see page 42), but their ability to bind DNA also enables group specific affinity purification using heparin as a ligand. Heparin is a highly sulphated glycosaminoglycan with the ability to bind a very wide range of biomolecules including:

- DNA binding proteins such as initiation factors, elongation factors, restriction endonucleases, DNA ligase, DNA and RNA polymerases.
- Serine protease inhibitors such as antithrombin III, protease nexins.
- Enzymes such as mast cell proteases, lipoprotein lipase, coagulation enzymes, superoxide dismutase.
- Growth factors such as fibroblast growth factor, Schwann cell growth factor, endothelial cell growth factor.
- Extracellular matrix proteins such as fibronectin, vitronectin, laminin, thrombospondin, collagens.
- Hormone receptors such as oestrogen and androgen receptors.
- Lipoproteins.

The structure of heparin is shown in Figure 34. Heparin has two modes of interaction with proteins and, in both cases, the interaction can be weakened by increases in ionic strength.

1. In its interaction with DNA binding proteins heparin mimics the polyanionic structure of the nucleic acid.
2. In its interaction with coagulation factors such as antithrombin III, heparin acts as an affinity ligand.

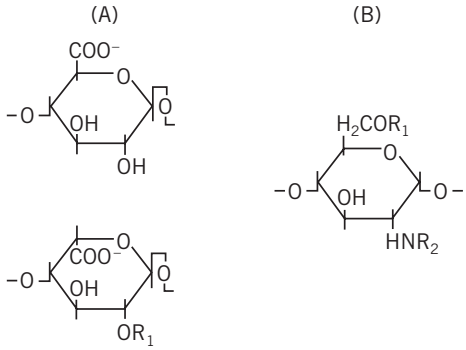


Fig. 34. Structure of a heparin polysaccharide consisting of alternating hexuronic acid (A) and D-glucosamine residues (B). The hexuronic acid can either be D-glucuronic acid (top) or its C-5 epimer, L-iduronic acid (bottom). R_1 = -H or $-SO_3^-$, R_2 = $-SO_3^-$ or $-COCH_3$.

Purification options

	Binding capacity	Maximum operating flow	Comments
HiTrap Heparin HP	Bovine antithrombin III, 3 mg/column Bovine antithrombin III, 15 mg/column	4 ml/min (1 ml column) 20 ml/min (5 ml column)	Prepacked columns.
HiPrep 16/10 Heparin FF	Bovine antithrombin III, 40 mg/column	10 ml/min	Prepacked 20 ml column.
Heparin Sepharose 6 Fast Flow	Bovine antithrombin III, 2 mg/ml medium	400 cm/h*	Supplied as a suspension ready for column packing.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Purification examples

Figures 35, 36 and 37 show examples of conditions used for the purification of different DNA binding proteins.

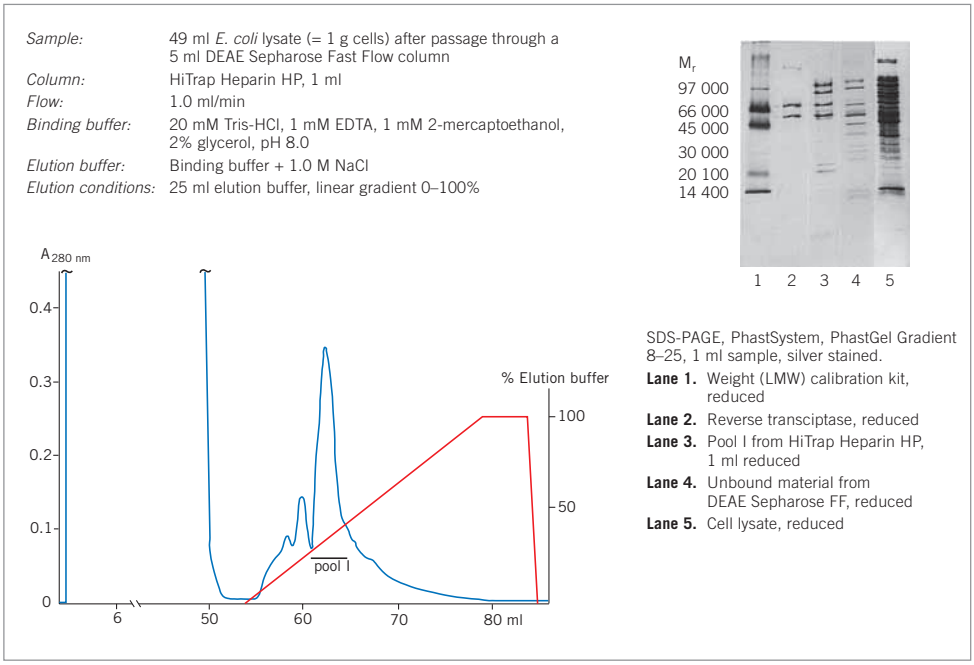


Fig. 35. Partial purification of recombinant HIV-reverse transcriptase on HiTrap Heparin HP.

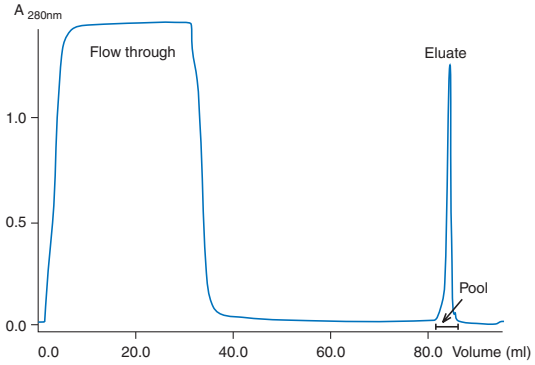
Sample: 30 ml extract containing Oct-1, filtered (0.45 µm) and transferred to binding buffer using a PD-10 desalting column

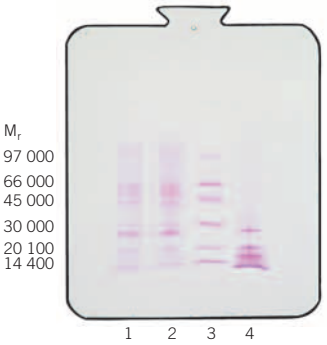
Column: HiTrap Heparin HP, 5 ml

Binding buffer: 20 mM Tris-HCl, 5% (v/v) glycerol, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM Pefabloc™, 0.5 M NaCl, pH 8

Elution buffer: 20 mM Tris-HCl, 5% (v/v) glycerol, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM Pefabloc, 2 M NaCl, pH 8

Flow: 1 ml/min (30 cm/h)





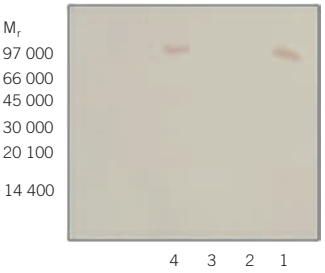
SDS-PAGE, PhastSystem, PhastGel 10–15, 1 µl sample, Coomassie Blue staining

Lane 1. Starting material, *E. coli* extract, dil. 4-fold

Lane 2. Flow-through

Lane 3. Low Molecular Weight Calibration Kit (LMW)

Lane 4. Eluate pool



Western blot of the electrophoresis gel using rabbit anti-Oct human-1 and alkaline phosphatase

Lane 1. Starting material

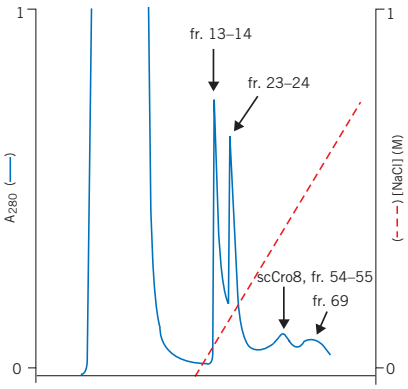
Lane 2. Flow-through

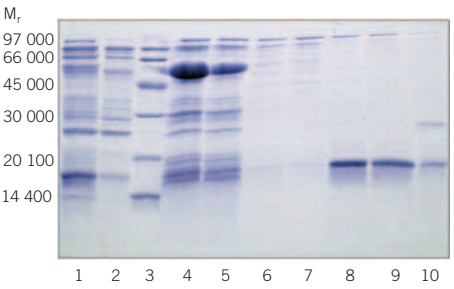
Lane 3. Low Molecular Weight Calibration Kit (LMW)

Lane 4. Eluate pool

Fig. 36. Partial purification of the recombinant DNA binding Oct-1 protein (courtesy of Dr Gunnar Westin, University Hospital, Uppsala, Sweden) using HiTrap Heparin HP, 5 ml.

Sample:	2000 ml partially purified sample from DEAE Sepharose CL-4B flow-through, pH 7.0
Column:	HiPrep 16/10 Heparin FF
Binding buffer:	50 mM sodium phosphate, pH 7.5
Elution buffer:	50 mM sodium phosphate, 1 M sodium chloride, pH 7.5
Flow:	1.5 ml/min (45 cm/h)
Chromatographic procedure:	Equilibration binding buffer: 80 ml Sample application: 2000 ml Wash with binding buffer: 100 ml Elution: 300 ml elution buffer as linear gradient 0–100%





Electrophoresis: SDS-PAGE, 12% gel, Coomassie Blue staining

Lane 1.	Pool from HiPrep 26/10 Desalting
Lane 2.	Flow-through pool from DEAE Sepharose CL-4B
Lane 3.	Low Molecular Weight Calibration Kit (LMW)
Lane 4-10.	Eluted fractions from HiPrep 16/10 Heparin FF
Lane 4.	Fraction 13
Lane 5.	Fraction 14
Lane 6.	Fraction 23
Lane 7.	Fraction 24
Lane 8.	Fraction 54
Lane 9.	Fraction 55
Lane 10.	Fraction 69

Fig. 37. scCro8 purification on HiPrep 16/10 Heparin FF.

Performing a separation

Binding buffers: 20 mM Tris-HCl, pH 8.0 or 10 mM sodium phosphate, pH 7.0

Elution buffer: 20 mM Tris-HCl, 1–2 M NaCl, pH 8.0 or 10 mM sodium phosphate, 1–2 M NaCl, pH 7.0

1. Equilibrate the column with 10 column volumes of binding buffer.
2. Apply the sample.
3. Wash with 5–10 column volumes of binding buffer or until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
4. Elute with 5–10 column volumes of elution buffer using a continuous or step gradient from 0–100% elution buffer.

Modify the selectivity of heparin by altering pH or ionic strength of the buffers. Elute using a continuous or step gradient with NaCl, KCl or $(NH_4)_2SO_4$ up to 1.5–2 M.

Cleaning

Remove ionically bound proteins by washing with 0.5 column volume 2 M NaCl for 10–15 minutes.

Remove precipitated or denatured proteins by washing with 4 column volumes 0.1 M NaOH for 1–2 hours or 2 column volumes 6 M guanidine hydrochloride for 30–60 minutes or 2 column volumes 8 M urea for 30–60 minutes.

Remove hydrophobically bound proteins by washing with 4 column volumes 0.1% – 0.5% Triton X-100 for 1–2 hours.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
HiTrap Heparin HP	10 mg/ml	Heparin coupled to Sepharose High Performance by reductive amination to give a stable attachment even in alkaline conditions.	Short term 5–10 Long term 5–10	34 µm
Heparin Sepharose 6 Fast Flow HiPrep 16/10 Heparin FF	5 mg/ml	Heparin coupled to Sepharose 6 Fast Flow by reductive amination to give a stable attachment even in alkaline conditions.	Short term 4–13 Long term 4–12	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

0.1 M NaOH (1 week at +20 °C), 0.05 M sodium acetate, pH 4.0, 4 M NaCl, 8 M urea, 6 M guanidine hydrochloride.

Storage

Wash media and columns with 0.05 M sodium acetate containing 20% ethanol (use approximately 5 column volumes for packed media) and store at +4 to +8 °C.

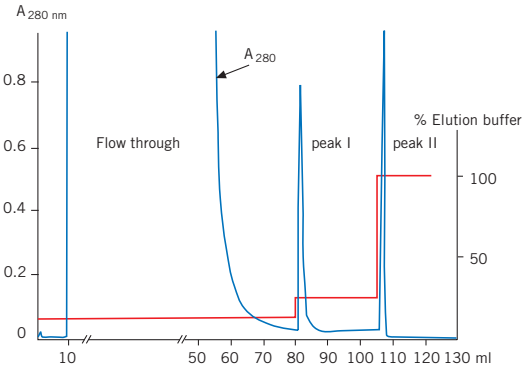
Coagulation factors

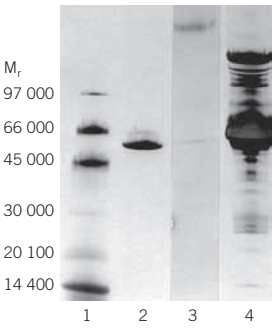
HiTrap Heparin HP, HiPrep 16/10 Heparin FF, Heparin Sepharose 6 Fast Flow

Blood coagulation factors form an extremely important group of proteins for research, medical and clinical applications. The information about the purification of DNA binding proteins (page 60) is applicable also to the purification of coagulation factors.

Purification examples

Sample: 30 ml bovine plasma diluted with 15 ml 0.1 M Tris, 0.01 M citrate, 0.225 M NaCl, pH 7.4
Column: HiTrap Heparin HP, 1 ml
Flow: 1.0 ml/min
Binding buffer: 0.1 M Tris, 0.01 M citric acid, 0.225 M NaCl, pH 7.4
Elution buffer: 0.1 M Tris, 0.01 M citric acid, 2 M NaCl, pH 7.4
Chromatographic procedure: 8 ml 12.5% elution buffer, 45 ml sample, 27 ml 12.5% elution buffer, 26 ml 25% elution buffer, 26 ml 100% elution buffer. 2.9 mg antithrombin-III was eluted in peak II
Electrophoresis: SDS-PAGE, PhastSystem, PhastGel Gradient 8–25, 1 µl sample, silver stained

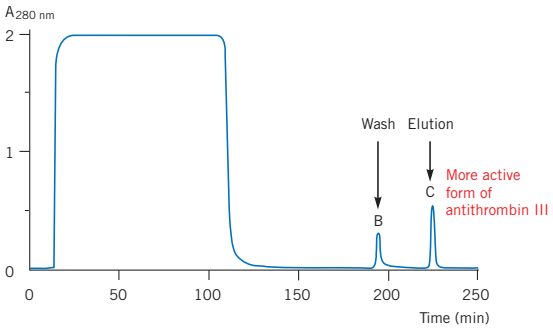


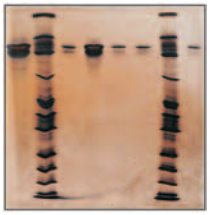


Lane 1. Low Molecular Weight Calibration Kit, reduced
Lane 2. Peak II, reduced, diluted 2-fold
Lane 3. Peak I, reduced, diluted 2-fold
Lane 4. Unbound material, reduced, diluted 15-fold

Fig. 38. Purification of antithrombin III from bovine plasma on HiTrap Heparin HP, 1 ml.

Sample:	Pooled and frozen human plasma from 5 donors. 50 ml of thawed plasma filtered (0.45 µM). Plasma and binding buffer mixed in ratio 2:1
Binding buffer:	0.1 M Tris-HCl, 0.01 M citric acid, 0.225 M NaCl, pH 7.4
Wash buffer:	0.1 M Tris-HCl, 0.01 M citric acid, 0.330 M NaCl, pH 7.4
Elution buffer:	0.1 M Tris-HCl, 0.01 M citric acid, 2.0 M NaCl, pH 7.4
Column:	Heparin Sepharose 6 Fast Flow packed in HR 5/5 column
Chromatographic procedure:	5 ml binding buffer, 45 ml sample, 40 ml binding buffer, 15 ml wash buffer, 9 ml elution buffer





Isoelectric focusing-PAGE analysis of the peaks B and C from the affinity chromatography.

Lanes 1 and 4. Peak C

Lanes 2 and 7. IEF calibration kit

Lanes 3 and 6. Antithrombin-III from Sigma (A7388)

Lanes 5 and 8. Peak B

The results show that pure antithrombin-III is present in the two peaks. NOR-Partigen-Antithrombin-III test of peaks B and C shows a more active form of antithrombin-III concentrated in peak C.

Fig. 39. Purification of antithrombin-III from human plasma on Heparin Sepharose 6 Fast Flow. Peak B elutes with wash buffer. Peak C elutes with elution buffer and includes a more active form of antithrombin-III.

Performing a separation

As for DNA binding proteins, see page 63.

Since the heparin acts as an affinity ligand for coagulation factors, it may be advisable to include a minimum concentration of 0.15 M NaCl in the binding buffer.

If an increasing salt gradient gives unsatisfactory results, use heparin (1–5 mg/ml) as a competing agent in the elution buffer.

Biotin and biotinylated substances

HiTrap Streptavidin HP, Streptavidin Sepharose High Performance

Biotin and biotinylated substances bind to streptavidin (a molecule isolated from *Streptomyces avidinii*) in a very strong interaction that requires denaturing conditions for elution. By coupling streptavidin to Sepharose a highly specific affinity medium is created and, using biotinylated antibodies, the strong interaction can be utilized for the purification of antigens. The biotinylated antibody-antigen complexes bind tightly to Streptavidin Sepharose and the antigen can then be eluted separately using milder elution conditions, leaving behind the biotinylated antibody. An alternative to labelling the antibody with biotin is to use 2-iminobiotin that binds to streptavidin above pH 9.5 and can be eluted at pH 4 (see Figure 40).

Purification options

	Binding capacity	Maximum operating flow	Comments
HiTrap Streptavidin HP	Biotin, > 300 nmol/column Biotinylated BSA, 6 mg/column	4 ml/min	Prepacked 1 ml column.
Streptavidin Sepharose High Performance	Biotin, > 300 nmol/medium Biotinylated BSA, 6 mg/medium	150 cm/h*	Supplied as a suspension ready for column packing.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Purification example

Sample: 9.0 ml of a mixture of BSA and iminobiotinylated BSA, filtered through a 0.45 µm filter
Column: HiTrap Streptavidin HP, 1 ml
Binding buffer: 50 mM ammonium carbonate buffer, 0.5 M NaCl, pH 10.0
Elution buffer: 50 mM ammonium acetate buffer, 0.5 M NaCl, pH 4.0
Flow: 1 ml/min (0.3 ml/min during sample application)
System: ÄKTAexplorer

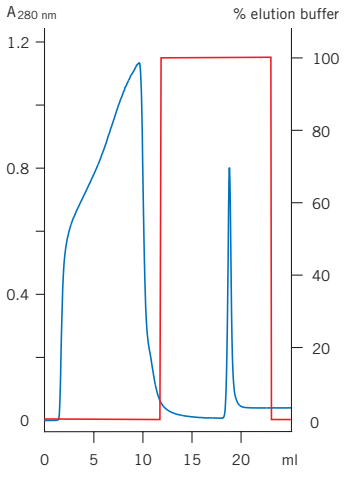


Fig. 40. Purification of iminobiotinylated BSA on HiTrap Streptavidin HP, 1 ml.

Performing a separation:

Biotinylated substances

Binding buffer: 20 mM sodium phosphate, 0.15 M NaCl, pH 7.5

Elution buffer: 8 M guanidine-HCl, pH 1.5

Iminobiotinylated substances

Binding buffer: 50 mM ammonium carbonate, 0.5 M NaCl, pH 10.0

Elution buffer: 50 mM ammonium acetate, 0.5 M NaCl, pH 4.0

1. Equilibrate the column with 10 column volumes of binding buffer.
2. Apply the sample. For the best results use a low flow rate, 0.1–0.5 ml/min, during sample application.
3. Wash with at least 10 column volumes of binding buffer or until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
4. Elute with 10–20 column volumes of elution buffer.*

**Since elution conditions can be quite harsh, it is recommended to collect fractions into neutralization buffer (100 µl – 200 µl 1 M Tris-HCl, pH 9.0 per ml fraction), so that the final pH of the fractions will be approximately neutral or perform a rapid buffer exchange on a desalting column (see page 133).*

The harsh conditions required to break the streptavidin-biotin bond may affect both the sample and the ligand. Streptavidin Sepharose columns cannot be re-used after elution under these conditions.

Antigen purification

Antigens can be purified from biotinylated antibody-antigen complexes bound to streptavidin. The following method was adapted for HiTrap Streptavidin HP from work published in *Anal. Biochem.* **163**, 270–277 (1987), Gretch, D.R., Suter, M. and Stinski, M.F.

Solubilization buffer: 20 mM sodium phosphate, 150 mM NaCl, pH 7.5 with 0.1% SDS, 1.0% Nonidet™-P-40, 0.5% sodium deoxycholate, 0.02% NaN_3, 100 µg/ml PMSF

Elution buffer: 0.1 M glycine-HCl, pH 2.2

1. Solubilize the antigen with an appropriate amount of solubilization buffer, clear the sample by centrifuging at 12 000 g for 15 min.
2. Add the biotinylated antibody and adjust the volume to 1 ml.
3. Incubate with end-over-end mixing, for at least 1 h or overnight.
4. Equilibrate the column with 10 column volumes of solubilization buffer.
6. Apply antibody-antigen solution to the column at a low flow rate such as 0.2 ml/min. If the sample volume is less than 1 ml, apply the sample, and leave for a few minutes to allow binding to take place.
7. Wash out unbound sample with 10 column volumes of solubilization buffer or until no material is found in eluent (monitored by UV absorption at $A_{280\ nm}$).
8. Elute with 5–10 column volumes of elution buffer.*

**Since elution conditions are quite harsh, it is recommended to collect fractions into neutralization buffer (100 µl – 200 µl 1 M Tris-HCl, pH 9.0 per ml fraction), so that the final pH of the fractions will be approximately neutral or perform a rapid buffer exchange on a desalting column (see page 133).*

Media characteristics

	Composition	pH stability*	Mean particle size
Streptavidin Sepharose High Performance HiTrap Streptavidin HP	Streptavidin is coupled to Sepharose High Performance using a N-hydroxysuccinimide coupling method.	Short term 2–10.5 Long term 4–9	34 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Storage

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

Purification or removal of fibronectin

Gelatin Sepharose 4B

Fibronectin is a high molecular weight glycoprotein found on the surfaces of many cell types and present in many extracellular fluids including plasma. Fibronectin binds specifically to gelatin at or around physiological pH and ionic strength.

Purification option

	Binding capacity/ml medium	Maximum operating flow	Comments
Gelatin Sepharose 4B	1 mg human plasma fibronectin	75 cm/h*	Supplied as a suspension ready for column packing.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Performing a separation

Binding buffer: PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4

Elution buffer alternatives:
- 0.05 M sodium acetate, 1.0 M sodium bromide (or potassium bromide), pH 5.0
- Binding buffer + 8 M urea
- Binding buffer + arginine

Fibronectin has a tendency to bind to glass. Use siliconized glass to prevent adsorption.

