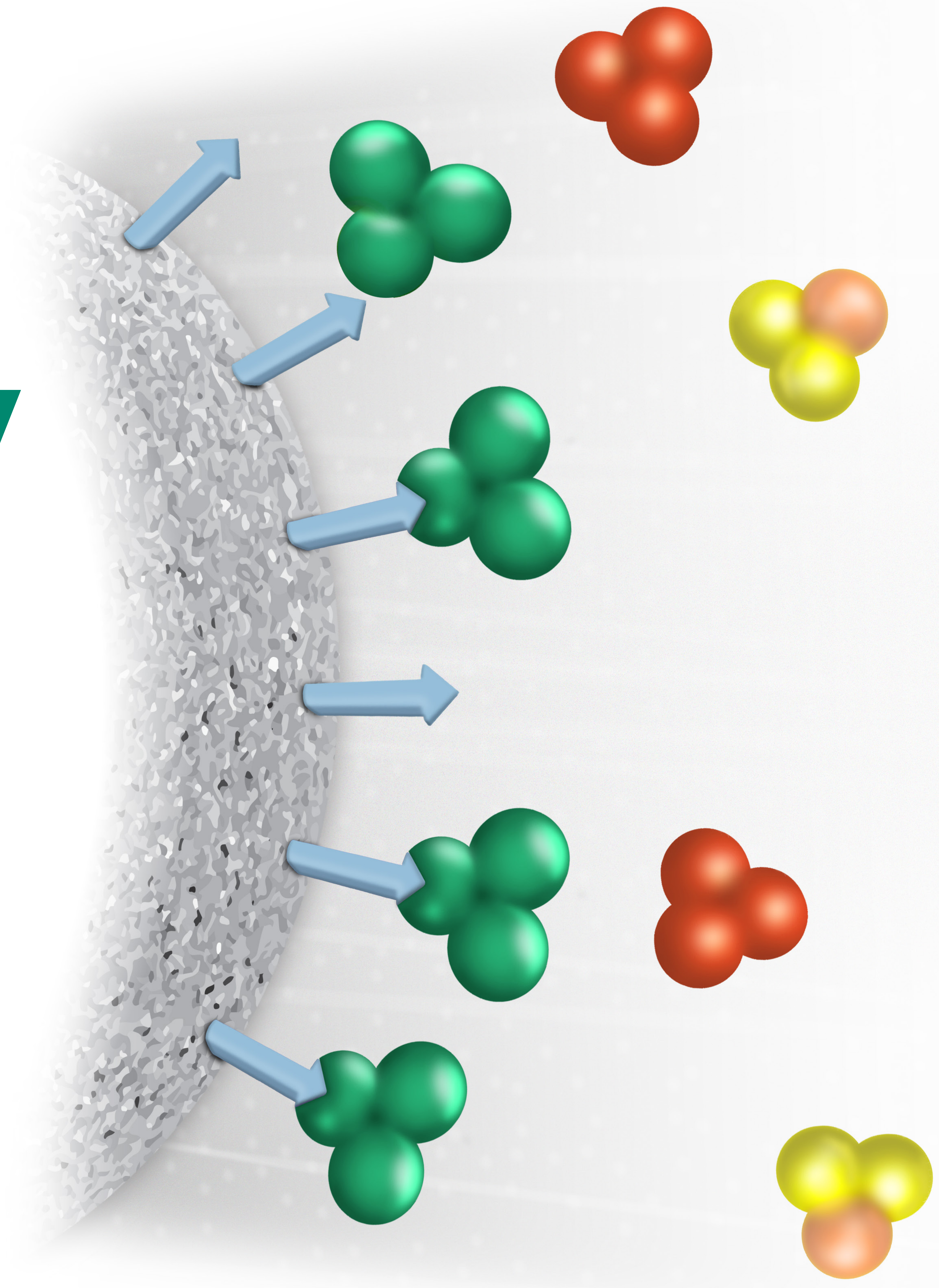
Vol. 3: Specific Groups of Biomolecules

Affinity Chromatography



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Contents

Introduction

Introduction

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

Affinity chromatography (AC) separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix. The technique 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.