Cleaning

Wash 3 times with 2–3 column volumes of buffer, alternating between high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5). Re-equilbrate immediately with 3–5 column volumes of binding buffer. Remove denatured proteins or lipids by washing the column with 0.1% Triton X-100 at +37 °C for one minute. Re-equilibrate immediately with 5 column volumes of binding buffer.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
Gelatin Sepharose 4B	4.5–8 mg gelatin/ml	Gelatin linked to Sepharose using the CNBr method	Short term 3–10 Long term 3–10	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

Stable in all commonly used aqueous buffers.

Storage

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

Purification or removal of albumin

HiTrap Blue HP, Blue Sepharose 6 Fast Flow

The same procedure can be used either to purify albumin or to remove albumin as a specific contaminant before or after other purification steps.

Albumin binds to Cibacron™ Blue F3G-A, a synthetic polycyclic dye that acts as an aromatic anionic ligand binding the albumin via electrostatic and/or hydrophobic interactions. Similar interactions are seen with coagulation factors, lipoproteins and interferon. Cibacron Blue F3G-A is linked to Sepharose to create Blue Sepharose affinity media.

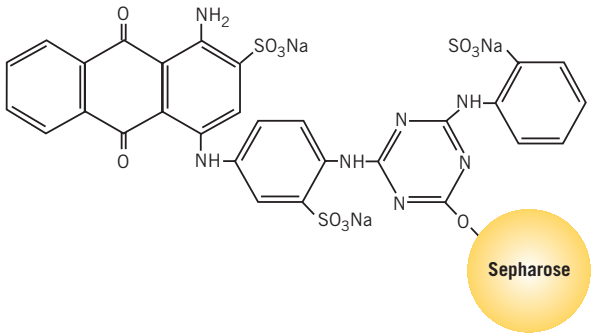


Fig. 41. Partial structure of Blue Sepharose Fast Flow and Blue Sepharose High Performance.

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.

Albumin can be a significant contaminant during the purification of immunoglobulins from ascites fluid, cell cultures or serum, chiefly because of its abundance in the original source material. Advice on the selection of techniques for the removal of albumin during antibody purification is given in *The Antibody Purification Handbook* from Amersham Biosciences.

Cibacron Blue F3G-A also shows certain structural similarities to naturally occurring molecules, such as the cofactor NAD^+, that enable it to bind strongly and specifically to a wide range of proteins including kinases, dehydrogenases and most other enzymes requiring adenylyl-containing cofactors (see page 73).

Purification options

	Binding capacity	Maximum operating flow	Comments
HiTrap Blue HP	Human serum albumin, 20 mg/column Human serum albumin, 100 mg/column	4 ml/min (1 ml column) 20 ml/min (5 ml column)	Prepacked columns.
Blue Sepharose 6 Fast Flow*	Human serum albumin, > 18 mg/ml medium	750 cm/h**	Supplied as a suspension ready for column packing.

**A convenient alternative to Blue Sepharose CL-6B, since rehydration is not required.*
***See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Purification examples

Figure 42 shows the use of HiTrap Blue HP for purification of increasing amounts of human serum albumin. The process is easily scaled up by connecting several 1 ml or 5 ml HiTrap columns in series.

Figure 43 shows the use of Blue Sepharose 6 Fast Flow for the separation of human serum albumin from interferon β.

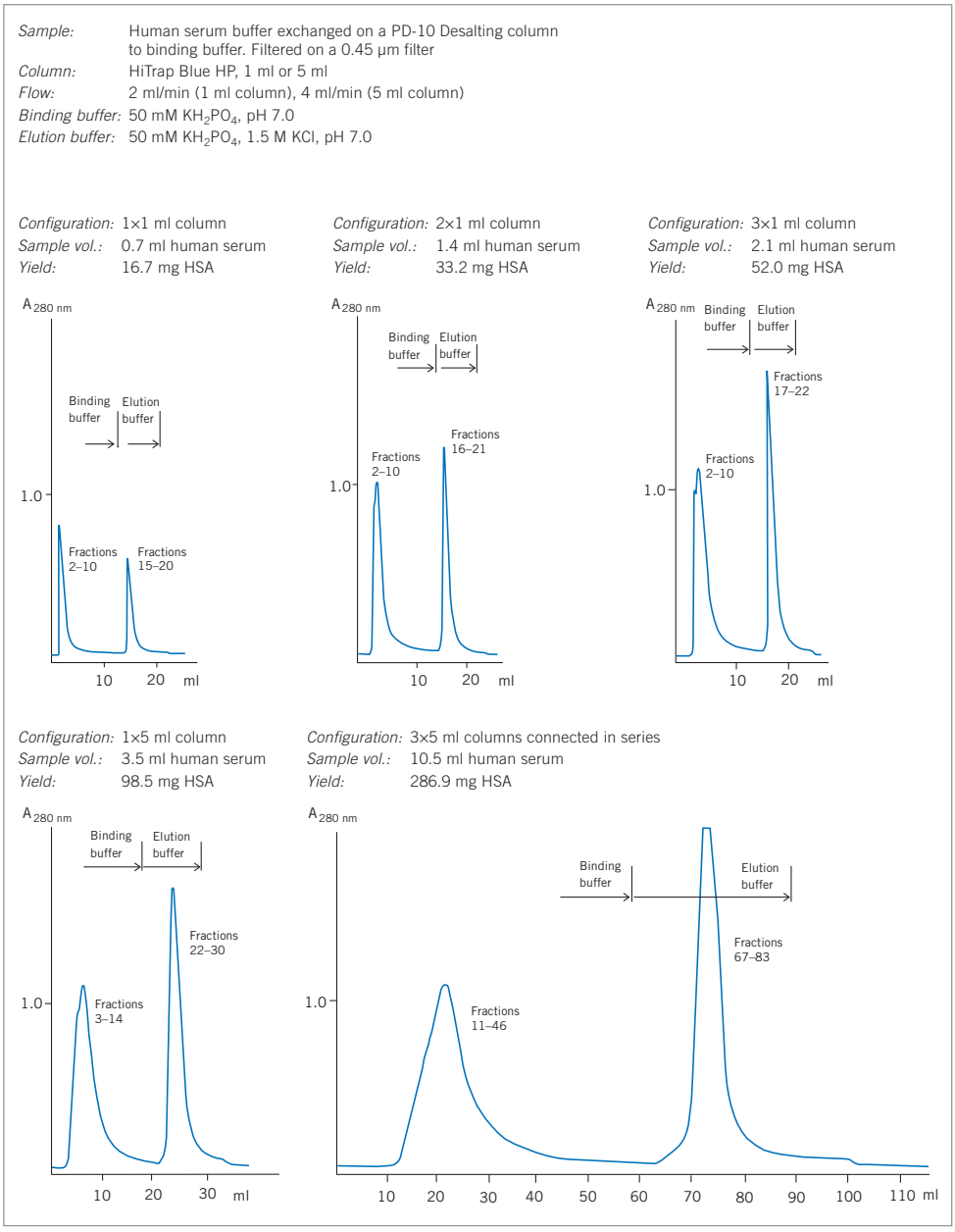


Fig. 42. Scaling up on HiTrap Blue HP gives predictable separations and quantitatively reproducible yields.

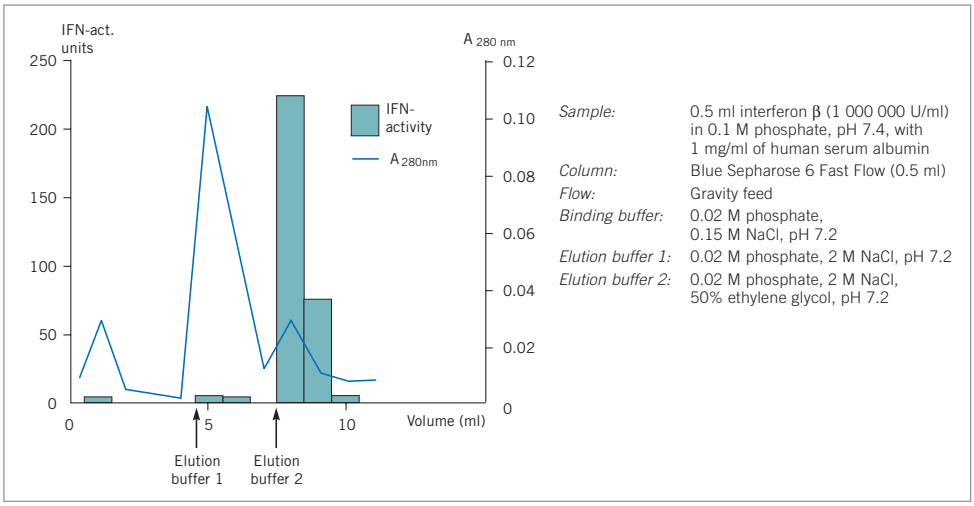


Fig. 43. Purification of human serum albumin and interferon β on Blue Sepharose 6 Fast Flow.

In these examples elution is achieved by increasing the ionic strength of the buffer or changing the polarity of the buffer. Changing the pH of the buffer can also work, but the correct co-factor is preferable for the elution of specifically bound proteins.

Performing a separation

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

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

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

Cleaning

Wash with 5 column volumes of high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) followed by low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5). Repeat 4–5 times. Re-equilibrate immediately with binding buffer.

Remove precipitated proteins with 4 column volumes of 0.1 M NaOH at a low flow rate, followed by washing with 3–4 column volumes of 70% ethanol or 2 M potassium thiocyanate. Alternatively, wash with 2 column volumes of 6 M guanidine hydrochloride. Re-equilibrate immediately with binding buffer.

Remove strongly hydrophobic proteins, lipoproteins and lipids by washing with 3–4 column volumes of up to 70% ethanol or 30% isopropanol. Alternatively, wash with 2 column volumes of detergent in a basic or acidic solution, e.g. 0.1% non-ionic detergent in 1 M acetic acid at a low flow rate, followed by 5 column volumes of 70% ethanol to remove residual detergent. Re-equilibrate immediately in binding buffer.

Media characteristics

	Ligand and density	Composition	pH stability*	Mean particl size
HiTrap Blue HP	Cibacron Blue F3G-A 4 mg/ml	Ligand coupled to Sepharose High Performance using the triazine method.	Short term 3–13 Long term 4–12	34 µm
Blue Sepharose 6 Fast Flow	Cibacron Blue F3G-A 6.7–7.9 µmoles/ml	Ligand coupled to Sepharose Fast Flw using the triazine method.	Short term 3–13 Long term 4–12	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

Stable in all commonly used aqueous buffers, 70% ethanol, 8 M urea and 6 M guanidine hydrochloride.

Storage

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

NAD^+-dependent dehydrogenases and ATP-dependent kinases

5' AMP Sepharose 4B, HiTrap Blue HP, Blue Sepharose 6 Fast Flow

NAD^+-dependent dehydrogenases and ATP-dependent kinases interact strongly with 5' AMP so that selective elution with gradients of NAD^+ or $NADP^+$ enables the resolution of complex mixtures of dehydrogenase isoenzymes, using 5' AMP Sepharos 4B.

Synthesis of 5' AMP Sepharose 4B takes place in several steps. Diaminohexane is linked to AMP via the N^6 of the purine ring. The derivatized AMP is then coupled to Sepharose 4B via the aminohexane spacer.

NAD^+-dependent dehydrogenases and ATP-dependent kinases are also members of a larger group of proteins that will interact with Cibacron Blue F3G-A, a synthetic polycyclic dye that shows certain structural similarities to the cofactor NAD^+. When used as an affinity ligand attached to Sepharose 6 Fast Flow or Sepharose HP, Cibacron Blue F3G-A will bind strongly and specifically to a wide range of proteins. Some proteins bind specifically due to their requirement for nucleotide cofactors, while others, such as albumin, lipoproteins, blood coagulation factors and interferon, bind in a less specific manner by electrostatic and/or hydrophobic interactions with the aromatic anionic ligand.

Purification options

	Binding capacity	Maximum operating flow	Comments
5' AMP Sepharose 4B	Lactate dehydrogenase, 10 mg/ml medium (0.1 M phosphate buffer, pH 7.0 at +20 °C)	75 cm/h*	High specificity for proteins with affinity for NAD^+. Supplied as a freeze-dried powder, rehydration required.
HiTrap Blue HP	Human serum albumin, 20 mg/column Human serum albumin, 100 mg/column	4 ml/min (1 ml column) 20 ml/min (5 ml column)	General specificity for proteins with affinity for $NADP^+$ and other proteins that react less specifically. Prepacked columns.
Blue Sepharose Fast Flow	Human serum albumin, > 18 mg/ml medium	750 cm/h*	General specificity for proteins with affinity for $NADP^+$ and other proteins that react less specifically. Supplied as a suspension ready for column packing.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

5' AMP Sepharose 4B

Performing a separation

Swell the required amount of powder for 15 min. in 0.1 M phosphate buffer, pH 7.0 (100 ml per gram dry powder) and wash on a sintered glass filter. Pack the column (see Appendix 3).

Binding buffer: 10 mM phosphate, 0.15 M NaCl, pH 7.3

If the protein of interest binds to the medium via ionic forces, it may be necessary to reduce the concentration of NaCl in the binding buffer.

Elution buffers:
- use low concentrations of the free cofactor, NAD^+ or $NADP^+$ (up to 20 mM) with step or gradient elution.

If detergent or denaturing agents have been used during purification, these can also be used in the high and low pH wash buffers.

Cleaning

Wash 3 times with 2–3 column volumes of buffers, alternating between high pH (0.5 M NaCl, 0.1 M Tris-HCl, pH 8.5) and low pH (0.5 M NaCl, 0.1 M sodium acetate, pH 4.5). Re-equilibrate immediately with 3–5 column volumes of binding buffer.

Remove denatured proteins or lipids by washing the column with 2 column volumes of detergent e.g. 0.1% Triton X-100 for 1 minute. Re-equilibrate immediately with 5 column volumes of binding buffer.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
5' AMP Sepharose 4B	2 µmoles/ml	N^6(6-aminohexyl-) 5' AMP coupled to Sepharose 4B using CNBr method**	Short term 4–10 Long term 4–10	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*
***The attachment of the ligand via an alkyl linkage to the N^6 amino group gives a stable product that is conformationally acceptable to most 5' AMP- or adenine nucleotide cofactor-requiring enzymes.*

Chemical stability

Stable to all commonly used aqueous buffers and additives such as detergents. Avoid high concentrations of EDTA, urea, guanidine hydrochloride, chaotropic salts and strong oxidizing agents. Exposure to pH >10 may cause loss of phosphate groups.

Storage

Store freeze-dried product below +8 °C under dry conditions.

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

HiTrap Blue HP, Blue Sepharose 6 Fast Flow

The information supplied for the purification or removal of albumin (page 70) is applicable also to the purification of enzymes with an affinity for NAD^+.

Performing a separation

As for albumin (see page 72), but note the following:

For elution use low concentrations of the free cofactor, NAD^+ or $NADP^+$ (1–20 mM), or increase ionic strength (up to 2 M NaCl or KCl, 1 M is usually sufficient).

For less specifically bound proteins: use higher concentrations of cofactor or salt or more severe eluents such as urea or potassium isothiocyanate. Polarity reducing agents such as dioxane (up to 10%) or ethylene glycol (up to 50%) may be used.

$NADP^+$-dependent dehydrogenases and other enzymes with affinity for $NADP^+$

2'5' ADP Sepharose 4B, Red Sepharose CL-6B

$NADP^+$-dependent dehydrogenases interact strongly with 2'5' ADP. Selective elution with gradients of NAD^+ or $NADP^+$ has allowed the resolution of complex mixtures of dehydrogenase isoenzymes using 2'5' ADP Sepharose 4B.

Synthesis of the medium takes place in several steps. Diaminohexane is linked to 2'5' ADP via the N^6 of the purine ring. The derivatized ADP is then coupled to Sepharose 4B via the aminohexane spacer. Figure 44 shows the partial structure of 2'5' ADP Sepharose 4B.

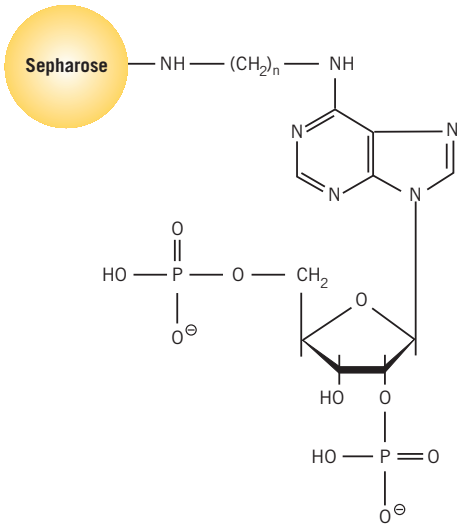


Fig. 44. Partial structure of 2'5' ADP Sepharose 4B.

$NADP^+$-dependent dehydrogenases are also members of a larger group of proteins that will interact with Procion™ Red, a synthetic polycyclic dye that shows certain structural similarities to naturally occurring $NADP^+$. When used as an affinity ligand attached to Sepharose CL-6B, Procion Red HE-3B will bind strongly and specifically to a wide range of proteins. Some proteins bind specifically due to their requirement for nucleotide cofactors, while others, such as albumin, lipoproteins, blood coagulation factors and interferon, bind in a less specific manner by electrostatic and/or hydrophobic interactions with the aromatic anionic ligand.

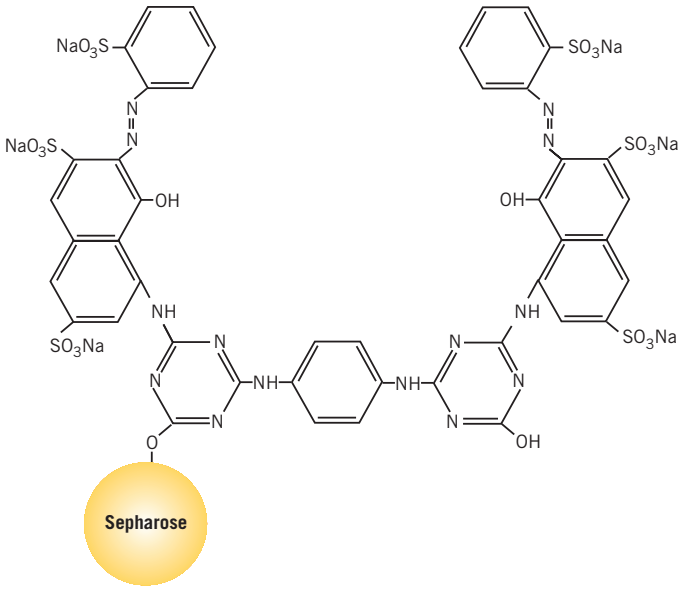


Fig. 45. Partial structure of Red Sepharose CL-6B.

Purification options

	Binding capacity/ml medium	Maximum operating flow	Comments
2'5' ADP Sepharose 4B	Glucose-6-phosphate, dehydrogenase, 0.4 mg (0.1 M Tris-HCl, 5 mM EDTA, 1 mM 2-mercaptoethanol buffer, pH 7.6).	75 cm/h*	High specificity for proteins with affinity for $NADP^+$. Supplied as a freeze-dried powder, rehydration required.
Red Sepharose CL-6B	Rabbit lactate dehydrogenase, 2 mg	150 cm/h*	General specificity for proteins with affinity for $NADP^+$ and other proteins that react less specifically. Supplied as dry powder, rehydration required.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

2'5' ADP Sepharose 4B

Purification example

Figure 46 shows a linear gradient elution used for the initial separation of $NADP^+$-dependent enzymes from a crude extract of *Candida utilis*.

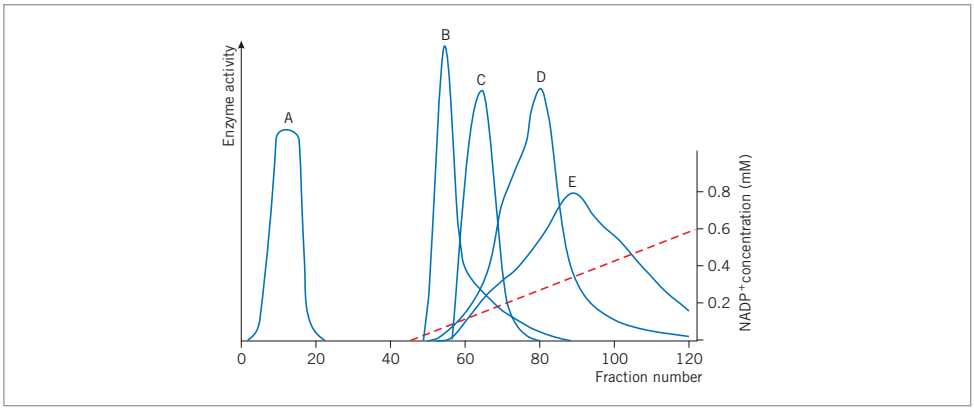


Fig. 46. Gradient elution with 0–0.6 mM $NADP^+$. A: non-interacting protein, B: glucose-6-phosphate dehydrogenase, C: glutamate dehydrogenase, D: glutathione reductase, E: 6-phosphogluconate dehydrogenase.
(Brodelius *et al.*, *Eur. J. Biochem.* **47**, 81–89 (1974)).

Performing a separation

Swell the required amount of powder for 15 min. in 0.1 M phosphate buffer, pH 7.3 (100 ml per gram dry powder) and wash on a sintered glass filter (porosity G3). Pack the column (see Appendix 3).

Binding buffer: 10 mM phosphate, 0.15 M NaCl, pH 7.3

If the protein of interest binds to the medium via ionic forces, it may be necessary to reduce the concentration of NaCl in the binding buffer.

Elution buffers:
- use low concentrations of the free cofactor, NAD^+ or $NADP^+$ (up to 20 mM) with step or gradient elution.

If detergent or denaturing agents have been used during purification, these can also be used in the low and high pH wash buffers.

Cleaning

Wash 3 times with 2–3 column volumes of buffers, alternating between high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5). Re-equilibrate immediately with 3–5 column volumes of binding buffer.

Remove denatured proteins or lipids by washing the column with 2 column volumes of detergent e.g. 0.1% Triton X-100 for 1 minute. Re-equilibrate immediately with 5 column volumes of binding buffer.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
2'5' ADP Sepharose 4B	2 µmoles/ml	N^6-(6-aminohexyl)adenosine 2'5' *bis*-phosphate coupled to Sepharose 4B by CNBr method**	Short term 4–10 Long term 4–10	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*
***Coupling via the N^6 position of the $NADP^+$ -analogue, adenosine 2'5' bisphosphate, gives a ligand that is stereochemically acceptable to most $NADP^+$-dependent enzymes.*

Chemical stability

Stable to all commonly used aqueous buffers and additives such as detergents. Avoid high concentrations of EDTA, urea, guanidine hydrochloride, chaotropic salts and strong oxidizing agents. Exposure to pH > 10 may cause loss of phosphate groups.

Storage

Store freeze-dried product below +8 °C under dry conditions.