AC is the only chromatography 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 AC. 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.

Cytiva's business offers a wide variety of prepacked columns, ready-to-use chromatography media, and preactivated media for ligand coupling.

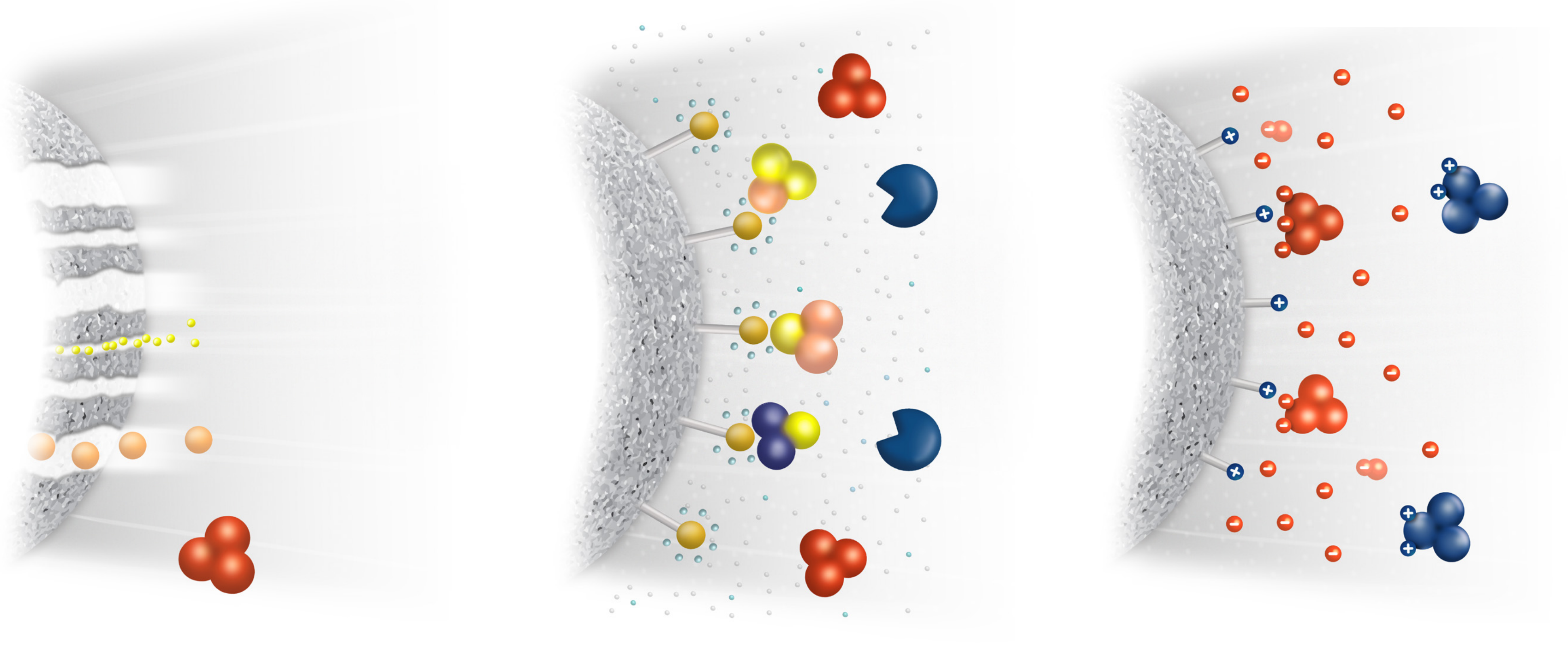
The Affinity Chromatography handbook is divided into three volumes:

Affinity Chromatography, Vol. 1: Antibodies

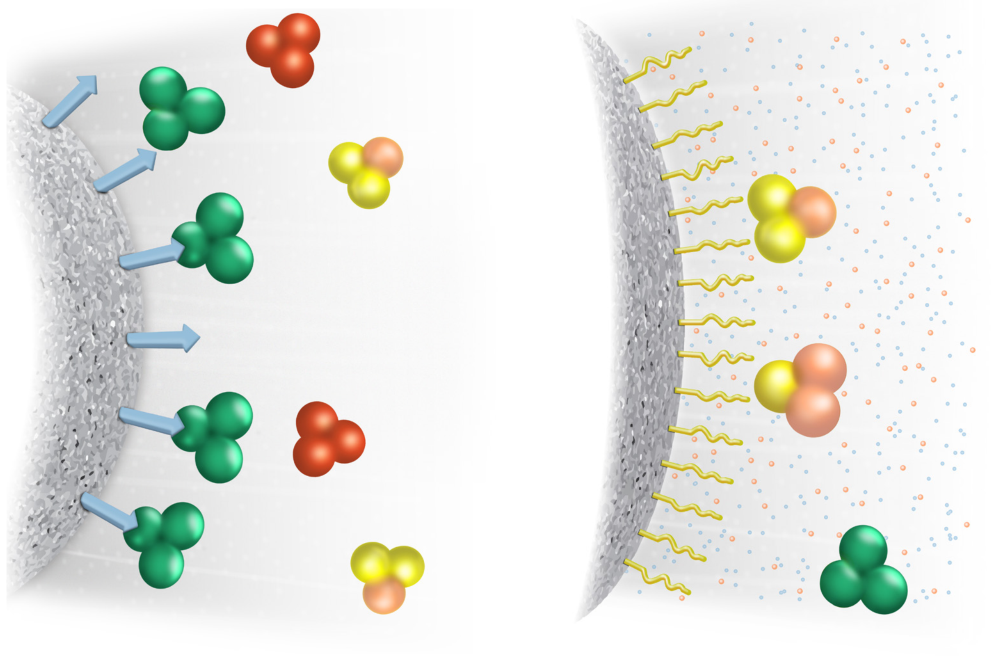
Affinity Chromatography, Vol. 2: Tagged Proteins

Affinity Chromatography, Vol. 3: Specific Groups of Biomolecules

Property	**Technique**
Biorecognition (ligand specificity)	Affinity chromatography (AC)
Charge	Ion exchange chromatography (IEX)
Size	Size exclusion chromatography (SEC), also called gel filtration (GF)
Hydrophobicity	Hydrophobic interaction chromatography (HIC) Reversed phase chromatography (RPC)



Size exclusion   Hydrophobic interaction   Ion exchange



Affinity   Reversed phase

Fig I.1. Separation principles in chromatographic purification.

This handbook describes the role of AC in the purification of specificgroups of biomolecules, the principle of the technique, the chromatography 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 desired results.

Visit cytiva.com/handbooks to see the range of handbooks that have been produced by Cytiva to ensure that purification with any chromatographic technique becomes a simple and efficient procedure at any scale and in any laboratory.

Symbols

☞	This symbol indicates general advice on how to improve procedures or recommends measures to take in specific situations
✋	This symbol indicates where special care should be taken
🟧	Highlights chemicals, buffers, and equipment
🟩	Outline of experimental protocol