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

Red Sepharose CL-6B

Performing a separation

Swell the required amount of powder for 15 min. and wash with distilled water on a sintered glass filter (porosity G3). Use 200 ml water for each gram of dry powder, adding in several aliquots. One gram of freeze-dried material gives a final volume of approximately 4 ml. Pack a column (see Appendix 3).

Binding buffer: Use a buffer at around neutral pH since proteins bind specifically to Red Sepharose CL-6B at this pH.

The binding capacity will depend upon parameters such as sample concentration, flow rate, pH, buffer composition and temperature. To obtain optimal purification with respect to capacity, determine the binding capacity over a range of different pH and flow rates.

Elution buffers:
- use low concentrations of the free cofactor, NAD^+ or $NADP^+$ (up to 20 mM), or increase ionic strength up to 2 M NaCl or 1 M KCl.

If detergent or denaturing agents have been used during purification, these can also be used in the low and high pH wash buffers.

Cleaning

Wash 3 times with 2–3 column volumes of buffers, alternating between high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5). Re-equilibrate immediately with 3–5 column volumes of binding buffer.

Remove denatured proteins or lipids by washing with 2 column volumes of 6 M guanidine hydrochloride or 8 M urea. Alternatively, wash the column with 2 column volumes of detergent in a basic or acidic solution, e.g. 0.1% Triton X-100 in 1 M acetic acid. Remove residual detergent by washing with 5 column volumes of 70% ethanol. In both cases wash immediately with 5 column volumes of binding buffer.

Media characteristics

	Ligand and density	Composition	pH stability*	Mean particle size
Red Sepharose CL-6B	Procion Red HE 3B 2 µmoles/ml	Ligand coupled to Sepharose CL-6B using the triazine method.	Short term 3–13 Long term 4–12	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

Stable with all commonly used aqueous buffers and additives such as 8 M urea and 6 M guanidine hydrochloride.

Storage

Store freeze-dried powders under dry conditions and below +8 °C.

Wash media and columns with 20% ethanol in 0.1 M KH_2PO_4, pH 8.0 (use approximately 5 column volumes for packed media) and store at +4 to +8 °C.

Glycoproteins or polysaccharides

Con A Sepharose 4B, Lentil Lectin Sepharose 4B, Agarose Wheat Germ Lectin

Glycoproteins and polysaccharides react reversibly, via specific sugar residues, with a group of proteins known as lectins.

As ligands for purification media, lectins are used to isolate and separate glycoproteins, glycolipids, polysaccharides, subcellular particles and cells, and to purify detergent-solubilized cell membrane components. Substances bound to the lectin are resolved by using a gradient of ionic strength or of a competitive binding substance.

Media screening

To select the optimum lectin for purification, it may be necessary to screen different media. The ligands, Concanavalin A (Con A), Lentil Lectin and Wheat Germ Lectin provide a spectrum of parameters for the separation of glycoproteins. Table 5 gives their specificity.

Table 5. Specificity of lectins.

Lectin	Specificity
Mannose/glucose binding lectins	
Con A, *Canavalia ensiformis*	Branched mannoses, carbohydrates with terminal mannose or glucose (αMan > αGlc > GlcNAc).
Lentil Lectin, *Lens culinaris*	Branched mannoses with fucose linked α(1,6) to N-acetyl-glucosamine, (αMan > αGlc > GlcNAc).
N-acetylglucosamine binding lectins	
Wheat Germ Lectin, *Triticum vulgare*	Chitobiose core of N-linked oligosaccharides, $[GlcNAc(\beta1,4GlcNAc)_{1-2} > \beta GlcNac]$.

Con A for binding of branched mannoses, carbohydrates with terminal mannose or glucose (αMan > αGlc > GlcNAc)

Concanavalin A (Con A) is a tetrameric metalloprotein isolated from *Canavalia ensiformis* (jack bean). Con A binds molecules containing α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. The binding sugar requires the presence of C-3, C-4 and C-5 hydroxyl groups for reaction with Con A. Con A can be used for applications such as:

- Separation and purification of glycoproteins, polysaccharides and glycolipids.
- Detection of changes in composition of carbohydrate-containing substances, e.g. during development.
- Isolation of cell surface glycoproteins from detergent-solubilized membranes.
- Separation of membrane vesicles into "inside out" and "right side out" fractions.

Purification options

	Binding capacity/ml medium	Maximum operating flow	Comments
Con A Sepharose 4B	Porcine thyroglobulin, 20–45 mg	75 cm/h**	Supplied as a suspension ready for column packing*.

**Supplied in acetate buffer solution (0.1 M, pH 6) containing 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20% ethanol.*
***See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Purification example

Figure 47 shows the purification of a human cell surface alloantigen on Con A Sepharose 4B.

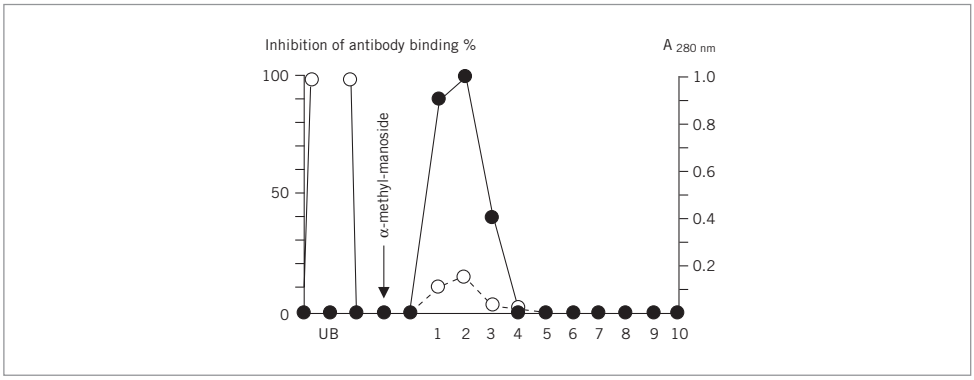


Fig. 47. Purification of a cell surface antigen on Con A Sepharose 4B. Solid circles represent antigen activity and open circles represent protein profile. Reproduced courtesy of the authors and publishers.
Reference: A novel heteromorphic human cell surface alloantigen, gp60, defined by a human monoclonal antibody. Schadendorf, D. *et al.*, *J. Immunol.* **142**, 1621 (1989).

Performing a separation

Binding buffer: 20 mM Tris-HCl, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4

Elution buffer: 0.1–0.5 M methyl-α-D-glucopyranoside (methyl-α-D-glucoside) or methyl-α-D-mannopyranoside (methyl-α-D-mannoside), 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4

1. Pack the column (see Appendix 3) and wash with at least 10 column volumes of binding buffer to remove preservative.
2. Equilibrate the column with 10 column volumes of binding buffer.
3. Apply the sample, using a low flow from 15 cm/h, during sample application (flow rate is the most significant factor to obtain maximum binding).
4. Wash with 5–10 column volumes of binding buffer or until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
5. Elute with 5 column volumes of elution buffer.

Recovery from Con A Sepharose 4B is decreased in the presence of detergents. If the glycoprotein of interest needs the presence of detergent and has affinity for either lentil lectin or wheat germ lectin, the media Lentil Lectin Sepharose 4B or Agarose Wheat Germ Lectin may provide a suitable alternative to improve recovery

For complex samples containing glycoproteins with different affinities for the lectin, a continuous gradient or step elution may improve resolution. Recovery can sometimes be improved by pausing the flow for some minutes during elution.

Elute tightly bound substances by lowering the pH. Note that elution below pH 4.0 is not recommended and that below pH 5.0 Mn^{2+} will begin to dissociate from the Con A and the column will need to be reloaded with Mn^{2+} before reuse.

Cleaning

Wash with 10 column volumes of 0.5 M NaCl, 20 mM Tris-HCl, pH 8.5, followed by 0.5 M NaCl, 20 mM acetate, pH 4.5. Repeat 3 times before re-equilibrating with binding buffer.

Remove strongly bound substances by:

- washing with 0.1 M borate, pH 6.5 at a low flow rate
- washing with 20% ethanol or up to 50% ethylene glycol
- washing with 0.1% Triton X-100 at +37 °C for one minute

Re-equilibrate immediately with 5 column volumes of binding buffer after any of these wash steps.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
Con A Sepharose 4B	10–16 mg/ml	Con A coupled to Sepharose 4B by CNBr method	Short term 4–9 Long term 4–9	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

Stable to all commonly used aqueous buffers. Avoid 8 M urea, high concentrations of guanidine hydrochloride, chelating agents such as EDTA, or solutions with pH < 4.0 as these remove the manganese from the lectin or dissociate Con A, resulting in loss of activity.

Storage

Wash media and columns with 20% ethanol in 0.1 M acetate, 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, pH 6 (use approximately 5 column volumes for packed media) and store at +4 to +8 °C.

Lentil lectin for binding of branched mannoses with fucose linked α(1,6) to the N-acetyl-glucosamine, (αMan > αGlc > GlcNAc) N-acetylglucosamine binding lectins

Lentil lectin binds α-D-glucose and α-D-mannose residues and is an affinity ligand used for the purification of glycoproteins including detergent-solubilized membrane glycoproteins, cell surface antigens and viral glycoproteins. Lentil lectin is the haemagglutinin from the common lentil, *Lens culinaris*. When compared to Con A, it distinguishes less sharply between glucosyl and mannosyl residues and binds simple sugars less strongly. It also retains its binding ability in the presence of 1% sodium deoxycholate. For these reasons Lentil Lectin Sepharose 4B is useful for the purification of detergent-solubilized membrane proteins, giving high capacities and extremely high recoveries.

Purification options

	Binding capacity/ml medium	Maximum operating flow	Comments
Lentil Lectin Sepharose 4B	Porcine thyroglobulin, 16–35 mg	75 cm/h*	Supplied as a suspension ready for column packing.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Performing a separation

Binding buffer: 20 mM Tris-HCl, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4.

Elution buffer: 0.1–0.5 M methyl-α-D-glucopyranoside (methyl-α-D-glucoside), 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4

Buffers for soluble glycoproteins:

Binding buffer: 20 mM Tris-HCl, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4

Elution buffer: 0.3 M methyl-α-D-mannopyranoside, 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4

Buffers for detergent-solubilized proteins:

Equilibrate column with the buffer 20 mM Tris-HCl, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4, to ensure saturation with Mn^{2+} and Ca^{2+}.

Binding buffer: 20 mM Tris-HCl, 0.5 M NaCl, 0.5% sodium deoxycholate, pH 8.3

Elution buffer: 0.3 M methyl-α-D-mannopyranoside, 20 mM Tris-HCl, 0.5 M NaCl, 0.5% sodium deoxycholate, pH 8.3

1. Pack the column (see Appendix 3) and wash with at least 10 column volumes of binding buffer to remove preservative.
2. Equilibrate the column with 10 column volumes of binding buffer.
3. Apply the sample, using a low flow from 15 cm/h, during sample application (flow rate is the most significant factor to obtain maximum binding).
4. Wash with 5–10 column volumes of binding buffer or until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
5. Elute with 5 column volumes of elution buffer using a step or gradient elution.

Below pH 5, excess Mn^{2+} and Ca^{2+} (1 mM) are essential to preserve binding activity. It is not necessary to include excess Ca^{2+} or Mn^{2+} in buffers if conditions that lead to their removal from the coupled lectin can be avoided.

For complex samples containing glycoproteins with different affinities for the lectin, a continuous gradient or step elution may improve resolution. Recovery can sometimes be improved by pausing the flow for some minutes during elution

Elute tightly bound substances by lowering the pH, but not below pH 3. In some cases strongly bound substances can be eluted with detergent, for example 1.0% deoxycholate.

Cleaning

Wash with 10 column volumes of 0.5 M NaCl, 20 mM Tris-HCl, pH 8.5, followed by 0.5 M NaCl, 20 mM acetate, pH 4.5. Repeat 3 times before re-equilibrating with binding buffer.

Remove strongly bound substances by:

- washing with 0.1 M borate, pH 6.5 at a low flow rate
- washing with 20% ethanol or up to 50% ethylene glycol
- washing with 0.1% Triton X-100 at +37 °C for one minute

Re-equilibrate immediately with 5 column volumes of binding buffer after any of these wash steps.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
Lentil Lectin Sepharose 4B	2.5 mg/ml	Lentil lectin coupled to Sepharose 4B by CNBr method.	Short term 3–10 Long term 3–10	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

To avoid loss of activity of the coupled lectin, avoid solutions having a pH below 3 or above 10, buffers that contain metal chelating agents such as EDTA, or high concentrations of guanidine hydrochloride or urea.

Storage

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

Wheat germ lectin for binding of chitobiose core of N-linked oligosaccharides, [GlcNAc(β1,4GlcNAc)$_{1-2}$ > β GlcNAc]

Wheat germ lectin can be used for group specific affinity purification of glycoproteins and polysaccharides. This lectin binds N-acetylglucosamine residues and reacts strongly with the chitobiose core of N-linked oligosaccharides. It also has affinity for N-acetylneuraminic acid. Wheat germ lectin is a dimeric, carbohydrate-free protein composed of two identical subunits, each with a molecular weight of approximately M_r 20 000.

Purification options

	Binding capacity/ml medium	Maximum operating flow	Comments
Agarose Wheat Germ Lectin	No data available	75 cm/h*	Supplied as a suspension ready for column packing.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Performing a separation

Binding buffer: 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4

Elution buffer: 0.5 M N-acetylglucosamine, 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4

Agarose Wheat Germ Lectin can be used with detergents, such as 1% deoxycholate or 0.5% Triton X-100.

1. Pack the column (see Appendix 3) and wash with at least 10 column volumes of binding buffer to remove preservative.
2. Equilibrate the column with 10 column volumes of binding buffer.
3. Apply the sample, using a low flow from 15 cm/h, during sample application (flow rate is the most significant factor for maximum binding).
4. Wash with 5–10 column volumes of binding buffer or until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
5. Elute with 5 column volumes of elution buffer.

Use 0–0.5 M N-acetylglucosamine, 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4 with a continuous gradient or step elution to improve resolution of complex samples containing glycoproteins with different affinities for the lectin.

Elute tightly bound substances with 20 mM acetate buffer, pH 4.5 or with an alternative sugar, for example triacetylchitotriose.

Higher concentrations of eluting substances may be necessary and recovery may be improved by pausing the flow for some minutes during elution.

Cleaning

Wash with 5–10 column volumes of 20 mM Tris-HCl, 1 M NaCl, pH 8.5 and re-equilibrate immediately with binding buffer. Low concentrations of non-ionic detergents in the Tris-HCl buffer can be used if necessary, for example 0.1% Nonidet P-40.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
Agarose Wheat Germ Lectin	1–2 mg/ml	Wheat Germ Lectin coupled to Sepharose 4B by CNBr method	Short term 4–9 Long term 4–9	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

Avoid exposure to conditions below pH 4.0 as this causes dissociation of the wheat germ lectin dimer.

Storage

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

Calmodulin binding proteins: ATPases, adenylate cyclases, protein kinases, phosphodiesterases, neurotransmitters

Calmodulin Sepharose 4B

Calmodulin is a highly conserved regulatory protein found in all eukaryotic cells. This protein is involved in many cellular processes such as glycogen metabolism, cytoskeletal control, neurotransmission, phosphate activity and control of $NAD^+/NADP^+$ ratios. Calmodulin Sepharose 4B provides a convenient method for the isolation of many of the calmodulin binding proteins involved in these pathways.

Calmodulin binds proteins principally through their interactions with hydrophobic sites on its surface. These sites are exposed after a conformational change induced by the action of Ca^{2+} on separate Ca^{2+}-binding sites. The binding of enzymes may be enhanced if the enzyme substrate is present and enzyme-substrate-calmodulin-Ca^{2+} complexes are particularly stable.

Purification options

	Binding capacity/ml medium	Maximum operating flow	Comments
Calmodulin Sepharose 4B	No data available	75 cm/h*	Supplied as a suspension ready for column packing.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Performing a separation

Binding buffer: 50 mM Tris-HCl, 0.05–0.2 M NaCl, 2 mM $CaCl_2$, pH 7.5

Elution buffer: 50 mM Tris-HCl, 0.05–0.2 M NaCl, 2 mM EGTA, pH 7.5

1. Pack the column (see Appendix 3) and wash with at least 10 column volumes of binding buffer to remove preservative.
2. Equilibrate the column with 10 column volumes of binding buffer.
3. Apply the sample, using a low flow from 15 cm/h, during sample application (flow rate is the most significant factor for maximum binding).
4. Wash with 5–10 column volumes of binding buffer or until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
5. Elute with 5 column volumes of elution buffer.

Remove proteases as quickly as possible from the sample as the calmodulin-binding sites on proteins are frequently very susceptible to protease action (see page 54).

Remove free calmodulin from the sample by hydrophobic interaction chromatography in the presence of Ca^{2+} on HiTrap Phenyl FF (high sub) or by ion exchange chromatography on HiTrap Q FF.

Since some non-specific ionic interactions can occur, a low salt concentration (0.05–0.20 M NaCl) is recommended to promote binding to the ligand while eliminating any non-specific binding.

Use chelating agents to elute the proteins. Chelating agents strip Ca^{2+} from the calmodulin, reversing the conformational change that exposed the protein binding sites. Calcium ions may also be displaced by a high salt concentration, 1 M NaCl.

Cleaning

Alternative 1

Wash with 3 column volumes of 0.05 M Tris-HCl, 1.0 M NaCl, 2 mM EGTA, pH 7.5 and re-equilibrate immediately with 5–10 column volumes of binding buffer.

Alternative 2

Wash with 3 column volumes of 0.1 M ammonium carbonate buffer, 2 mM EGTA, pH 8.6 followed by 3 column volumes of 1 M NaCl, 2 mM $CaCl_2$. Continue washing with 3 column volumes of 0.1 M sodium acetate buffer, 2 mM $CaCl_2$, pH 4.4 followed by 3 column volumes of binding buffer.

Remove severe contamination by washing with non-ionic detergent such as 0.1% Triton X-100 at +37 °C for 1 min.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
Calmodulin Sepharose 4B	0.9–1.3 mg/ml	Bovine testicular calmodulin coupled to Sepharose 4B by the CNBr method.	Short term 4–9 Long term 4–9	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

Stable in all commonly used aqueous solutions.

Storage

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

Proteins and peptides with exposed amino acids: His, Cys, Trp, and/or with affinity for metal ions (also known as IMAC, immobilized metal chelate affinity chromatography)

HiTrap Chelating HP, Chelating Sepharose Fast Flow, His MicroSpin Purification Module, HisTrap Kit

Proteins and peptides that have an affinity for metal ions can be separated using metal chelate affinity chromatography. The metals are immobilized onto a chromatographic medium by chelation. Certain amino acids, e.g. histidine and cysteine, form complexes with the chelated metals around neutral pH (pH 6–8) and it is primarily the histidine-content of a protein which is responsible for its binding to a chelated metal.

Metal chelate affinity chromatography is excellent for purifying recombinant $(His)_6$ fusion proteins (see page 47) as well as many natural proteins. Chelating Sepharose, the medium used for metal chelate affinity chromatography, is formed by coupling a metal chelate forming ligand (iminodiacetic acid) to Sepharose.

Before use the medium is loaded with a solution of divalent metal ions such as Ni^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+} or Fe^{2+}. The binding reaction with the target protein is pH dependent and bound sample is, most commonly, eluted by reducing the pH and increasing the ionic strength of the buffer or by including EDTA or imidazole in the buffer. The structure of the ligand, iminodiacetic acid, is shown in Figure 48.

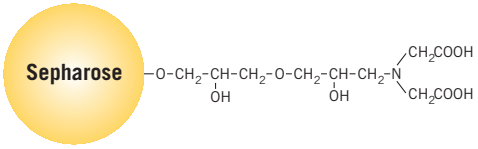


Fig. 48. Partial structure of Chelating Sepharose High Performance and Chelating Sepharose Fast Flow.

Metalloproteins are not usually suitable candidates for purification by chelating chromatography since they tend to scavenge the metal ions from the column.

Purification options

	Binding capacity	Maximum operating flow	Comments
His MicroSpin Purification Module	100 µg/column	Not applicable	Ready to use, prepacked columns, buffers and chemicals for purification of $(His)_6$ fusion proteins.
HiTrap Chelating HP 1 ml	12 mg/column	4 ml/min	Prepacked column, ready to use.
HiTrap Chelating HP 5 ml	60 mg/column	20 ml/min	Prepacked column, ready to use.
HisTrap Kit	12 mg/column*	4 ml/min	Ready to use, prepacked columns, buffers and chemicals for purification of $(His)_6$ fusion proteins for up to 12 purifications using a syringe.
Chelating Sepharose Fast Flow	12 mg/ml medium	400 cm/h**	Supplied as suspension for packing columns and scale up.

**Estimate for a $(His)_6$ fusion protein of M_r 27 600, binding capacity varies according to specific protein.*
***See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Purification example

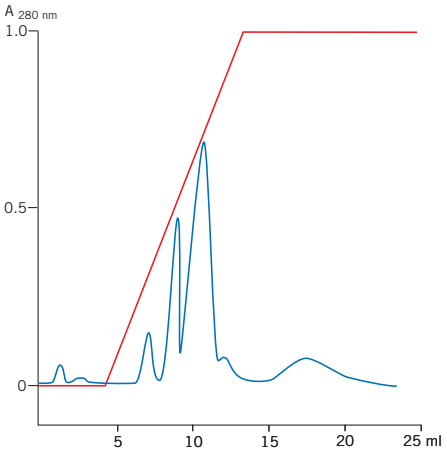


Fig. 49. Purification of egg white proteins on HiTrap Chelating HP 1 ml, using the metal ion Cu^{2+}.

Development of a separation protocol

Details of a specific purification protocol are given on page 50. This protocol can be used as a base from which to develop purification methods for other proteins and peptides with affinity for metal ions, as shown in Figure 49.

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

Selecting the metal ion

The following guidelines may be used for preliminary experiments to select the metal ion that is most useful for a given separation:

- Cu^{2+} gives strong binding and some proteins will only bind to Cu^{2+}. Load solution equivalent to 60% of the packed column volume to avoid leakage of metal ions during sample application. Alternatively, the medium can be saturated and a short secondary uncharged column of HiTrap Chelating HP or packed Chelating Sepharose Fast Flow should be connected in series after the main column to collect excess metal ions.
- Zn^{2+} gives a weaker binding and this can, in many cases, be exploited to achieve selective elution of a protein mixture. Load solution equivalent to 85% of the packed column volume to charge the column.
- Ni^{2+} is commonly used for poly (His) fusion proteins. Ni^{2+} solution equivalent to half the column volume is usually sufficient to charge the column.
- Co^{2+} and Ca^{2+} are also alternatives.

Charge the column with metal ions by passing through a solution of the appropriate salt through the column, e.g. 0.1 M $ZnCl_2$, $NiSO_4$ or $CuSO_4$ in distilled water. Chloride salts can be used for other metals.

Several methods can be used to determine when the column is charged. If a solution of metal salt in distilled water is used during charging, the eluate initially has a low pH and returns to neutral pH as the medium becomes saturated with metal ions. The progress of charging with Cu^{2+} is easily followed by eye (the column contents become blue). When charging a column with zinc ions, sodium carbonate can be used to detect the presence of zinc in the eluate. Wash the medium thoroughly with binding buffer after charging the column.

Choice of binding buffer

A neutral or slightly alkaline pH will favor binding. Tris-acetate (0.05 M), sodium phosphate (0.02–0.05 M) and Tris-HCl (0.02–0.05 M) are suitable buffers. Tris-HCl tends to reduce binding and should only be used when metal-protein affinity is fairly high.

High concentrations of salt or detergents in the buffer normally have no effect on the adsorption of protein and it is good practice to maintain a high ionic strength (e.g. 0.5–1 M NaCl) to avoid unwanted ion exchange effects.

Chelating agents such as EDTA or citrate should not be included, as they will strip the metal ions from the medium.

Choice of elution buffers

Differential elution of bound substances may be obtained using a gradient of an agent that competes for either the ligand or the target molecules. An increased concentration of imidazole (0–0.5 M), ammonium chloride (0–0.15 M), or substances such as histamine or glycine with affinity for the chelated metal can be used. The gradient is best run in the binding buffer at constant pH.

Since pH governs the degree of ionization of charged groups at the binding sites, a gradient or step-wise reduction in pH can be used for non-specific elution of bound material. A range of pH 7.0–4.0 is normal, most proteins eluting between pH 6.0 and 4.2. Deforming eluents such as 8 M urea or 6 M guanidine hydrochloride can be used.

Elution with EDTA (0.05 M) or other strong chelating agents will strip away metal ions and other material bound. This method does not usually resolve different proteins.

If harsh elution conditions are used, it is recommended to transfer eluted fractions immediately to milder conditions (either by collecting them in neutralization buffer or by passing directly onto a desalting column for buffer exchange (see page 133).

The loss of metal ions is more pronounced at lower pH. The column does not have to be stripped between consecutive purifications if the same protein is going to be purified, as shown in Figure 50.

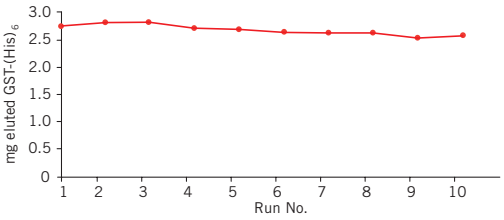
Samples: 2.5 ml cell extract containing expressed GST-$(His)_6$

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

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

Flow: 2 ml/min, 312 cm/h

Note: No Ni^{2+} re-loading of the column between the runs



Result:

Run No.	Eluted GST-$(His)_6$, total mg
1	2.76
2	2.82
3	2.83
4	2.72
5	2.71
6	2.65
7	2.64
8	2.63
9	2.54
10	2.59

Fig. 50. 10 repetitive purifications of GST-$(His)_6$ without reloading the column with Ni^{2+} between the runs.

Although metal leakage is very low, the presence of any free metal in the purified product can be avoided by connecting an uncharged HiTrap Chelating HP column in series after the first column and before the protein is eluted. This column will bind any metal ions removing them from the protein as it passes through the second column.

Scale of operation

To increase capacity use several HiTrap Chelating HP columns (1 ml or 5 ml) in series (note that back pressure will increase) or, for even larger capacity, pack Chelating Sepharose Fast Flow into a suitable column (see Appendix 3).

Cleaning

Remove metal ions by washing with 5 column volumes 20 mM sodium phosphate, 0.5 M NaCl, 0.05 M EDTA, pH 7.4.

Remove precipitated proteins by filling the column with 1 M NaOH and incubate for 2 hours. Wash out dissolved proteins with 5 column volumes of water and a buffer at pH 7.0 until the pH of the flow-through reaches pH 7.0.

Alternatively wash with a non-ionic detergent such as 0.1% Triton X-100 at +37 °C for 1 min.

Remove lipid and very hydrophobic proteins by washing with 70% ethanol, or with a saw-tooth gradient 0%–30%–0% isopropanol/water.

Media characteristics

	Composition	Metal ion capacity	pH stability*	Mean particle size
Chelating Sepharose High Performance	Iminodiacetic acid coupled to Sepharose High Performance via an ether bond.	23 µmoles Cu^{2+}/ml	Short term 2–14 Long term 3–13	34 µm
Chelating Sepharose Fast Flow	Iminodiacetic acid coupled Sepharose Fast Flow via a spacer arm using epoxy coupling.	22–30 µmoles Zn^{2+}/ml	Short term 2–14 Long term 3–13	90 µm

**Long term refers to the pH interval over which the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.*

Chemical stability

Stable in all commonly used aqueous buffers and denaturants such as 6 M guanidine hydrochloride, 8 M urea and other chaotropic agents.

Storage

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

Before long term storage, remove metal ions by washing with five column volumes 20 mM sodium phosphate, 0.5 M NaCl, 0.05 M EDTA, pH 7.4.

The column must be recharged with metal ions after long term storage.

Thiol-containing substances (purification by covalent chromatography)

Activated Thiol Sepharose 4B, Thiopropyl Sepharose 6B

Thiol-containing substances can be isolated selectively by covalent binding to an activated thiolated matrix via thiol-disulphide exchange to form a mixed disulphide bond. After washing away unbound material, the thiol-containing substance is eluted by reducing the disulphide bond. This technique is also known as covalent chromatography. The reaction scheme is shown in Figure 51.

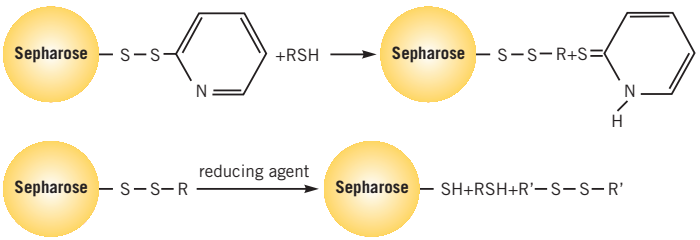


Fig. 51. Reaction scheme purification of a thiolated substance (RSH) on Activated Thiol Sepharose 4B or Thiopropyl Sepharose 6B. The reducing agent is a low molecular weight thiol such as dithiothreitol.

In Activated Thiol-Sepharose 4B the hydrophilic glutathione residue acts as a spacer group thereby decreasing steric interference with exchange reactions at the terminal thiol group. The partial structure is shown in Figure 52.

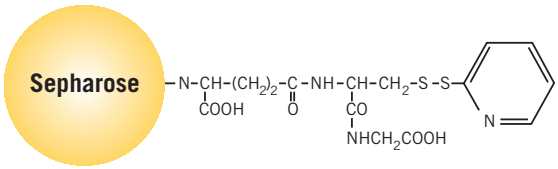


Fig. 52. Partial structure of Activated Thiol Sepharose 4B.

In Thiopropyl Sepharose 6B the 2-hydroxypropyl residue acts as a hydrophilic spacer group. The partial structure of Thiopropyl Sepharose 6B is shown in Figure 53.

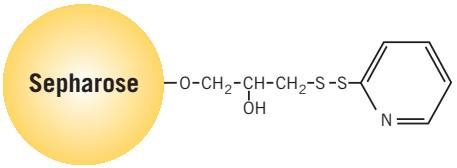


Fig. 53. Partial structure of Thiopropyl Sepharose 6B.

Purification options

	Binding capacity/ml medium	Coupling conditions	Maximum operating flow	Comments
Activated Thiol Sepharose 4B	Mercaptalbumin, 2–3 mg	pH 4–8, 3–16 hours, +4 °C - room temp.	75 cm/h*	Low capacity derivative suitable for coupling of high molecular weight substances. Supplied as dry powder, rehydration required.
Thiopropyl Sepharose 6B	Ceruloplasmin, 14 mg	pH 4–8, 3–16 hours, +4 °C - room temp.	75 cm/h*	High capacity derivative suitable for coupling of low molecular weight substances. Supplied as dry powder, rehydration required.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Both media react spontaneously and reversibly under mild reducing conditions or in the presence of denaturing agents with substances containing thiol groups.

Performing a separation

Binding buffer: 20 mM Tris-HCl, 0.1–0.5 M NaCl, pH 7.0.

If required, include 8 M urea or 6 M guanidine HCl to ensure that the protein is denatured and all thiol groups are accessible for the reaction.

1 mM EDTA can be added to remove trace amounts of catalytic heavy metals.

Elution buffer alternatives:

For covalently bound proteins: 0.025 M cysteine, 50 mM Tris-HCl, pH 7–8.

To minimize reduction of intramolecular disulphide bridges: 5–20 mM L-cysteine, 50 mM Tris-HCl, 1 mM EDTA, pH 8.0 or 20–50 mM 2-mercaptoethanol, 50 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Note: When using Thiopropyl Sepharose, 2-thiopyridyl groups must be removed after the protein has bound. Wash the column with sodium acetate 0.1 M, 2-mercaptoethanol 5 mM, pH 4.0 before beginning elution.

N.B. Degas all buffers to avoid oxidation of free thiol groups.

If the proteins to be purified contain disulphide bonds, the disulphide bridges must be reduced, for example with 2-mercaptoethanol (5 mM).

Analyze the thiol content of the sample by thiol titration to ensure that the capacity of the medium will not be exceeded.

Use preliminary titration studies with 2,2'-dipyridyl disulphide to provide a guide to optimal coupling conditions. A spectrophotometer can be used to determine the release of 2-thiopyridone (absorbance coefficient = 8.08×10^3 M^{-1} cm^{-1} at 343 nm) when the sample (1–5 mg in 1–3 ml binding buffer) reacts with 2, 2'-dipyridyl disulphide. Choose the conditions to suit the specific sample. Under standard conditions at pH 7.5, a few minutes is usually enough for a complete reaction.

1. Use a desalting column to transfer pre-dissolved sample into the binding buffer (see page 133) and to remove any low molecular weight thiol compounds and reducing agents that might interfere with the coupling reaction.
2. Weigh out the required amount of powder (1 g gives about 3 ml for Activated Thiol Sepharose 4B and 4 ml for Thiopropyl Sepharose 6B).
3. Wash and re-swell on a sintered glass filter (porosity G3), using degassed, distilled water or binding buffer (200 ml/g, 15 min at room temperature) to remove additives.
4. Prepare the slurry with binding buffer in a ratio of 75% settled medium to 25% buffer.
5. Pack the column (see Appendix 3) and equilibrate with binding buffer.
6. Load the sample at a low flow (5–10 cm/h) and leave in contact with the medium for at least one hour to ensure maximum binding.
7. Wash the column with binding buffer until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
8. Elute the target molecules with elution buffer using a low flow (5–10 cm/h).

The coupling reaction can be monitored and, in some cases, quantified by following the appearance of 2-thiopyridone in the eluent at 343 nm during the purification.

Sodium phosphate or ammonium acetate can be used as an alternative to Tris-HCl.

Resolve different thiol proteins by sequential elution: 5–25 mM L-cysteine < 0.05 M glutathione < 0.02–0.05 M 2-mercaptoethanol < and 0.02–0.05 M dithiothreitol in 50 mM Tris-HCl, 1 mM EDTA, pH 7–8.

Reactivation

Pass one to two column volumes of a saturated solution (approximately 1.5 mM) of 2,2'-dipyridyl disulphide, pH 8.0 through the medium.

Prepare 2,2'-dipyridyl disulphide:

1. Make a stock solution by adding 40 mg 2,2'-dipyridyl disulphide to 50 ml buffer at room temperature and stirring the suspension for several hours.
2. Filter off insoluble material.
3. Adjust the pH. The solution will be approximately 1.5 mM with respect to 2,2'-dipyridyl disulphide.

Cleaning

Wash with non-ionic detergent such as 0.1% Triton X-100 at +37 °C for 1 minute. Re-equilibrate immediately with a minimum of 5 column volumes of binding buffer.

Media characteristics

	Density of thiol groups	Composition	pH stability*	Mean particle size
Thiopropyl Sepharose 6B	25 µmoles/ml	Mixed disulphide containing 2-thiopyridyl protecting groups attached to Sepharose 6B through a chemically stable ether linkage.	Short term 2–8 Long term 2–8	90 µm
Activated Thiol Sepharose 4B	1 µmole/ml	Mixed disulphide formed between 2,2'-dipyridyl disulphide and glutathione coupled to CNBr-activated Sepharose 4B.	Short term 2–8 Long term 2–8	90 µm

**Long term refers to the pH interval over which the matrix is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. When a molecule has been coupled to the thiolated matrix, the long term and short term pH stability of the medium will be dependent upon the nature of that molecule.*

Chemical stability

Stable to all commonly used aqueous buffers and additives such as detergents. Avoid azides.

Storage

Store freeze-dried powders below +8 °C.

Wash media and columns with 20% ethanol at neutral pH (use approximately 5 column volumes for packed media) and store at +4 to +8 °C. Storage under nitrogen is recommended to prevent oxidation of thiol groups by atmospheric oxygen.

Avoid using sodium azide, merthiolate or phenyl mercuric salts as bacteriostatic agents. Azide ions will react with the 2,2'-dipyridyl disulphide groups, although low concentrations (0.04%) have been used.

Do not store the suspension for long periods in the free thiol form. Thiol groups are susceptible to oxidation by atmospheric oxygen, especially at alkaline pH. Figure 54 shows the decrease in free thiol content of Thiopropyl Sepharose 6B on storage for moderate periods at three different pH values. The thiol content of partially oxidized medium is restored by treatment with reducing agent under conditions used for removing protecting groups (see below).

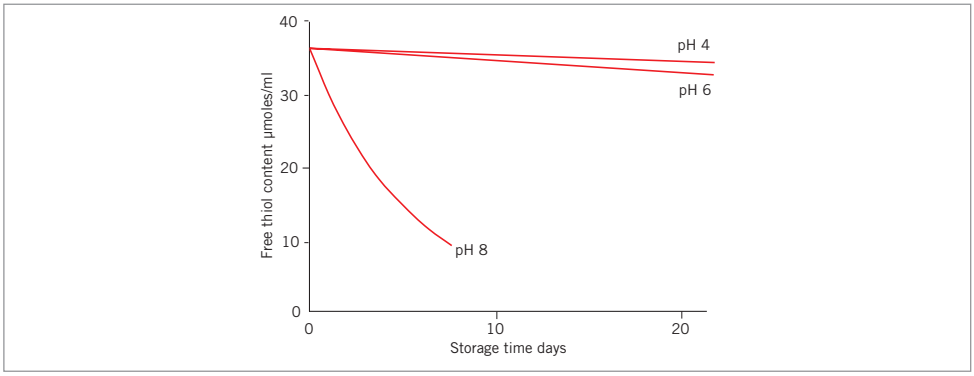


Fig. 54. Loss of free thiol content of reduced Thiopropyl Sepharose 6B on storage at +4 °C. The reduced medium was stored in 0.1 M sodium acetate or phosphate, 0.3 M NaCl, 1 mM EDTA at the indicated pH values.

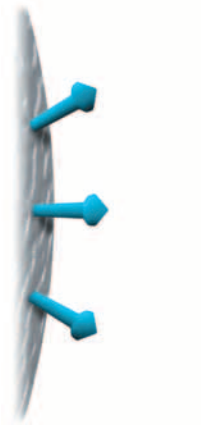
Removal of protecting groups

Activated Thiol Sepharose 4B and Thiopropyl Sepharose 6B may easily be converted into the free thiol form (i.e. reduced) by removing the 2-thiopyridyl protecting groups with a reducing agent.

1. Prepare the medium as described earlier. Gently remove excess liquid on a glass filter (porosity G3).
2. Suspend the medium in a solution containing 1% (w/v) dithiothreitol or 0.5 M 2-mercaptoethanol, 0.3 M sodium bicarbonate, 1 mM EDTA, pH 8.4.
3. Use 4 ml of solution per gram of freeze-dried powder.
4. React for 40 minutes at room temperature, mixing gently.
5. Wash the medium thoroughly with 0.5 M NaCl, 1 mM EDTA in 0.1 M acetic acid. Use a total of 400 ml of solution per gram of original freeze-dried powder. Perform the washing in several steps.

Estimate the content of free thiol groups by measuring the absorption increase at 343 nm (see above) due to the 2-thiopyridone liberated in the wash solutions. The amount of thiol groups on the medium can be estimated by reacting an excess of 2,2'-dipyridyl disulphide with the medium and measuring the liberated 2-thiopyridone at 343 nm.

Chapter 4
Components of an affinity medium



Matrix: for ligand attachment. Matrix should be chemically and physically inert.

Spacer arm: used to improve binding between ligand and target molecule by overcoming any effects of steric hindrance.

Ligand: molecule that binds reversibly to a specific target molecule or group of target molecules.

The matrix

The matrix is an inert support to which a ligand can be directly or indirectly coupled. The list below highlights many of the properties required for an efficient and effective chromatography matrix.

- Extremely low non-specific adsorption, essential since the success of affinity chromatography relies on specific interactions.
- Hydroxyl groups on the sugar residues are easily derivatized for covalent attachment of a ligand, providing an ideal platform for the development of affinity media.
- An open pore structure ensures high capacity binding even for large biomolecules, since the interior of the matrix is available for ligand attachment.
- Good flow properties for rapid separation.
- Stability under a range of experimental conditions such as high and low pH, detergents and dissociating agents.

Sepharose, a bead-form of agarose (Figure 55), provides many of the these properties.

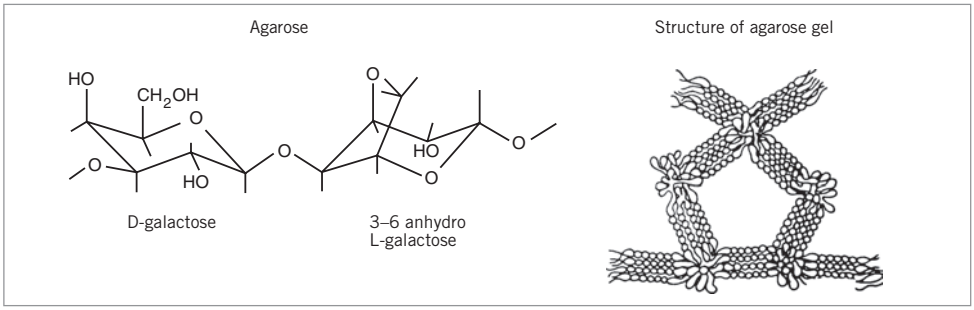


Fig. 55. Partial structure of agarose.

Sepharose has been modified and developed to further enhance these excellent properties, resulting in a selection of matrices chosen to suit the particular requirements for each application (see Table 6).

In affinity chromatography the particle size and porosity are designed to maximize the surface area available for coupling a ligand and binding the target molecule. A small mean particle size with high porosity increases the surface area. Increasing the degree of cross-linking of the matrix improves the chemical stability, in order to tolerate potentially harsh elution and wash conditions, and creates a rigid matrix that can withstand high flow rates. These high flow rates, although not always used during a separation, save considerable time during column equilibration and cleaning procedures.

Table 6. Sepharose matrices used with Amersham Biosciences affinity media.

	Form	Mean particle size
Sepharose High Performance	6% highly cross-linked agarose	34 µm
Sepharose 6 Fast Flow	6% highly cross-linked agarose	90 µm
Sepharose 4 Fast Flow	4% highly cross-linked agarose	90 µm
Sepharose CL-6B	6% cross-linked agarose	90 µm
Sepharose CL-4B	4% cross-linked agarose	90 µm
Sepharose 6B	6% agarose	90 µm
Sepharose 4B	4% agarose	90 µm

The ligand

The ligand is the molecule that binds reversibly to a specific molecule or group of molecules, enabling purification by affinity chromatography.

The selection of the ligand for affinity chromatography is influenced by two factors: the ligand must exhibit specific and reversible binding affinity for the target substance(s) and it must have chemically modifiable groups that allow it to be attached to the matrix without destroying binding activity.

The dissociation constant (k_D) for the ligand - target complex should ideally be in the range 10^{-4} to 10^{-8} M in free solution.

Interactions involving dissociation constants greater than 10^{-4} M, for example the binding reaction between an enzyme and a weak inhibitor, are likely to be too weak for successful affinity chromatography. Conversely, if the dissociation constant is lower than approximately 10^{-8} M, for example the affinity between a hormone and hormone receptor, elution of the bound substance without causing inactivation is likely to be difficult. If no information on the strength of the binding complex is available, a trial and error approach must be used. Refer to Appendix 7 for further details on the kinetics involved in affinity chromatography.

Altering elution methods may help to promote successful affinity chromatography when the dissociation constant is outside the useful range (see Appendix 7).

It is important to consider the region of the ligand that will be used for attachment to the matrix. For example, many proteins have several equivalent groups through which coupling can take place resulting in a random orientation of the ligand on the matrix. This may reduce the number of ligand molecules that are available in the correct orientation to bind during an affinity purification.

If several functional groups are available, couple the ligand via the group least likely to be involved in the specific affinity interaction. A range of pre-activated matrices for attachment of the ligand through different functional groups is available (see Table 7).

Spacer arms

The binding site of a target protein is often located deep within the molecule and an affinity medium prepared by coupling small ligands, such as enzyme cofactors, directly to Sepharose may exhibit low binding capacity due to steric interference i.e. the ligand is unable to access the binding site of the target molecule, as shown in Figure 56a. In these circumstances a "spacer arm" is interposed between the matrix and the ligand to facilitate effective binding. Spacer arms must be designed to maximize binding, but to avoid non-specific binding effects. Figure 56 shows the improvement that can be seen in a purification as the spacer arm creates a more effective environment for binding.

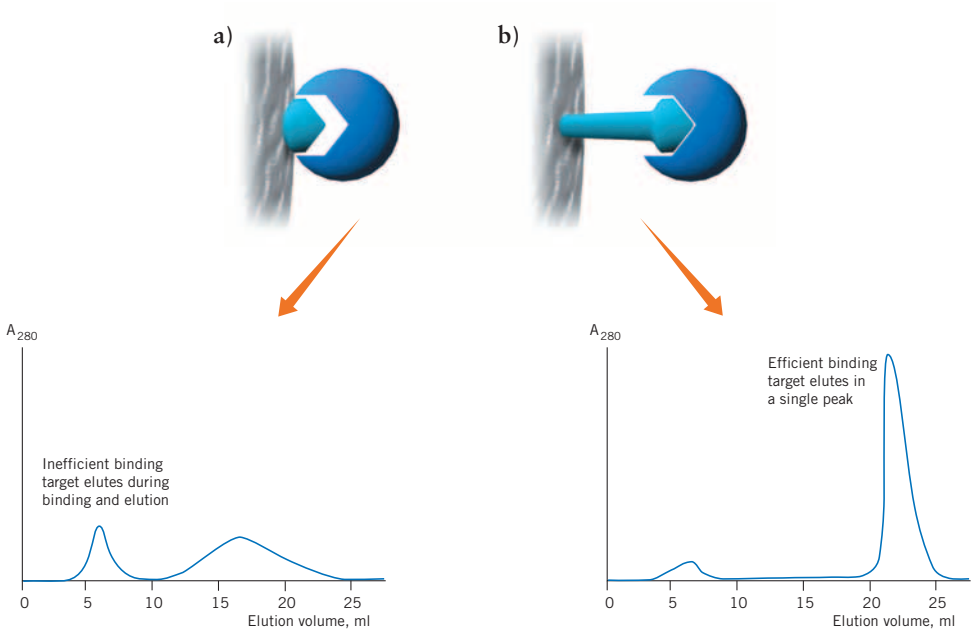


Fig. 56. Using spacer arms. a) Ligand attached directly to the matrix. b) Ligand attached to the matrix via a spacer arm.