Common acronyms and abbreviations

A_{280}	UV absorbance at specified wavelength (in this example, 280 nm)
AC	affinity chromatography
AIEX	anion exchange chromatography
APMSF	4-aminophenyl-methylsulfonyl fluoride
AU	absorbance units
BSA	bovine serum albumin
cGMP	current good manufacturing practice
CF	chromatofocusing
CHO	Chinese hamster ovary
CIEX	cation exchange chromatography
CIP	cleaning-in-place
CIPP	capture, intermediate purification, polishing
CV	column volume
Dab	domain antibody, the smallest functional entity of an antibody
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DOC	deoxycholate
DoE	design of experiments
DS	desalting (group separation by size exclusion chromatography; buffer exchange)
EDAC	1-ethyl-(3-dimethylaminopropyl)carboiimide
EDTA	ethylene diaminetetraacetic acid
EGTA	ethylene glycol-O, O'-bis-[2-amino-ethyl]-N,N,N',N',-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
$F(ab')_2$ fragment	fragment with two antigen binding sites, obtained by pepsin digestion
Fab fragment	antigen binding fragment obtained by papain digestion
Fc fragment	crystallizable fragment obtained by papain digestion
Fv fragment	unstable fragment containing the antigen binding domain
GF	gel filtration; also called size exclusion chromatography
GST	glutathione S-transferase
HCP	host cell protein
HIC	hydrophobic interaction chromatography
HMW	high molecular weight
HSA	human serum albumin
IEF	isoelectric focusing
IEX	ion exchange chromatography
IgA, IgG, etc.	different classes of immunoglobulin
IMAC	immobilized metal ion affinity chromatography
LC-MS	liquid chromatography–mass spectrometry

LMW	low molecular weight
MAb	monoclonal antibody
MALDI-ToF	Matrix-assisted laser desorption/ionization time-of-flight
mo	month
MPa	megaPascal
M_r	relative molecular weight
MS	mass spectrometry
n	native, as in nProtein A
NC	nitrocellulose
NHS	N-hydroxysuccinimide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
pI	isoelectric point, the pH at which a protein has zero net surface charge
PMSF	phenylmethylsulfonyl fluoride
psi	pounds per square inch
PVDF	polyvinylidene fluoride
PVP	polyvinylpyrrolidine
r	recombinant, as in rProtein A
RNA	ribonucleic acid
RNAse	ribonuclease
RPC	reversed phase chromatography
scFv	single chain Fv fragment
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
TCEP	tris(2-carboxyethyl) phosphine hydrochloride
TFA	Trifluoroacetic acid
Tris	tris-(hydroxymethyl)-aminomethane
UV	ultraviolet
v/v	volume to volume
w	week
w/v	weight to volume

01

Principles of affinity chromatography

Principles of affinity chromatography

Affinity chromatography (AC) separates biomolecules on the basis of a reversible interaction between a biomolecule (or group of biomolecules) and a specific ligand coupled to a chromatography matrix. Figure 1.1 shows the key stages in an affinity purification. The technique is an excellent choice for a capture or intermediate step in a purification protocol and can be used whenever a suitable ligand is available for the target molecule(s) of interest. With high selectivity, hence high resolution, and high capacity, purification levels in the order of several thousand-fold with high recovery of active material are achievable. Target biomolecule(s) is collected in a purified, concentrated form.