The length of the spacer arm is critical. If it is too short, the arm is ineffective and the ligand fails to bind substances in the sample. If it is too long, proteins may bind non-specifically to the spacer arm and reduce the selectivity of the separation.

As a general rule, use spacer arms when coupling molecules M_r < 1 000. Spacer arms are not generally needed for larger molecules. Table 7 shows the pre-activated media with different types of spacers arms that are available from Amersham Biosciences.

Ligand coupling

Several methods are available to couple a ligand to a pre-activated matrix. The correct choice of coupling method depends on the ligand characteristics. The use of commercially available, pre-activated media is recommended to save time and avoid the use of the potentially hazardous reagents that are required in some cases.

Table 7. Examples of pre-activated media.

NHS-activated Sepharose High Performance	12-atom hydrophilic spacer arm to couple via amino groups.
NHS-activated Sepharose 4 Fast Flow	As above.
CNBr-activated Sepharose 4 Fast Flow	Coupling via primary amino groups.
EAH Sepharose 4B	10-atom spacer arms to couple via amino groups.
ECH Sepharose 4B	9-atom spacer arms to couple via carboxyl groups.
Epoxy-activated Sepharose 6B	12-atom hydrophilic spacer arm to couple through hydroxyl, amino or thiol groups.
Activated Thiol Sepharose 4B	10-atom spacer arm for reversible coupling through free thiol groups.
Thiopropyl Sepharose 6B	4-atom hydrophilic spacer arm for reversible coupling of proteins and small thiolated ligands through thiol groups. Also reacts with heavy metal ions, alkyl and aryl halides and undergoes addition reactions with compounds containing C=O, C=C and N=N bonds.

Ligand specificity

For purification of specific molecules or groups of molecules, many ligands are available coupled to an appropriate matrix (see Chapter 3). Ligands can also be isolated and purified to prepare a specific affinity medium for a specific target molecule. Coupling of ligands to pre-activated matrices is described in Chapter 5.

Chapter 5
Designing affinity media using pre-activated matrices

Earlier chapters in this handbook have covered a wide range of ligands that have been coupled to Sepharose to provide ready to use affinity media for specific groups of molecules. However, it is also possible to design new media for special purposes. When a ready to use affinity medium is not available, a medium can be designed for the purification of one or more target molecules by coupling a specific ligand onto a pre-activated chromatography matrix. For example, antibodies, antigens, enzymes, receptors, small nucleic acids or peptides can be used as affinity ligands to enable the purification of their corresponding binding partners.

There are three key steps in the design of an affinity medium:

- Choosing the matrix.
- Choosing the ligand and spacer arm.
- Choosing the coupling method.

Choosing the matrix

Sepharose provides a macroporous matrix with high chemical and physical stability and low non-specific adsorption to facilitate a high binding capacity and sample recovery and to ensure resistance to potentially harsh elution and wash conditions. The choice of a pre-activated Sepharose matrix depends on the functional groups available on the ligand and whether or not a spacer arm is required. Table 8 reviews the pre-activated matrices available.

Choosing the ligand and spacer arm

The ligand must selectively and reversibly interact with the target molecule(s) and must be compatible with the anticipated binding and elution conditions. The ligand must carry chemically modifiable functional groups through which it can be attached to the matrix without loss of activity (see Table 8).

If possible, test the affinity of the ligand: target molecule interaction. Too low affinity will result in poor yields since the target protein may wash through or leak from the column during sample application. too high affinity will result in low yields since the target molecule may not dissociate from the ligand during elution.

Use a ligand with the highest possible purity since the final purity of the target substance depends on the biospecific interaction.

As discussed in Chapter 4, when using small ligands (M_r < 5 000) there is a risk of steric hindrance between the ligand and the matrix that restricts the binding of target molecules. In this case, select a pre-activated matrix with a spacer arm. For ligands with M_r > 5 000 no spacer arm is necessary.

Choosing the coupling method

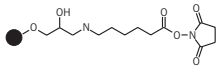
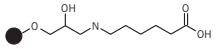
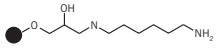
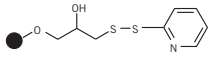
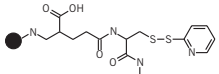
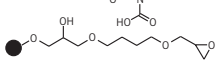
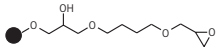
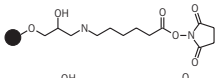
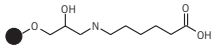
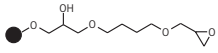
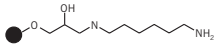
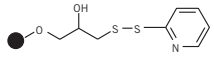
Ligands are coupled via reactive functional groups such as amino, carboxyl, hydroxyl, thiol and aldehyde moieties. In the absence of information on the location of binding sites in the ligand, a systematic trial and error approach should be used.

Couple a ligand through the least critical region of the ligand to minimize interference with the normal binding reaction. For example, an enzyme inhibitor containing amino groups can be attached to a matrix through its amino groups, provided that the specific binding activity with the enzyme is retained. However, if the amino groups are involved in the binding reaction, an alternative, non-essential, functional group must be used.

Avoid using a functional group that is close to a binding site or that plays a role in the interaction between the ligand and target molecule.

If a suitable functional group does not exist, consider derivatizing the ligand to add a functional group.

Table 8.

Chemical group on ligand	Length of spacer arm	Structure of spacer arm	Product
Proteins, peptides, amino acids			
amino	10-atom		HiTrap NHS-activated HP NHS-activated Sepharose 4 Fast Flow
	None	–	CNBr-activated Sepharose 4B CNBr-activated Sepharose 4 Fast Flow
	10-atom		ECH Sepharose 4B
carboxyl	11-atom		EAH Sepharose 4B
thiol	4-atom		Thiopropyl Sepharose 6B
	10-atom		Activated Thiol Sepharose 4B
	12-atom		Epoxy-activated Sepharose 6B
Sugars			
hydroxyl	12-atom		Epoxy-activated Sepharose 6B
amino	10-atom		HiTrap NHS-activated HP
	10-atom		ECH Sepharose 4B
	12-atom		Epoxy-activated Sepharose 6B
carboxyl	11-atom		EAH Sepharose 4B
Polynucleotides			
amino	None		CNBr-activated Sepharose 4B CNBr-activated Sepharose 4 Fast Flow
mercurated base	4-atom		Thiopropyl Sepharose 6B
Coenzymes, cofactors, antibiotics, steroids			
amino, carboxyl, thiol or hydroxyl			use matrix with spacer arm (see above)

Coupling the ligand

1. Prepare the ligand solution in coupling buffer, either by dissolving the ligand in coupling buffer or exchanging the solubilized ligand into the coupling buffer using a desalting column.
2. Prepare the pre-activated matrix according to the manufacturer's instructions.
3. Mix the ligand solution and the matrix in the coupling buffer until the coupling reaction is completed.
4. Block any remaining active groups.
5. Wash the coupled matrix alternately at high and low pH to remove excess ligand and reaction by-products.
6. Equilibrate in binding buffer or transfer to storage solution.

It is not usually necessary to couple a large amount of ligand to produce an efficient affinity medium. After coupling, wash the medium thoroughly using buffers of alternating low and high pH to remove non-covalently bound ligand.

A high concentration of coupled ligand is likely to have adverse effects on affinity chromatography. The binding efficiency of the medium may be reduced due to steric hindrance between the active sites (particularly important when large molecules such as antibodies, antigens and enzymes interact with small ligands).Target substances may become more strongly bound to the ligand making elution difficult. The extent of non-specific binding increases at very high ligand concentrations thus reducing the selectivity of the medium.

Remember that the useful capacity of an affinity medium may be significantly affected by flow rate.

For applications that require operating at high pH, the amide bond formed when using NHS-activated Sepharose is stable up to pH 13.

Figure 57 shows the effect of ligand concentration on the final amount of ligand coupled to a matrix.

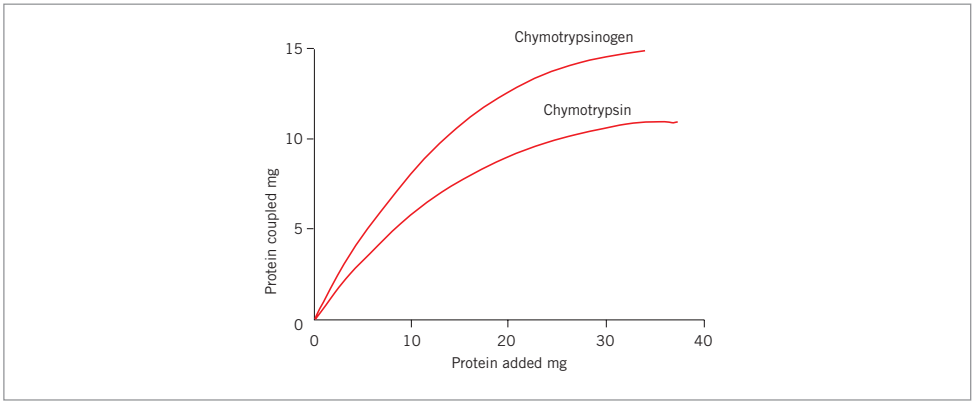


Fig. 57. Effect of protein concentration on amount of protein coupled. Protein was coupled to 2 ml CNBr-activated Sepharose 4B in $NaHCO_3$, NaCl solution, pH 8.

Table 9 summarizes recommended ligand concentrations according to the experimental conditions.

Table 9.

Experimental condition	Recommended concentration for coupling
Readily available ligands	10–100 fold molar excess of ligand over available groups
Small ligands	1–20 µmoles/ml medium (typically 2 µmoles/ml medium)
Protein ligands	5–10 mg protein/ml medium
Antibodies	5 mg protein/ml medium
Very low affinity systems	Maximum possible ligand concentration to increase the binding

For certain pre-activated matrices agents are used to block any activated groups that remain on the matrix after ligand coupling. These blocking agents such as ethanolamine and glycine may introduce a small number of charged groups into the matrix. The effect of these charges is overcome by the use of a relatively high salt concentration (0.5 M NaCl) in the binding buffer for affinity purification. A wash cycle of low and high pH is essential to ensure that no free ligand remains ionically bound to the coupled ligand. This wash cycle does not cause loss of covalently bound ligand.

Binding capacity, ligand density and coupling efficiency

Testing the binding capacity of the medium after coupling will give an indication of the success of the coupling procedure and establish the usefulness of the new affinity medium.

Several different methods can be used to determine the ligand density (µmoles/ml medium) and coupling efficiency.

- The fastest and easiest, but least accurate, way to quantify the free ligand in solution is by spectrophotometry. Measure the ligand concentration before coupling and compare this with the concentration of the unbound ligand after coupling. The difference is the amount that is coupled to the matrix.
- Spectroscopic methods or scintillation counting can also be used if the ligand has been suitably pre-labelled. The coupled ligand can be quantified by direct spectroscopy of the affinity medium suspended in a solution with the same refractive index, such as 50% glycerol or ethylene glycol. By-products of the coupling reaction, such as N-hydroxy-succinimide in the case of NHS-activated matrices, can be quantified by spectroscopy.
- The medium can be titrated to determine ligand concentration. The titrant must be relevant to the ligand.
- The most accurate method to determine ligand concentration is direct amino acid analysis or determination of characteristic elements. Note that these are destructive techniques.

If the binding capacity for the target is insufficient there are several ways to try to increase the coupling efficiency:

- Ensure that the ligand is of high purity. There may be contaminants present that are preferentially coupled.

- Increase the ligand concentration to increase the ligand density on the matrix, but avoid overloading the matrix as this may cause steric hindrance and so reduce the binding capacity again.
- Modify reaction conditions such as pH, temperature, buffers or contact time. Most pre-activated matrices are supplied with details of the preferred conditions for a coupling reaction that can be used as a basis for further optimization.

Binding and elution conditions

Binding and elution conditions will depend on the nature of the interaction between the ligand and target. As for any affinity purification, the general guidelines outlined in Chapter 2 can be applied during development.

For the first run, perform a blank run to ensure that any loosely bound ligand is removed.

Immunospecific interactions can be 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, try alternative elution buffers such as:

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

The following protocol can be used as a guideline for a preliminary separation:

1. Prepare the column (blank run)
    a. Wash with 2 column volumes binding buffer.
    b. Wash with 3 column volumes elution buffer.
2. Equilibrate with 10 column volumes of binding buffer.
3. Apply sample. The optimal flow rate is dependent 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 $A_{280\ nm}$.
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 133).
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.

Coupling through the primary amine of a ligand

HiTrap NHS-activated HP, NHS-activated Sepharose 4 Fast Flow

NHS-activated Sepharose is designed for the covalent coupling of ligands (often antigens or antibodies) containing primary amino groups (the most common form of attachment) and is the first choice for the preparation of immunospecific media. The matrix is based on highly cross-linked agarose beads with 10-atom spacer arms (6-aminohexanoic acid) attached by epichlorohydrin and activated by N-hydroxysuccinimide (Figure 58). Non-specific adsorption of proteins (which can reduce binding capacity of the target protein) is negligible due to the excellent hydrophilic properties of the base matrix. The matrix is stable at high pH to allow stringent washing procedures (subject to the pH stability of the coupled ligand).

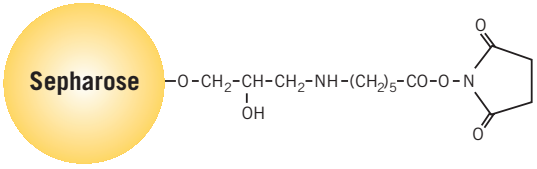


Fig. 58. Partial structure of NHS-activated Sepharose bearing activated spacer arms.

Ligands containing amino groups couple rapidly and spontaneously by nucleophilic attack at the ester linkage to give a very stable amide linkage (Figure 59). The amide bond is stable up to pH 13 making NHS-activated Sepharose suitable for applications that require conditions at high pH.

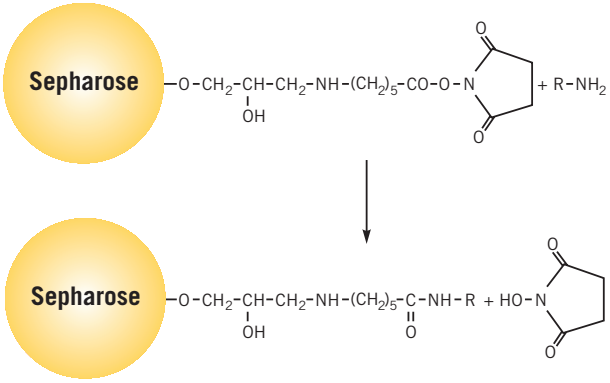


Fig. 59. Coupling a ligand to NHS-activated Sepharose.

Options

Product	Spacer arm	Coupling conditions	Maximum operating flow	Comments
HiTrap NHS-activated HP	10-atom	pH 6.5–9, 15–30 min at room temp. or 4 hours at +4 °C.	4 ml/min (1 ml column) 20 ml/min (5 ml column)	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	10-atom	pH 6–9, 2–16 hours, +4 °C - room temp.	300 cm/h*	Supplied as a suspension ready for column packing.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Figure 60 shows that over 30 mg IgG can be coupled to a 1 ml HiTrap NHS-activated HP column. The coupling process takes less than 15 minutes. The affinity medium is then ready to use for antigen purification.

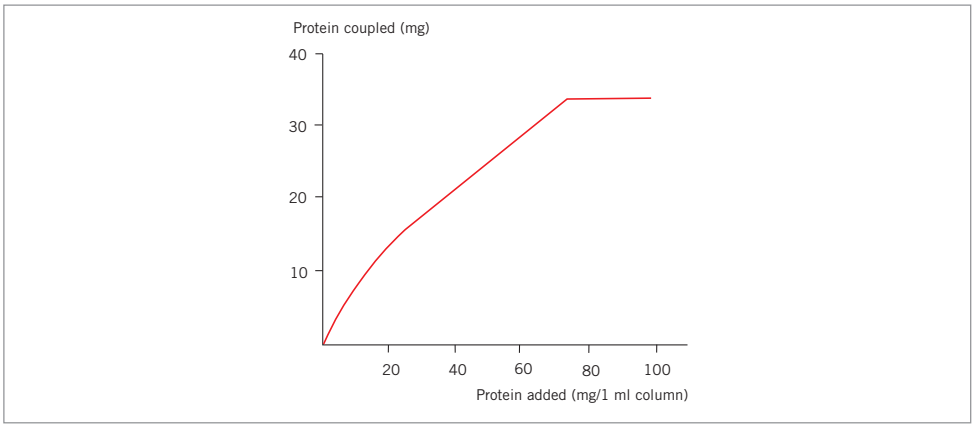


Fig. 60. Ligand coupling to HiTrap NHS-activated HP.

Preparation of HiTrap NHS-activated HP

The protocol below describes the preparation of a prepacked HiTrap NHS-activated HP column and is generally applicable to NHS-activated Sepharose media. A general column packing procedure is described in Appendix 3.

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

Buffer preparation

Acidification solution:	1 mM HCl (kept on ice)
Coupling buffer:	0.2 M $NaHCO_3$, 0. M NaCl, pH 8.3
Blocking buffer:	0.5 M ehanolamine, 0.5 M NaCl, pH 8.3
Wash buffer:	0.1 M acetate, 0.5 M NaCl, pH 4.0

Couping within pH range 6.5–9, maximum yield is achieved at around pH 8.

Ligand and 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 133). The optimal concentration depends on the ligand. Dissolve the ligand in one column volume of buffer.
2. Remove the top cap from the column 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. Leave for 15–30 minutes at +25 °C (or 4 hours at +4 °C).

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, circulate the ligand solution through the column.

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.

Measure the efficiency of protein ligand by comparing the A_{280} values of the ligand solution before and after coupling. Note that the N-hydroxy-succinimide, released during the coupling procedure, absorbs strongly at 280 nm and should be removed from the used coupling solution before measuring the concentration of the remaining ligand. Use a small desalting column (see page 133) to remove N-hydroxy-succinimide from protein ligands. Alternative methods for the measurement of coupling efficiency are described on page 104 and in the HiTrap NHS-activated HP instructions.

Washing and deactivation

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

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

The column is now ready for use.

Media characteristics

Product	Ligand density	Composition	pH stability*	Mean particle size
HiTrap NHS-activated HP	10 µmoles/ml	6-aminohexanoic acid linked by epoxy coupling to highly cross linked agarose, terminal carboxyl group esterified with NHS.	Short term 3–12 Long term 3–12	34 µm
NHS-activated Sepharose 4 Fast Flow	16–23 µmoles/ml	As above	Short term 3–13 Long term 3–13	90 µm

**Long term refers to the pH interval over which the matrix is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-plac and sanitization procedures. Stability data refers to the coupled medium provided that the ligand can withstand the pH.*

Storage

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

pH stability of the media when coupled to the chosen ligand will depend upon the stability of the ligand itself.

Sodium azide can interfere with many coupling methods and some biological assays. It can be removed by using a desalting column (see page 133).

CNBr-activated Sepharose

CNBr-activated Sepharose offers a well-established option for the attachment of larger ligands and as an alternative to NHS-activated Sepharose.

Cyanogen bromide reacts with hydroxyl groups on Sepharose to form reactive cyanate ester groups. Proteins, peptides, amino acids or nucleic acids can be coupled to CNBr-activated Sepharose, under mild conditions, via primary amino groups or similar nucleophilic groups. The activated groups react with primary amino groups on the ligand to form isourea linkages (Figure 61). The coupling reaction is spontaneous and requires no special chemicals or equipment. The resulting multi-point attachment ensures that the ligand does not hydrolyze from the matrix. The activation procedure also cross-links Sepharose and thus enhances its chemical stability, offering considerable flexibility in the choice of elution conditions.

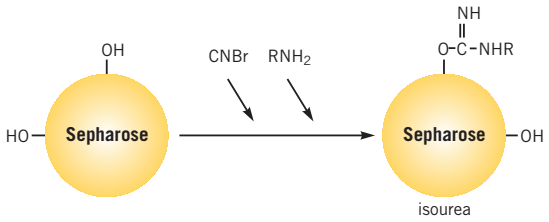


Fig. 61. Activation by cyanogen bromide and coupling to the activated matrix.

Options

Product	Spacer arm	Coupling conditions	Maximum operating flow	Comments
CNBr-activated Sepharose 4 Fast Flow	None	pH 7–9, 2–16 hours, +4 °C - room temp.	400 cm/h*	Supplied as a freeze-dried powder.
CNBr-activated Sepharose 4B	None	pH 8–10, 2–16 hours, +4 °C - room temp.	75 cm/h*	Supplied as a freeze-dried powder.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

There are many examples in the literature of the use of CNBr-activated Sepharose. Figure 62 shows the separation of a native outer envelope glycoprotein, gp120, from HIV-1 infected T-cells. Galanthus nivalis agglutinin (GNA), a lectin from the snowdrop bulb, was coupled to CNBr-activated Sepharose 4 Fast Flow to create a suitable affinity medium.

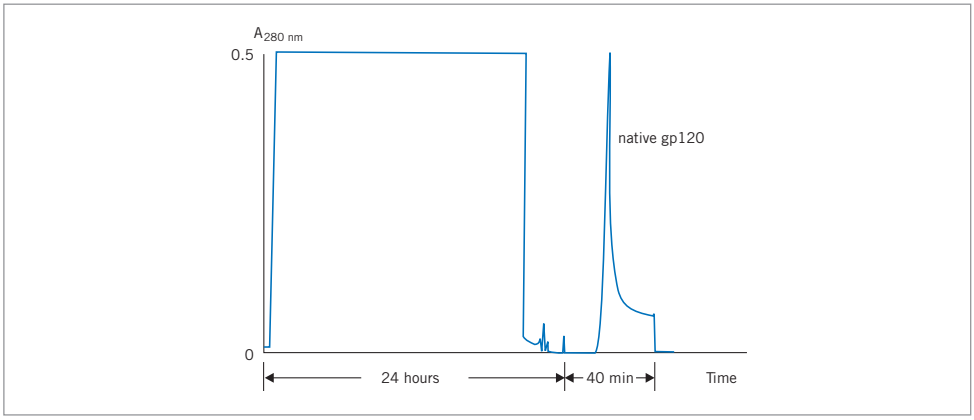


Fig. 62. Separation of native gp120 protein on GNA coupled to CNBr-activated Sepharose 4 Fast Flow. From Gilljam, G. *et al.*, Purification of native gp120 from HIV-1 infected T-cells. Poster presented at Recovery of Biological Products VII, Sept. 25-30, 1994, San Diego, CA, USA. Further details are available in the CNBr-activated Sepharose 4 Fast Flow datafile, from Amersham Biosciences.

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:	1 M ethanolamine or 0.2 M glycine, pH 8.0
Wash buffer:	0.1 M acetate, 0.5 M NaCl, pH 4

Preparation of CNBr-activated Sepharose 4 Fast Flow and CNBr-activated Sepharose 4B

1. Suspend the required amount of freeze-dried powder in ice-cold 1 mM HCl (HCl preserves the activity of the reactive groups that hydrolyze at high pH).
2. Wash for 15 min. on a sintered glass filter (porosity G3), using a total of 200 ml 1 mM HCl per gram dry powder, added and sucked off in several aliquots. The final aliquot of 1 mM HCl is sucked off until cracks appear in the cake.
3. Transfer the matrix immediately to the ligand solution.

Preparation of the matrix should be completed without delay since reactive groups on the matrix hydrolyze at the coupling pH.

Do not use buffers containing amino groups at this stage since they will couple to the matrix.

Ligand preparation

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 133). The optimal concentration depends on the ligand. Use a matrix:buffer ratio of 1:2.

Ligand coupling

1. Mix the ligand solution with suspension in an end-over-end or similar mixer for 2 hours at room temperature or overnight at +4 °C. A matrix: buffer ratio of 1:2 gives a suitable suspension for coupling.
2. Transfer the medium to blocking buffer for 16 hours at +4 °C or 2 hours at room temperature to block any remaining active groups. Alternatively, leave the medium for 2 hours in Tris-HCl buffer, pH 8.
3. Remove excess ligand and blocking agent by alternately washing with coupling buffer followed by wash buffer. Repeat four or five times. A general column packing procedure is described in Appendix 3.

Do not use magnetic stirrers as they may disrupt the Sepharose matrix.

The coupling reaction proceeds most efficiently when the amino groups on the ligand are predominantly in the unprotonated form. A buffer at pH 8.3 is most frequently used for coupling proteins. The high salt content of the coupling buffer minimizes protein-protein adsorption caused by the polyelectrolyte nature of proteins.

Coupling of α-chymotrypsinogen by the method described here typically yields about 90% coupled protein. It may be necessary to reduce the number of coupling groups on the matrix to preserve the structure of binding sites in a labile molecule, or to facilitate elution when steric effects reduce the binding efficiency of a large ligand. Reduced coupling activity may be achieved by controlled hydrolysis of the activated matrix before coupling, or by coupling at a lower pH. Pre-hydrolysis reduces the number of active groups available for coupling and reduces the number of points of attachment between the protein and matrix as well as the amount of protein coupled. In this way a higher binding activity of the product may be obtained. At pH 3, coupling activity is lost only slowly, whereas at pH 8.3 activity is lost fairly rapidly. A large molecule is coupled at about half as many points after 4 hours pre-hydrolysis at pH 8.3 (Figure 63).

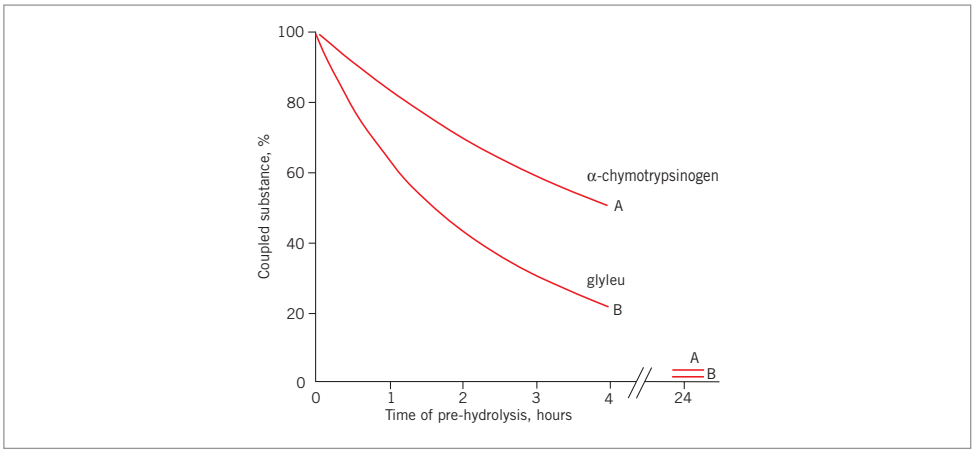


Fig. 63. Variation of coupling activity with time of pre-hydrolysis at pH 8.3. CNBr-activated Sepharose 4B was washed at pH 3 and transferred to 0.1 M $NaHCO_3$, pH 8.3 for pre-hydrolysis. Samples were removed after different times and tested for coupling activity towards α-chymotrypsinogen (A) and glycyl-leucine (B).

Coupling at low pH is less efficient, but may be advantageous if the ligand loses biological activity when it is fixed too firmly, e.g. by multi-point attachment, or because of steric hindrance between binding sites which occurs when a large amount of high molecular weight ligand is coupled. Use a buffer of approximately pH 6.

IgG is often coupled at a slightly higher pH, for example in 0.2–0.25 M $NaHCO_3$, 0.5 M NaCl, pH 8.5–9.0.

Media characteristics

Product	Composition	Binding capacity per ml medium	pH stability*	Mean particle size
CNBr-activated Sepharose 4 Fast Flow	Cyanogen bromide reacts with hydroxyl groups on Sepharose to give a reactive product for coupling ligands via primary amino groups or similar nucleophilic groups.	α-chymotrypsinogen, 13–26 mg	Short term 3–11 Long term 3–11	90 µm
CNBr-activated Sepharose 4B		α-chymotrypsinogen, 25–60 mg	Short term 2–11 Long term 2–11	

**Long term refers to the pH interval over which the matrix is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. Stability data refers to the coupled medium provided that the ligand can withstand the pH.*

Storage

Store the freeze-dried powder below +8 °C in dry conditions.

Store the column in a solution that maintains the stability of the ligand and contains a bacteriostatic agent, for example, PBS, 0.05% NaN_3, pH 7.2 or 20% ethanol in a suitable buffer.

The pH stability of the medium when coupled to the chosen ligand will depend upon the stability of the ligand itself.

Immunoaffinity chromatography

Immunoaffinity chromatography utilizes antigens or antibodies as ligands (sometimes referred to as adsorbents, immunoadsorbents or immunosorbents) to create highly selective media for affinity purification.

Antibodies are extremely useful as ligands for antigen purification, especially when the substance to be purified has no other apparent complementary ligand.

Similarly, highly purified antigens or anti-antibodies can provide highly specific ligands for antibody purification. *The Antibody Handbook* from Amersham Biosciences covers the purification and application of antibodies in greater detail.

Immunoaffinity media are created by coupling the ligand (a pure antigen, an antibody or an anti-antibody) to a suitable matrix. The simplest coupling is via the primary amine group of the ligand, using NHS-activated Sepharose or CNBr-activated Sepharose. Figure 64 illustrates a typical immunoaffinity purification.

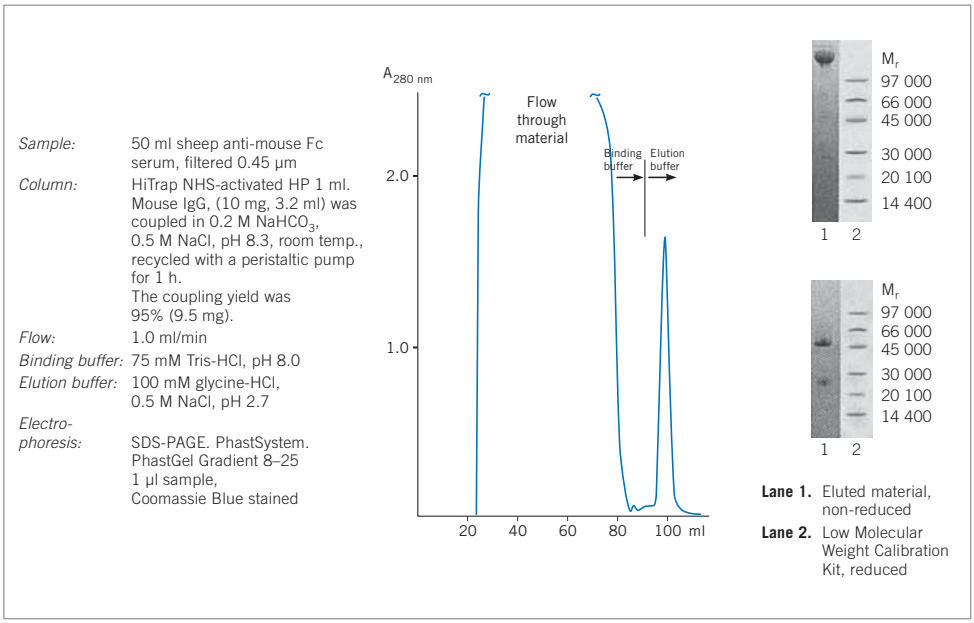


Fig. 64. Purification of anti-mouse Fc-IgG from sheep antiserum.

If there is no primary amine available (for example, 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.

The guidelines given in Chapter 2, Affinity chromatography in practice, and Chapter 3, Purification of immunoglobulins, are applicable to immunoaffinity chromatography. Optimal binding and elution conditions will be different for each immunospecific reaction according to the strength of interaction and the stability of the target proteins.

Coupling small ligands through amino or carboxyl groups via a spacer arm

EAH Sepharose 4B and ECH Sepharose 4B

The partial structures of EAH Sepharose 4B and ECH Sepharose 4B are shown in Figure 65.

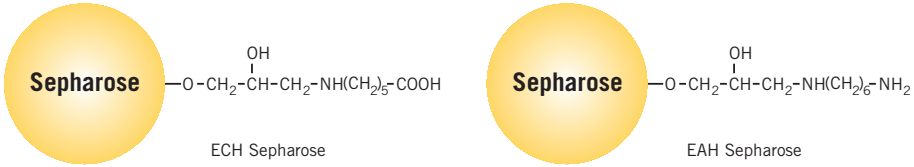


Fig. 65. Partial structures of ECH Sepharose 4B and EAH Sepharose 4B.

Ligands are coupled in a simple one-step procedure in the presence of a coupling reagent, carbodiimide. The carbodiimides may be regarded as anhydrides of urea. The N,N' di-substituted carbodiimides promote condensation between a free amino and a free carboxyl group to form a peptide link by acid-catalyzed removal of water. Thus EAH Sepharose 4B can be coupled with carboxyl-containing ligands and ECH Sepharose 4B can be coupled with ligands containing amino groups. The carbodiimide yields an isourea upon hydration. The coupling reaction is shown in Figure 66.

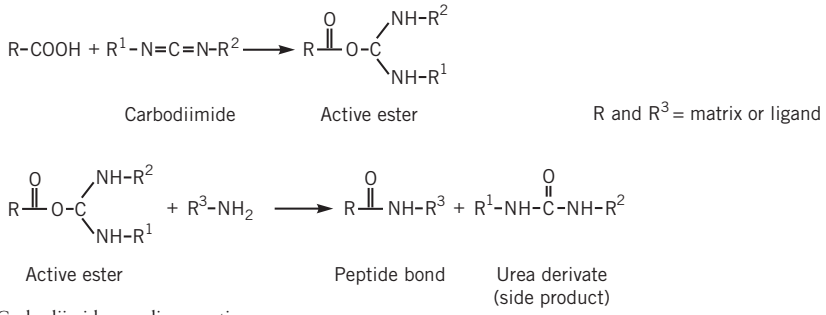


Fig. 66. Carbodiimide coupling reaction.

Options

Product	Spacer arm	Substitution per ml matrix	Coupling conditions	Maximum operating flow	Comments
EAH Sepharose 4B	10-atom	7–11 µmoles amino groups	pH 4.5, 1.5–24 hours, +4 °C - room temp.	75 cm/h*	Couple ligands containing free carboxyl groups. Supplied as a suspension ready for use.
ECH Sepharose 4B	9-atom	12–16 µmoles carboxyl groups	pH 4.5, 1.5–24 hours, +4 °C - room temp.	75 cm/h*	Couple ligands containing free amino groups. Supplied as a suspension ready for use.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Preparation of coupling reagent

Use a water-soluble carbodiimide such as N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) or N-cyclohexyl-N'-2-(4'-methyl-morpholinium) ethyl carbodiimide p-toluene sulphonate (CMC). These two carbodiimides have been used in a variety of experimental conditions and at a wide range of concentrations (Table 10). EDC often gives better coupling yields than CMC.

Table 10. Examples of conditions used during coupling via carbodiimides.

Coupled ligand	Carbodiimide	Conc. of carbodiimide mg/ml	pH	Reaction time
Methotrexate	EDC	18	6.4	1.5 h
UDP-glucuronic acid	EDC	32	4.8	24 h
p-amino-benzamidine	CMC	2	4.75	5 h
Folic acid	EDC	5	6	2 h
Mannosylamine	EDC	19	4.5–6.0	24 h

Use a concentration of carbodiimide greater than the stoichiometric concentration, usually 10–100 times greater than the concentration of spacer groups.

The coupling reaction is normally performed in distilled water adjusted to pH 4.5–6.0 to promote the acid-catalyzed condensation reaction. Blocking agents are not usually required after the coupling reaction if excess ligand has been used.

Always use freshly prepared carbodiimides.

Coupling buffer: Dissolve the carbodiimide in water and adjust to pH 4.5

Wash buffer: 0.1 M acetate, 0.5 M NaCl, pH 4

Avoid the presence of amino, phosphate or carboxyl groups, as these will compete with the coupling reaction.

Preparation of EAH and ECH Sepharose 4B

Wash the required amount of matrix on a sintered glass filter (porosity G3) with distilled water adjusted to pH 4.5 with HCl, followed by 0.5 M NaCl (80 ml in aliquots/ml sedimented matrix).

Ligand preparation

Dissolve the ligand and adjust to pH 4.5. The optimal concentration depends on the ligand. Organic solvents can be used to dissolve the ligand, if necessary. If using a mixture of organic solvent and water, adjust the pH of the water to pH 4.5 before mixing it with the organic solvent. Solvents such as dioxane (up to 50%), ethylene glycol (up to 50%), ethanol, methanol and acetone have been used.

If organic solvents have been used, use pH paper to measure pH since solvents may damage pH electrodes.

Ligand coupling

1. Add the ligand solution followed by the carbodiimide solution to the matrix suspension and leave on an end-over-end or similar mixer. Use a matrix: ligand solution ratio of 1:2 to produce a suspension that is suitable for coupling. Typically the reaction takes place overnight either at +4 °C or room temperature.
2. Adjust the pH of the reaction mixture during the first hour (pH will decrease) by adding 0.1 M sodium hydroxide.
3. Wash at pH 8 and pH 4 to remove excess reagents and reaction by-products.

If a mixture of aqueous solution and organic solvent has been used, use this mixture to wash the final product as in Step 3. After Step 3 wash in distilled water, followed by the binding buffer to be used for the affinity purification.

Do not use magnetic stirrers as they may disrupt the Sepharose matrix.

Media characteristics

Product	Composition	pH stability*	Mean particle size
EAH Sepharose 4B	Covalent linkage of 1,6-diamino-hexane by epoxy coupling creates a stable, uncharged ether link between a 10-atom spacer arm and Sepharose 4B.	Short term 3–14 Long term 3–14	90 µm
ECH Sepharose 4B	Covalent linkage of 6-aminohexanoic acid by epoxy coupling creates a stable, uncharged ether link between the 9-atom spacer arm and Sepharose 4B.	Short term 3–14 Long term 3–14	90 µm

**Long term refers to the pH interval over which the matrix is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. Stability data refers to the coupled medium provided that the ligand can withstand the pH.*

Storage

Store pre-activated matrices at +4 to +8 °C in 20% ethanol.

Store the column in a solution that maintains the stability of the ligand and contains a bacteriostatic agent, for example, PBS, 0.05% NaN_3, pH 7.2 or 20% ethanol in a suitable buffer.

The pH stability of the media when coupled to a ligand will depend upon the stability of the ligand.

Performing a separation

See page 105 for a preliminary separation protocol and Chapter 2 for general guidelines.

Coupling through hydroxy, amino or thiol groups via a 12-carbon spacer arm

Epoxy-activated Sepharose 6B

Epoxy-activated Sepharose 6B is used for coupling ligands that contain hydroxyl, amino or thiol groups. Because of the long hydrophilic spacer arm, it is particularly useful for coupling small ligands such as choline, ethanolamine and sugars. The pre-activated matrix is formed by reacting Sepharose 6B with the *bis* oxirane, 1,4 *bis*-(2,3-epoxypropoxy-)butane. The partial structure is shown in Figure 67.

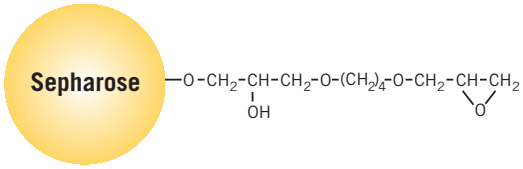


Fig. 67. Partial structure of Epoxy-activated Sepharose 6B.

A stable ether linkage is formed between the hydrophilic spacer and the matrix. Free oxirane groups couple via stable ether bonds with hydroxyl-containing molecules such as sugars, via alkylamine linkages with ligands containing amino groups, and via thioether linkages with ligands containing thiol groups.

Options

Product	Spacer arm	Substitution per ml matrix	Coupling conditions	Maximum operating flow	Comments
Epoxy-activated Sepharose 6B	12-atom	19–40 µmoles epoxy groups	pH 9–13, 16 hours - several days, +20 - +40 °C	75 cm/h*	Supplied as a freeze-dried powder.

**See Appendix 4 to convert linear flow (cm/h) to volumetric flow rate. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.*

Purification example

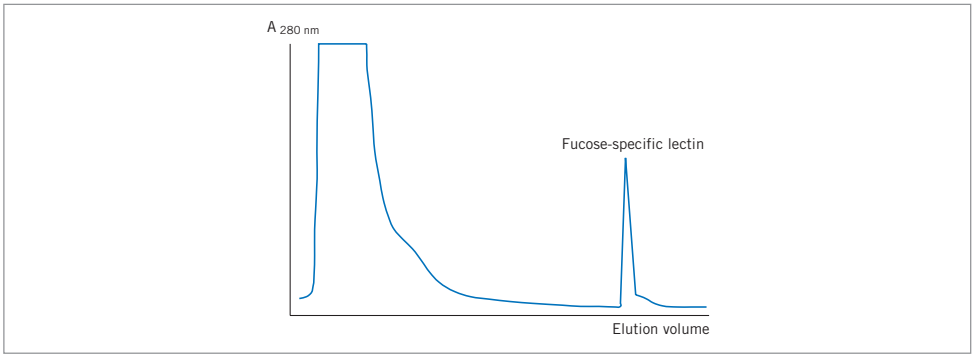


Fig. 68. Chromatography of a crude extract of *Ulex europaeus* on fucose coupled to Epoxy-activated Sepharose 6B, column volume 11 ml. Extract was applied in 0.9% NaCl. Fucose-specific lectin was eluted with 5 ml fucose (50 mg/ml).

Alternative coupling solutions:

Distilled water or aqueous buffers with sugars and carbohydrates are preferable. Carbonate, borate or phosphate buffers can be used.

Sodium hydroxide may be used for solutions of high pH.

Organic solvents such as dimethylformamide (up to 50%) and dioxane (up to 50%) may be used to dissolve the ligand. The same concentration of organic solvent should be included in the coupling solution.

Coupling procedure

1. Suspend the required amount of freeze-dried powder in distilled water (1 g freeze-dried powder gives about 3.0 ml final matrix volume).
2. Wash immediately for 1 hour on a sintered glass filter (porosity G3), using approximately 200 ml distilled water per gram freeze-dried powder, added in several aliquots.
3. Dissolve the ligand in the coupling buffer to a final concentration of 0.5–10 mg/ml (for protein ligands) or transfer solubilized ligands into the coupling buffer using a desalting column (see page 133). Adjust the pH of the aqueous phase.
4. Use a matrix:buffer ratio of 1:2, mix the matrix suspension with the ligand solution for 16 h at +25 to +40 °C in a shaking water bath.
5. Block remaining excess groups with 1 M ethanolamine for at least 4 h or overnight, at +40 to +50 °C.
6. Wash away excess ligand with coupling solution followed by distilled water, 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0 and 0.1 M NaCl, 0.1 M acetate, pH 4.0.

If organic solvents have been used, use pH paper to measure pH since solvents may damage pH electrodes.

Using the higher temperatures can decrease coupling times.

Do not use Tris, glycine or other nucleophilic compounds as these will couple to the oxirane groups.

Do not use magnetic stirrers as they may disrupt the Sepharose matrix.

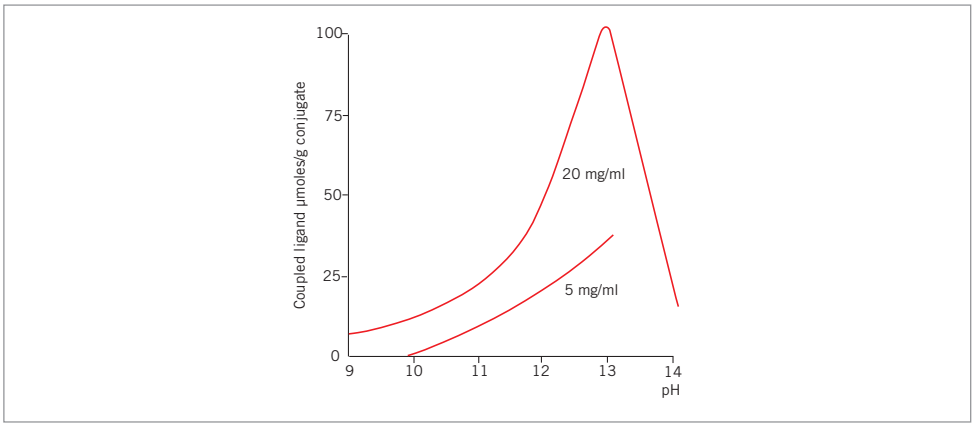


Fig. 69. pH dependence of coupling N-acetyl-D-galactosamine to Epoxy-activated Sepharose 6B. Carbonate/bicarbonate buffers were used in the range pH 9–11, sodium hydroxide solution in the range pH 12–14.
Ligand concentrations: 5 mg/ml and 20 mg/ml.

When a ligand contains more than one kind of group (thiol, amino and hydroxyl), the coupling pH will determine which of these groups is coupled preferentially. As a general rule, the order of coupling is ε-amino > thiol > α-amino > hydroxyl although the exact result will depend on the detailed structure of the ligand.