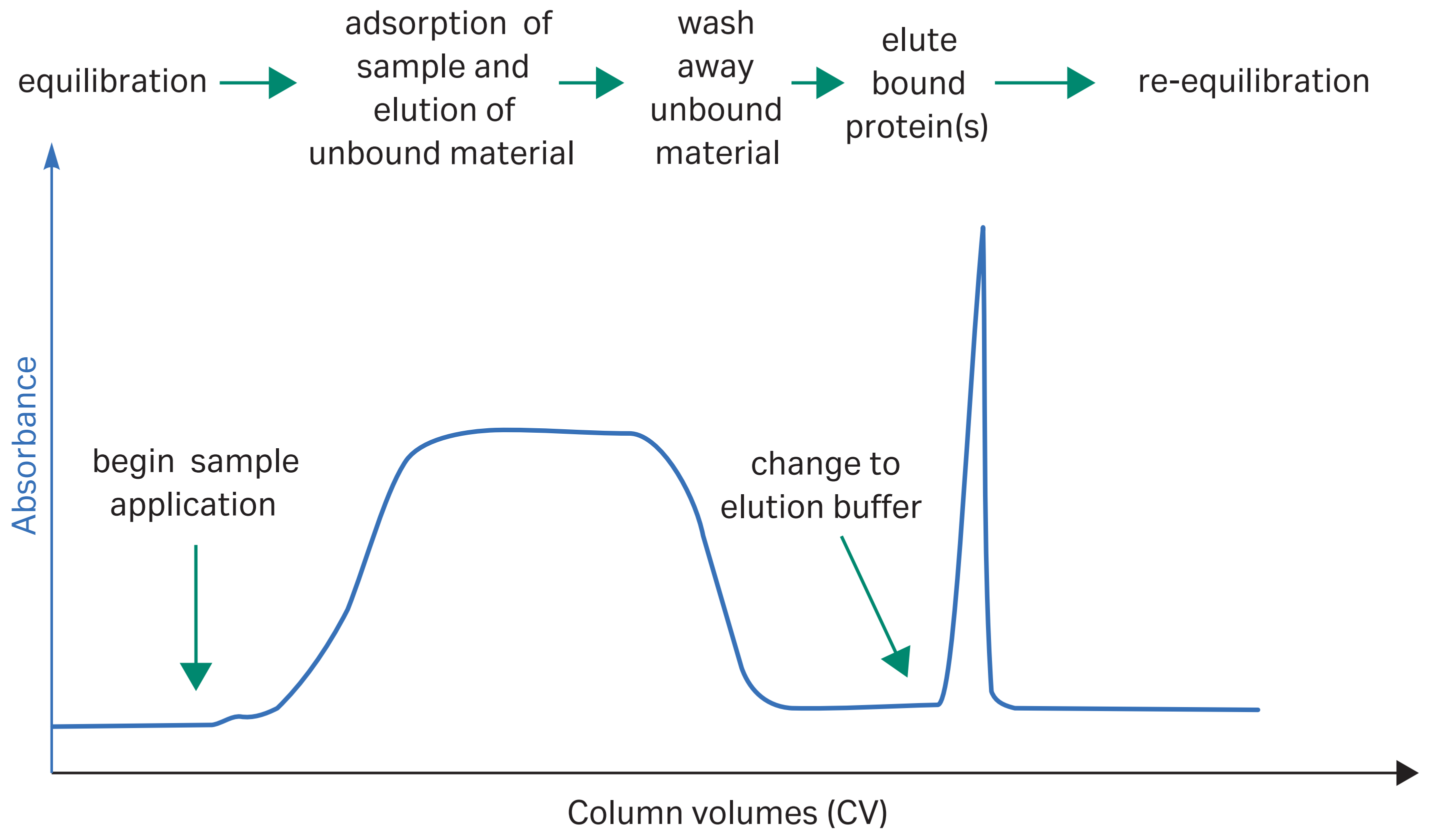
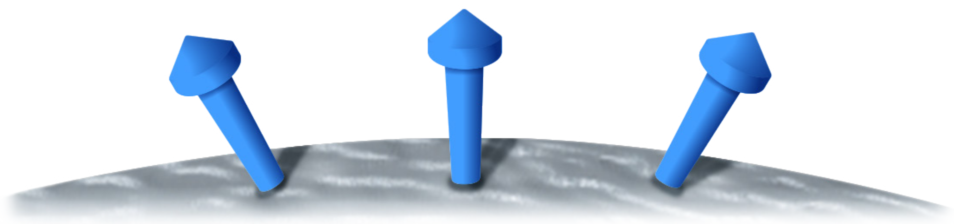
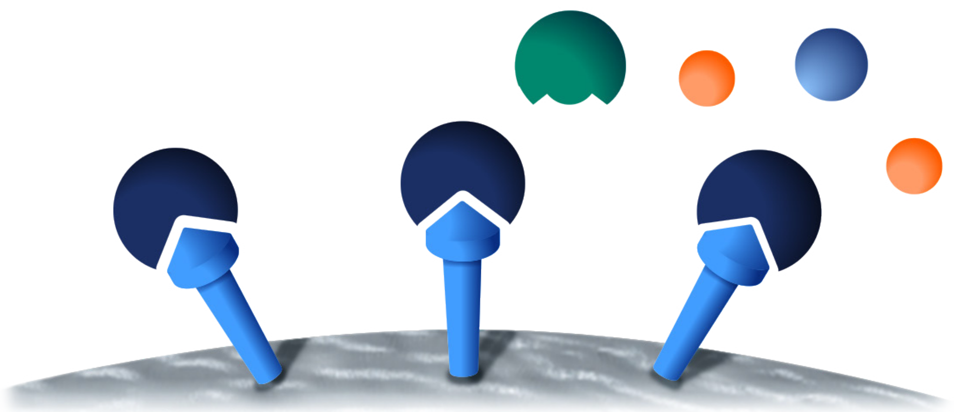