The time of reaction depends greatly on the pH of the coupling solution, properties of the ligand and the coupling temperature. The stability of the ligand and the carbohydrate chains of the matrix limit the maximum pH that can be used. Coupling is performed in the range pH 9–13 as shown in Figure 69 and the efficiency of coupling is pH and temperature dependent (Figure 70).

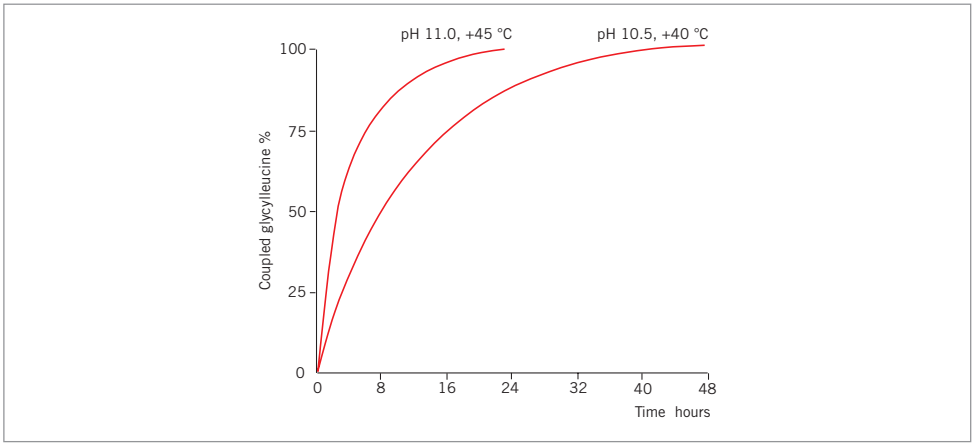


Fig. 70. Efficiency of coupling glycyl-leucine to Epoxy-activated Sepharose 6B.

Media characteristics

Product	Composition	pH stability*	Mean particle size
Epoxy-activated Sepharose 6B	Sepharose 6B reacts with 1,4 *bis*-(2,3 epoxypropoxy-) butane to form a stable ether linkage.	Short term 2–14 Long term 2–14	90 µm

**Long term refers to the pH interval over which the matrix is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. Stability data refers to the coupled medium provided that the ligand can withstand the pH.*

Storage

Store the freeze-dried powder dry below +8 °C.

Store the column in a solution that maintains the stability of the ligand and contains a bacteriostatic agent, for example, PBS, 0.05% NaN_3, pH 7.2 or 20% ethanol in a suitable buffer.

The pH stability of the media when coupled to a ligand will depend upon the stability of the ligand.

Coupling through a thiol group

Thiopropyl Sepharose 6B

The active thiol groups of Thiopropyl Sepharose 6B (see page 92 for product details) can be used to couple many types of small ligands to synthesize affinity media.

- Heavy metal ions and derivatives can be used as ligands to react with thiol groups forming mercaptides.
- Alkyl or aryl halide ligands give thioether derivatives.
- Ligands containing C=O, N=N and, under certain conditions, C=C bonds undergo addition reactions.

The medium is converted into the free thiol form, as described earlier, before ligands can be coupled. The hydroxypropyl group acts as a small spacer arm. Reactions of free thiol groups are shown in Figure 71.

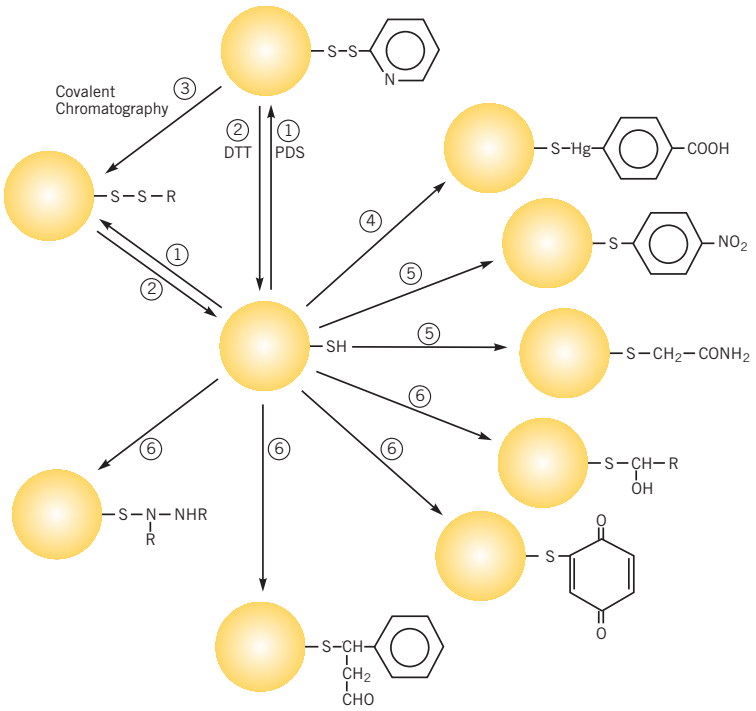


Fig. 71. Reactions of thiol groups. Mixed disulphide formation (1), reversible by reducing agents such as dithiothreitol (DTT) (2). Mixed disulphide formation with 2, 2'-dipyridyl disulphide gives a 2-thiopyridyl derivative suitable for use in covalent chromatography (3). Reaction with heavy metals and their derivatives e.g. p-chloromercuribenzoate (4) leads to mercaptide formation. Treatment with alkyl or aryl halides gives thioether derivatives (5). Addition reactions (6) are possible with a wide variety of compounds containing C=O, C=C and N=N bonds.

Use Thiopropyl Sepharose 6B in the activated form to couple thiol-containing low molecular weight ligands, such as coenzyme A. If the ligand:protein interaction is so strong that elution requires denaturing conditions, the entire ligand-protein complex may be eluted by reduction with dithiothreitol or 2-mercaptoethanol.

Ligands containing amino groups can be attached to Thiopropyl Sepharose 6B or Activated Thiol Sepharose 4B by multi-point attachment or coupling through a small number of groups using the heterobifunctional thiolating reagent, SPDP. The coupled molecules may be recovered by eluting with a reducing agent. This may be extremely useful when elution is difficult using other methods. The entire ligand-protein complex is eluted from the medium.

Coupling other functional groups

EAH Sepharose 4B may be used as a starting material for coupling via alternative functional groups (Figure 72). Phenolic groups may be attached via diazonium derivatives (VII) or via the bromoacetamidoalkyl derivative (V) prepared by treating EAH Sepharose 4B with O-bromoacetyl-N-hydroxysuccinimide. This derivative also couples via primary amino groups. The spacer arm of EAH Sepharose 4B may be extended by reaction with succinic anhydride at pH 6 (VI) to form a derivative to which amino groups can be coupled by carbodiimide reaction. Carboxyl groups are coupled to EAH Sepharose 4B by the carbodiimide reaction (III). Thiol derivatives, prepared by reaction (IV), couple carboxyl groups in the presence of carbodiimide and the thiol ester bond may be cleaved specifically using hydroxylamine, thus providing a simple and gentle method for eluting the intact ligand-protein complex.

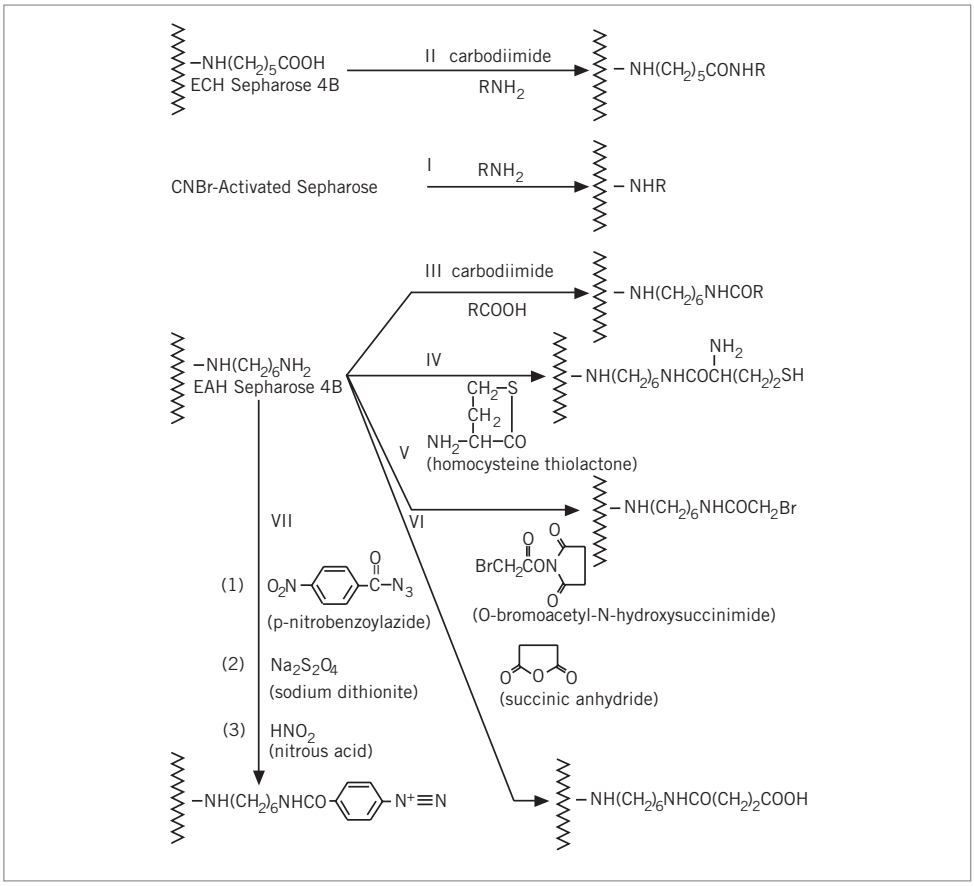


Fig. 72. Reactions used to couple ligands to Sepharose.

Chapter 6
Affinity chromatography and CIPP

Affinity chromatography 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. With such high selectivity and hence high resolution for the protein(s) of interest, purification levels in the order of several thousand-fold with high recovery of active material are achievable. Samples are concentrated during binding and the target protein(s) is collected in a purified, concentrated form.

Affinity purification can therefore offer immense time-saving over less selective multi-step procedures. Common operations such as the purification of antibodies or tagged fusion proteins can be performed in a single step. The concentrating effect enables large volumes to be processed. Target molecules can be purified from complex biological mixtures, native forms separated from denatured forms of the same substance, small amounts of biological material purified from high levels of contaminating substances. Affinity chromatography can also be used to remove specific contaminants, such as proteases.

In many cases, the high level of purity achievable requires, at most, only a second step on a gel filtration column to remove unwanted small molecules, such as salts or aggregates.

For an even higher degree of purity, or when there is no suitable ligand for affinity purification, an efficient multi-step process must be developed using the purification strategy of Capture, Intermediate Purification and Polishing (CIPP), shown in Figure 73. When applying this strategy affinity chromatography offers an ideal capture or intermediate step in any purification protocol and can be used whenever a suitable ligand is available for the protein(s) of interest.

CIPP 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. Affinity chromatography can be used, in combination with other chromatography techniques, as an effective capture or intermediate step in a CIPP strategy.

This chapter gives a brief overview of the approach recommended for any multi-step protein purification. *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.

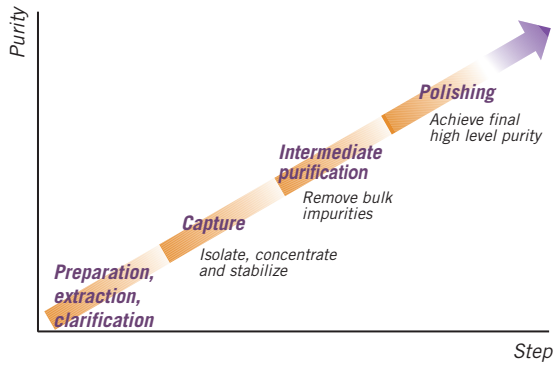


Fig. 73. Preparation and CIPP.

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 73, an important first step for any purification is correct *sample preparation* and this is covered in detail in Appendix 1.

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.

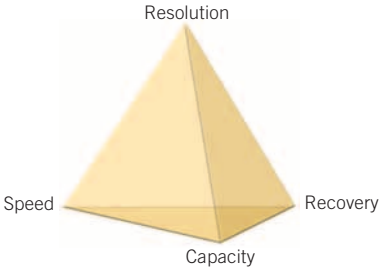
Selection and combination of purification techniques

Proteins are purified using purification techniques that separate according to differences in specific properties, as shown in Table 11.

Table 11. Protein properties used during purification.

Protein property	Technique*
Biorecognition (ligand specificity)	Affinity (AC)
Charge	Ion exchange (IEX)
Size	Gel filtration (GF)
Hydrophobicity	Hydrophobic interaction (HIC), Reversed phase (RPC)

**Expanded bed adsorption is a technique used for large-scale purification. Proteins can be purified from crude sample without the need for separate clarification, concentration and initial purification to remove particulate matter. The STREAMLINE adsorbents, used for expanded bed adsorption, capture the target molecules using the same principles as affinity, ion exchange or hydrophobic interaction chromatography.*



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

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. The high selectivity of affinity chromatography typically gives a high resolution result.

Capacity, in the simple model shown, refers to the amount of target protein that can be loaded during purification. In some cases the amount of sample that can be loaded will be limited by volume (as in gel filtration) or by large amounts of contaminants, rather than by the amount of the target protein. Since affinity chromatography is a binding technique the separation is unaffected by sample volume as long as the correct binding conditions are maintained during sample application and the total amount of target protein loaded onto the column does not exceed the binding capacity of the affinity medium.

Speed is most important at the beginning of purification where contaminants such as proteases must be removed as quickly as possible. Modern affinity matrices enable high flow rates to be used for sample application as well as washing and reequilibration steps. For each application a flow rate can be selected to achieve an optimal balance between efficient binding and elution of the target protein and a fast separation.

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. Affinity media provided with optimized separation protocols can give extremely high recoveries of target protein.

Select the technique that 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 12.

Table 12. Suitability of purification techniques for CIPP.

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

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 buffer conditions suitable for the start conditions of the next column (see Table 12).

Ammonium sulphate, often used for sample clarification and concentration (see Appendix 1), 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) 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 74.

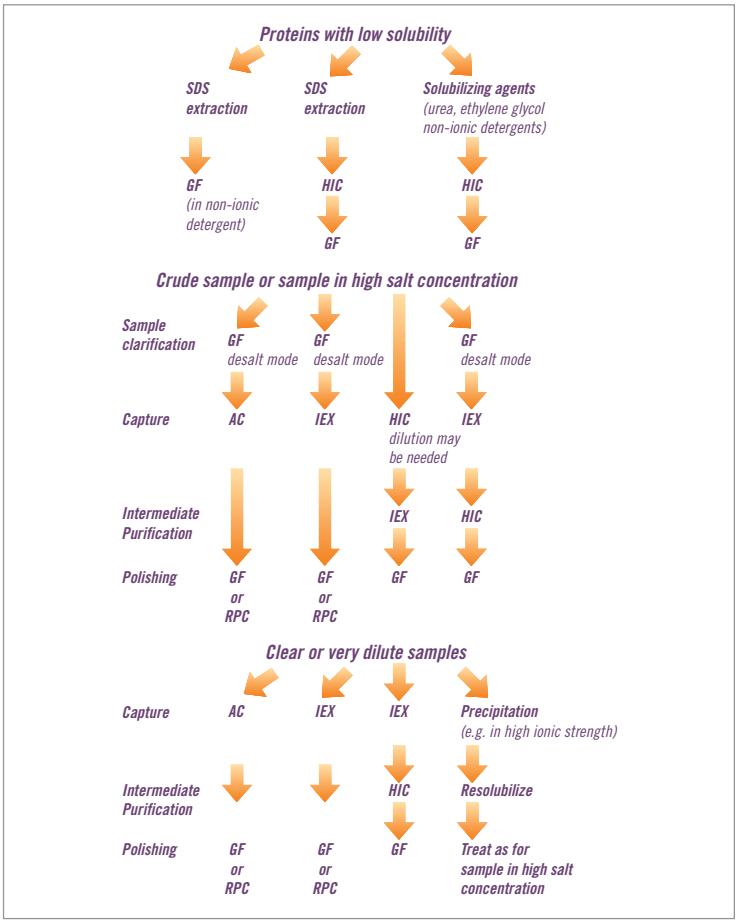


Fig. 74. Logical combinations of chromatography techniques.

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 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, RPC is not generally recommended for protein purification because recovery of activity and return to a native tertiary structure may be compromised. However, in the polishing phase, when the majority of protein impurities have been removed, RPC can be excellent, particularly for small target proteins that are 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.

Appendix 1

Sample preparation

Samples for chromatographic purification should be clear and free from particulate matter. Simple steps to clarify a sample before beginning purification will avoid clogging the column, may reduce the need for stringent washing procedures and can extend the life of the chromatographic medium.

Sample extraction procedures and the selection of buffers, additives and detergents are determined largely by the source of the material, the stability of the target molecule, the chromatographic techniques that will be employed and the intended use of the product. These subjects are dealt with in general terms in the *Protein Purification Handbook* and more specifically according to target molecule in the *Recombinant Protein Handbook, Protein Amplification and Simple Purification* and *Antibody Purification Handbook*, available from Amersham Biosciences.

Sample stability

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

Proteins generally contain a high degree of tertiary structure, kept together by van der Waals' forces, ionic and hydrophobic interactions and hydrogen bonding. Any conditions capable of destabilizing these forces may cause denaturation and/or precipitation. By contrast, peptides contain a low degree of tertiary structure. Their native state is dominated by secondary structures, stabilized mainly by hydrogen bonding. For this reason, peptides tolerate a much wider range of conditions than proteins. This basic difference in native structures is also reflected in that proteins are not easily renatured, while peptides often renature spontaneously.

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

- Test pH stability in steps of one pH unit between pH 2 and pH 9.
- Test salt stability with 0–2 M NaCl and 0–2 M $(NH_4)_2SO_4$ in steps of 0.5 M.
- Test the stability towards acetonitrile and methanol in 10% steps between 0 and 50%.
- Test the temperature stability in +10 °C steps from +4 to +40 °C.
- Test the stability and occurrence of proteolytic activity by leaving an aliquot of the sample at room temperature overnight. Centrifuge each sample and measure activity and UV absorbance at 280 nm in the supernatant.

Sample clarification

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

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

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 as a first step and one of the filters below as a second step filter.

- For small sample volumes or proteins that adsorb to filters, centrifuge at 10 000 g for 15 minutes.
- For cell lysates, centrifuge at 40 000–50 000 g for 30 minutes.
- Serum samples can be filtered through glass wool after centrifugation to remove any remaining lipids.

Filtration

Filtration removes particulate matter. 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.

Desalting

Desalting columns are suitable for any sample volume and will rapidly remove low molecular weight contaminants in a single step at the same time as transferring the sample into the correct buffer conditions. Centrifugation and/or filtration of the sample before desalting is still recommended. Detailed procedures for buffer exchange and desalting are given on page 133.

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

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.

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

Specific sample preparation steps

Specific sample preparation steps may be required if the crude sample is known to contain contamininants such as lipids, lipoproteins or phenol red that may build up on a column or if certain gross impurities, such as bulk protein, should be removed before any chromatographic step.

Fractional precipitation

Fractional precipitation is frequently used at laboratory scale to remove gross impurities from small sample volumes, and occasionally used in small-scale commercial production. 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 75.

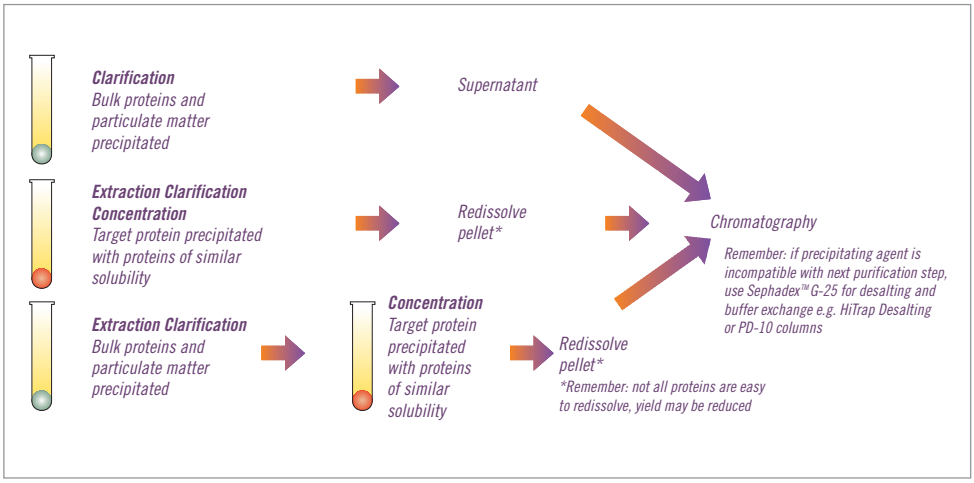


Fig. 75. Three ways to use precipitation.

Examples of precipitation agents are reviewed in Table 13. The most common precipitation method using ammonium sulphate is described in more detail.

Table 13. Examples of precipitation techniques.

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

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

Ammonium sulphate precipitation

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

For routine, reproducible purification, precipitation with ammonium sulphate should be avoided in favor of chromatography.

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

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 the sample (10 000 g at +4 °C).
2. Add 1 part 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently. Add ammonium sulphate solution, drop by drop. Add up to 50% saturation*. Stir for 1 hour.
4. Centrifuge 20 minutes at 10 000 g.
5. Remove supernatant. Wash the 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.
6. Dissolve pellet in a small volume of the buffer to be used for the next step.
7. Ammonium sulphate is removed during clarification/buffer exchange steps with Sephadex G-25, using desalting columns (see page 133).

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

The quantity of ammonium sulphate required to reach a given degree of saturation varies according to temperature. Table 14 shows the quantities required at +20 °C.

Table 14. Quantities of ammonium sulphate required to reach given degrees of saturation at +20 °C.

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

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 15 gives examples of common denaturing agents.

Table 15.

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.

Buffer exchange and desalting

Dialysis is frequently mentioned in the literature as a technique to remove salt or other small molecules and to exchange the buffer composition of a sample. However, dialysis is generally a very slow technique, requiring large volumes of buffer. During handling or as a result of proteolytic breakdown or non-specific binding to the dialysis membranes, there is a risk of losing material. A simpler and much faster technique is to use a desalting column, packed with Sephadex G-25, to perform a group separation between high and low molecular weight substances. Proteins are separated from salts and other small molecules.

In a fast, single step, the sample is desalted, transferred into a new buffer and low molecular weight materials are removed.

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

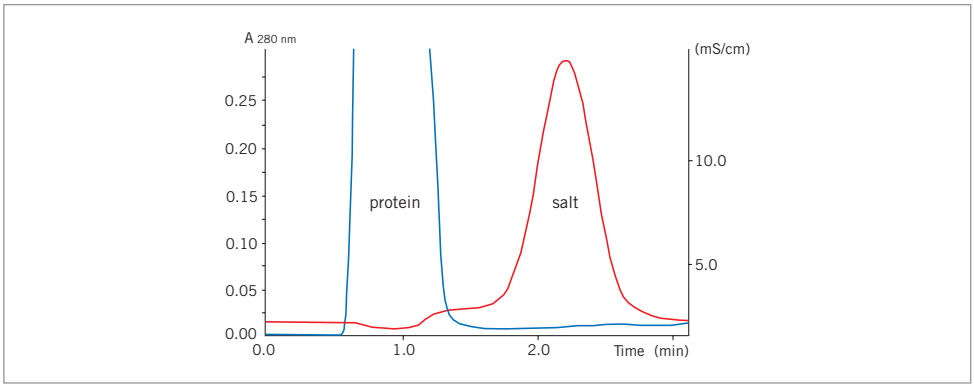


Fig. 76. Buffer exchange of mouse plasma (10 ml) on HiPrep 26/10 Desalting.

For laboratory scale operations, Table 16 shows a selection guide for prepacked, ready to use desalting and buffer exchange columns.

Table 16. Selection guide for desalting and buffer exchange.

Column	Sample volume	Sample elution volume
MicroSpin G-25	0.1–0.15 ml	0.1–0.15 ml
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

To desalt larger sample volumes:

- connect up to 5 HiTrap Desalting 5 ml columns 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.

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

Alternative 1: Manual desalting with HiTrap Desalting 5 ml using a syringe

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 provided).
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 16.

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. A simple peristaltic pump can also be used to apply sample and buffers.

The maximum recommended sample volume is 1.5 ml. See Table 17 for the effect of reducing the sample volume applied to the column.

Table 17. Recommended sample and elution volumes using a syringe or Multipipette™.

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

A simple peristaltic pump can also be used to apply sample and buffers.

Alternative 2: Simple desalting with ÄKTAprime

ÄKTAprime 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 77 shows a typical result obtained from ÄKTAprime. The UV (protein) and conductivity (salt) traces enable pooling of the desalted fractions.

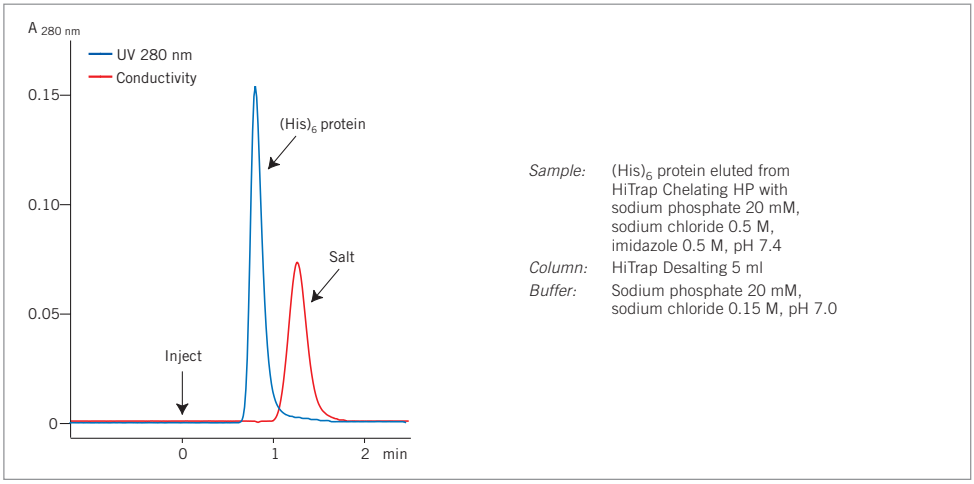


Fig. 77. Desalting of a $(His)_6$ fusion protein on ÄKTAprime.

Removal of lipoproteins

Lipoproteins and other lipid material can rapidly clog chromatography columns and it is advisable to remove them before beginning purification. Precipitation agents such as dextran sulphate and polyvinylpyrrolidine, described under Fractional precipitation, are recommended to remove high levels of lipoproteins from samples such as ascitic fluid.

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.

Removal of phenol red

Phenol red is frequently 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, as described under Buffer exchange and desalting.

Removal of low molecular weight contaminants

If samples contain a high level of low molecular weight contaminants, use a desalting column before the first chromatographic purification step, as described under Buffer exchange and desalting.

Appendix 2

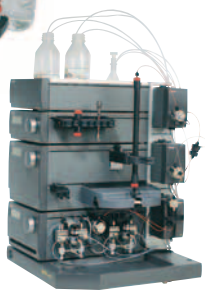
Selection of purification equipment

Many affinity chromatography experiments may be carried out using the simplest methods and equipment, for example step-gradient elution using a syringe together with prepacked HiTrap columns. When more complex elution methods are necessary, large sample volumes are being applied or the same column is to be used for many runs in series, it is wise to use a dedicated system.

	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

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*).

Table 18.

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 4

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 5

Conversion data: proteins, column pressures

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

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

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

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

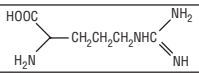
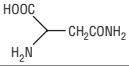
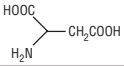
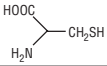
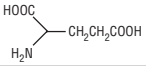
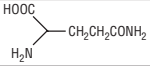
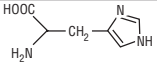
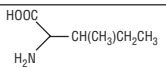
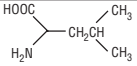
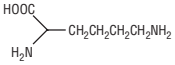
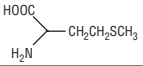
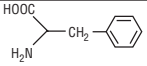
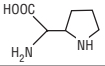
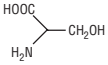
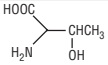
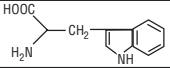
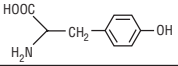
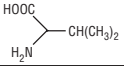
Column pressures

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

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

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	

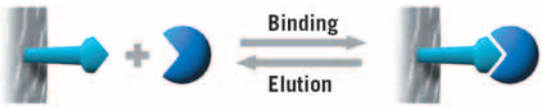
Formula	M_r	Middle unit residue (-H_2O) Formula	M_r	Charge at pH 6.0–7.0	Hydrophobic (non-polar)	Uncharged (polar)	Hydrophilic (polar)
$C_3H_7NO_2$	89.1	C_3H_5NO	71.1	Neutral	■		
$C_6H_{14}N_4O_2$	174.2	$C_6H_{12}N_4O$	156.2	Basic (+ve)			■
$C_4H_8N_2O_3$	132.1	$C_4H_6N_2O_2$	114.1	Neutral		■	
$C_4H_7NO_4$	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

Kinetics in affinity chromatography

The binding (adsorption) and elution (desorption) of a target protein (T) to and from an affinity ligand (L) can be considered in terms of the binding equilibria involved and the kinetics of adsorption and desorption.

Binding equilibria: Non-selective elution by changing K_D



$$L + T \rightleftharpoons LT$$

The standard definition of the equilibrium dissociation constant K_D is shown below. Free ligand is the ligand that is not bound to a target protein and free target is the target which is not bound to a ligand.

At equilibrium

$$K_D = \frac{[L][T]}{[LT]}$$

K_D is the equilibrium dissociation constant
[L] is the concentration of free ligand
[T] is the concentration of free target
[LT] is the concentration of the ligand/target complex

Standard definition of the equilibrium constant.

Graves and Wu in *Methods in Enzymology* **34,** 140–163 (1974) have shown that:

$$\frac{\text{Bound target}}{\text{Total target}} \approx \frac{L_0}{K_D + L_0}$$

K_D is the equilibrium dissociation constant
L_0 is the concentration of ligand, usually 10^{-4} - 10^{-2} M

There are many assumptions and simplifications behind the derivation of this equation, but, although it is not an exact description, it does give a reasonable qualitative description. The ratio of bound to total target should be close to 1 during binding, i.e. almost all the target binds to the ligand. K_D should be small compared to the ligand concentration, i.e. K_D is 10^{-6} - 10^{-4} M when L_0 is 10^{-4} - 10^{-2} M, to achieve efficient binding.

Since K_D can be changed by altering pH, temperature, ionic strength and other parameters, these parameters can be modified to cause elution in affinity chromatography. If the conditions are changed the binding equilibrium changes and to get a reasonable elution the dissociation constant must be increased by quite a large factor (Figure 78).

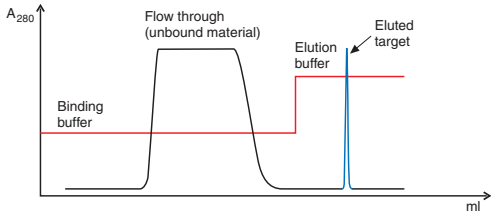
During binding K_D 10^{-6} - 10^{-4} M $\quad L + T \rightleftharpoons LT$

During elution K_D 10^{-1} - 10^{-2} M $\quad LT \rightleftharpoons L + T$

Fig. 78. Changes in binding and elution alter K_D.

Expected results when changing conditions to alter K_D

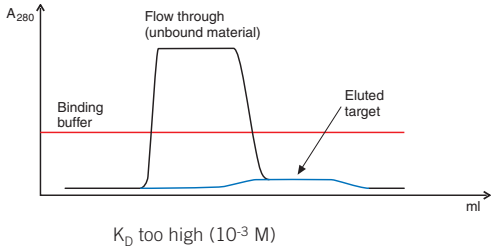
K_D changes when pH, ionic strength or temperature is changed.



Target elutes as a sharp peak.

Unexpected results when changing conditions to alter K_D

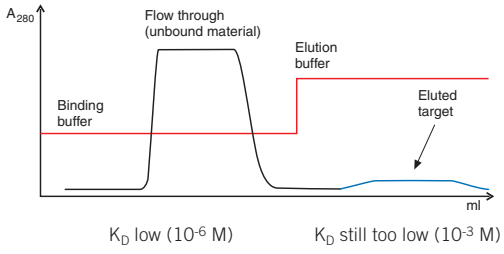
K_D too high during binding.



Target binds as a broad peak and elutes as a broad, low peak while binding buffer is being applied.

- Find better binding conditions to reduce K_D.

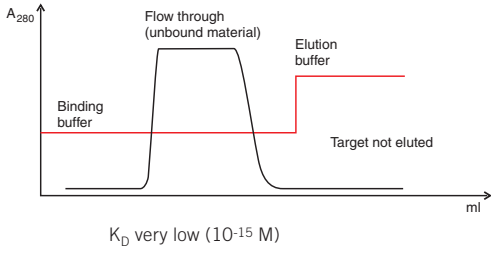
K_D too low during elution.



Target elutes in a long, low peak.

- Try different elution conditions to increase K_D.

K_D too low during binding.

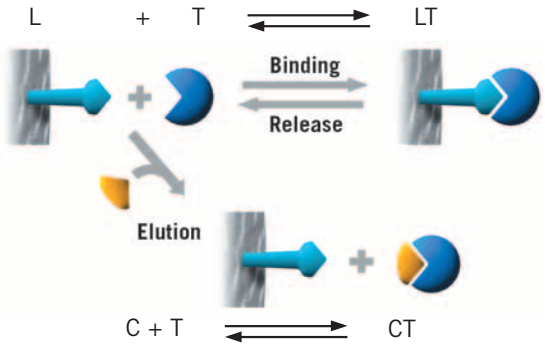


Difficult or impossible to increase K_D enough to elute the target without destroying it.

- Change ligand.

Binding equilibria: Selective elution or competitive elution

The examples shown have related to the changes in K_D caused by non-selective elution techniques for affinity chromatography. However, competitive elution can also be interpreted in terms of changes in the binding equilibrium, as in the illustration below showing elution by adding a competing free ligand. A similar situation applies when adding a competing binding substance.



Binding equilibrium for competing ligand.

At equilibrium

$$K_{DComp} = \frac{[C][T]}{[CT]}$$

K_{DComp} is the equilibrium dissociation constant
[C] is the concentration of free competing ligand
[T] is the concentration of free target
[CT] is the concentration of the competing ligand/target complex

Graves and Wu in *Methods in Enzymology* **34,** 140–163 (1974) have shown that:

$$\frac{\text{Eluted target}}{\text{Total bound target}} \approx \left(\frac{\rho}{\rho + 1}\right)\left[\frac{\rho C_0}{\rho C_0 + \frac{K_{DComp} L_0}{K_D}}\right]$$

ρ is the ratio between the volume of competitor added and the pore volume in the gel, assumed to be in the range 1–10
K_D is the dissociation constant, coupled ligand
K_{DComp} is the dissociation constant, free competing ligand
C_0 is the concentration of competing ligand, usually 10^{-2} - 10^{-1} M
L_0 is the concentration of coupled ligand, usually 10^{-4} - 10^{-2} M

Again the derivation of the equation relies on some assumptions and simplifications and can only be expected to give a qualitative picture of what happens during binding.

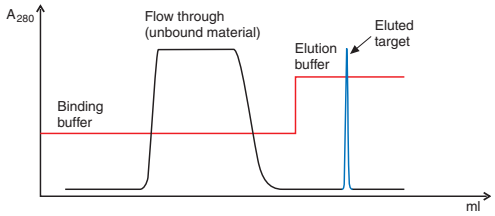
If K_{DComp} and K_D are similar then the concentrations of competing and coupled ligand should be similar to achieve effective elution.

If K_{DComp} is 10 x K_D (i.e. the free competing ligand binds more weakly) then the concentration of competing ligand will need to be 10 x higher to achieve effective elution.

If the competing ligand is not very effective in capturing the target protein at low concentrations so that the target is eluted from the column as a very broad peak, then a higher concentration of the competing ligand will be required to achieve elution. Since competing ligands are often expensive this is not a desirable situation.

Expected results with competitive elution

K_{DComp} too high or C_0 too low.

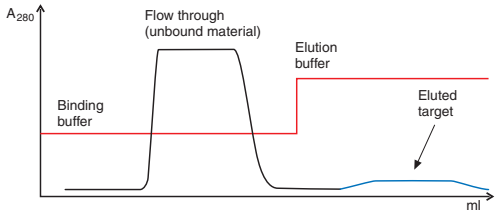


K_D for coupled form not too low (10^{-5} M)

K_{DComp} for soluble competitor not too high (10^{-3} M)

Target elutes as a sharp peak.

Unexpected results with competitive elution



K_D low (10^{-6} M)

K_{DComp} too high or C_0 too low

Target elutes in a long, low peak.

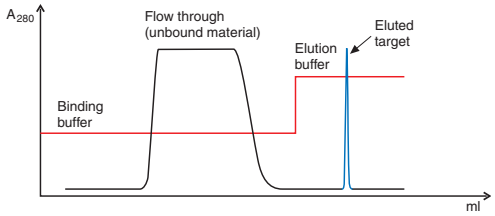
- Increase competitor concentration or use a more effective competitor.

Kinetics of adsorption and desorption

Overall kinetics are influenced by diffusion processes and slow kinetics during adsorption or desorption may create problems during an affinity separation. The effects of diffusion become noticeable for target molecules which are relatively large - they will diffuse more slowly than smaller target molecules thus taking longer to reach ligands in the interior of the gel and so slowing down the whole process.

Desorption is a first order reaction, i.e. the rate is not affected by ligand concentration.

Expected results with fast on/off kinetics

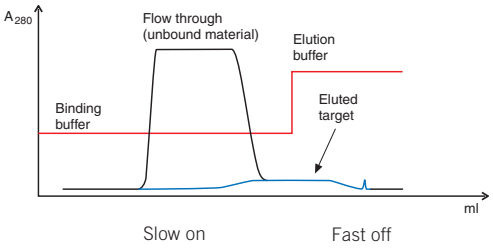


Fast on

Fast off

Target elutes as a sharp peak.

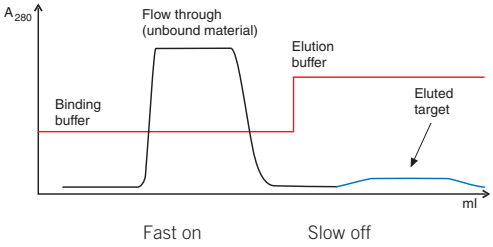
Unexpected results - slow binding (adsorption)



Some of the target elutes under binding conditions as a broad, low peak.

- Apply sample in aliquots to allow time for binding to take place.

Unexpected results - slow elution (desorption)



Target elutes as a long, low peak.

- Change elution scheme.

- Use pulsed elution (see page 22).

Appendix 8

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 (PlusOne Silver Staining Kit, Protein).

The percentage of acrylamide in the SDS-gel should be selected according to the expected molecular weight of the protein of interest (see Table 19).

Table 19.

% Acrylamide in resolving gel		Separation size range
Single percentage:	5%	36 000–200 000
	7.5%	24 000–200 000
	10%	14 000–200 000
	12.5%	14 000–100 000
	15%	14 000–60 000*
Gradient:	5–15%	14 000–200 000
	5–20%	10 000–200 000
	10–20%	10 000–150 000

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

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

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 9

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 133).

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 133).

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.

Product index

Additional reading

	Code No.
Purification	
Antibody Purification Handbook	18-1037-46
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
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
ÄKTA design Brochure	18-1158-77
GST Gene Fusion System Brochure	18-1159-30
Column Packing CD, "The Movie"	18-1165-33
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 GSTrap FF	18-1156-67
Reference list HisTrap Kit	18-1156-68
Reference list HiTrap Chelating HP	18-1156-69
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 Heparin HP	18-1156-75
Reference list HiTrap Blue HP	18-1156-76
Reference list HiTrap NHS-activated	18-1156-77
Reference list HiTrap Desalting	18-1156-70
Reference list HiPrep 26/10 Desalting	18-1156-89

Ordering information

Product	Quantity	Code No.
Prepacked columns		
HiTrap rProtein A FF	2 x 1 ml	17-5079-02
	5 x 1 ml	17-5079-01
	1 x 5 ml	17-5080-01
HiTrap Protein A HP	2 x 1 ml	17-0402-03
	5 x 1 ml	17-0402-01
	1 x 5 ml	17-0403-01
HiTrap Protein G HP	2 x 1 ml	17-0404-03
	5 x 1 ml	17-0404-01
	1 x 5 ml	17-0405-01
HiTrap Blue HP	5 x 1 ml	17-0412-01
	1 x 5 ml	17-0413-01
HiTrap Heparin HP	5 x 1 ml	17-0406-01
	1 x 5 ml	17-0407-01
HiTrap NHS-activated HP	5 x 1 ml	17-0716-01
	1 x 5 ml	17-0717-01
HiTrap Chelating HP	5 x 1 ml	17-0408-01
	1 x 5 ml	17-0409-01
HiTrap Streptavidin HP	5 x 1 ml	17-5112-01
HiTrap IgM Purification HP	5 x 1 ml	17-5110-01
HiTrap IgY Purification HP	1 x 5 ml	17-5111-01
GSTrap FF	2 x 1 ml	17-5130-02
	5 x 1 ml	17-5130-01
	1 x 5 ml	17-5131-01
HiTrap Benzamidine FF (high sub)	2 x 1 ml	17-5143-02
	5 x 1 ml	17-5143-01
	1 x 5 ml	17-5144-01
GSTPrep FF 16/10	1 x 20 ml	17-5234-01
HiPrep 16/10 Heparin FF	1 x 20 ml	17-5189-01
Kits		
MAbTrap Kit	HiTrap Protein G HP (1 x 1 ml), accessories, pre-made buffers for 10 purifications	17-1128-01
HisTrap Kit	3 x 1 ml HiTrap Chelating HP columns, pre-made buffers and accessories for up to 12 purifications	17-1880-01
Media		
Protein A Sepharose CL-4B	1.5 g	17-0780-01
	25 ml	17-0963-03
Protein A Sepharose 4 FF	5 ml	17-0974-01
	25 ml	17-0974-04
rProtein A Sepharose FF	5 ml	17-1279-01
	25 ml	17-1279-02
MabSelect	25 ml	17-5199-01
	200 ml	17-5199-02
Protein G Sepharose 4 FF	5 ml	17-0618-01
	25 ml	17-0618-02
2´5´ ADP Sepharose 4B	5 g	17-0700-01
5´ AMP Sepharose 4B	5 g	17-0620-01
Agarose Wheat Germ Lectin	5 ml	27-3608-02
Arginine Sepharose 4B	25 ml	17-0524-01
Benzamidine Sepharose 4 FF (high sub)	25 ml	17-5123-01

Product	Quantity	Code No.
Blue Sepharose 6 FF	50 ml	17-0948-01
Calmodulin Sepharose 4B	10 ml	17-0529-01
Chelating Sepharose FF	50 ml	17-0575-01
Con A Sepharose 4B	5 ml 100 ml	17-0440-03 17-0440-01
Gelatin Sepharose 4B	25 ml	17-0956-01
Glutathione Sepharose 4 FF	25 ml 100 ml 500 ml	17-5132-01 17-5132-02 17-5132-03
Glutathione Sepharose 4B	10 ml	17-0756-01
Heparin Sepharose 6 FF	50 ml 250 ml	17-0998-01 17-0998-25
IgG Sepharose 6 FF	10 ml	17-0969-01
Lentil Lectin Sepharose 4B	25 ml	17-0444-01
Red Sepharose CL-6B	10 g	17-0528-01
Streptavidin Sepharose HP	5 ml	17-5113-01
Pre-activated media and columns for ligand coupling		
HiTrap NHS-activated HP	5 x 1 ml 1 x 5 ml	17-0716-01 17-0717-01
NHS-activated Sepharose 4 FF	25 ml	17-0906-01
CNBr-activated Sepharose 4 FF	10 g	17-0981-01
CNBr-activated Sepharose 4B	15 g	17-0430-01
Activated CH Sepharose 4B	15 g	17-0490-01
ECH Sepharose 4B	50 ml	17-0571-01
Epoxy-activated Sepharose 6B	15 g	17-0480-01
EAH Sepharose 4B	50 ml	17-0569-01
Activated Thiol Sepharose 4B	15 g	17-0640-01
Thiopropyl Sepharose 6B	15 g	17-0420-01
Immunoprecipitation		
Immunoprecipitation Starter Pack (Protein A Sepharose 4 Fast Flow and Protein G Sepharose 4 Fast Flow)	2 x 2 ml	17-6002-35

See *Antibody Purification Handbook* from Amersham Biosciences for more details on immunoprecipitation.

Amersham Biosciences AB, Björkgatan 30, SE-751 84 Uppsala, Sweden
Amersham Biosciences UK Limited, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England
Amersham Biosciences Corp., 800 Centennial Avenue, PO Box 1327, Piscataway NJ 08855, USA
Amersham Biosciences Europe GmbH, Munzinger Strasse 9, D-79111 Freiburg, Germany
Amersham Biosciences K.K., Sanken Bldg. 3-25-1, Hyakunincho Amersham Shinjuku-ku, Tokyo 169-0073, Japan

Production: RAK Design AB

www.chromatography.amershambiosciences.com

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