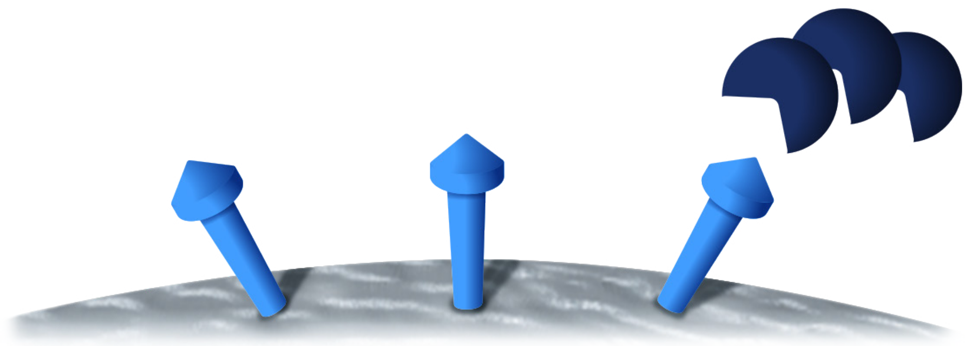
Fig 1.1. Principles of affinity purification.



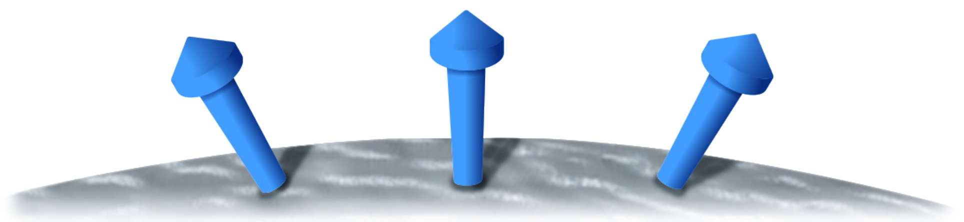
1. Equilibration
AC medium is equilibrated in binding buffer.



2. Sample application and wash
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. Elution
Target protein is recovered by changing conditions to favor elution of the bound molecules. Target protein is collected in a purified, concentrated form.



4. Re-equilibration
AC medium is re-equilibrated with binding buffer.

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 AC medium, the interaction can be reversed, either specifically using a competitive ligand, or nonspecifically, by changing the pH, ionic strength, or polarity.

In a single step, affinity purification can offer immense time-saving over less selective multistep 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.

Any component can be used as a ligand to purify its respective binding partner. Some typical biological interactions, frequently used in AC, are listed below:

- Antibody ⟷ antigen, virus, cell (see the handbook *Affinity Chromatography, Vol. 1: Antibodies*, 18103746)
- Metal ions ⟷ Histidine- (his)-tagged proteins (see the handbook *Affinity Chromatography, Vol. 2: Tagged Proteins*, 18114275)
- Glutathione ⟷ glutathione-S-transferase or GST-tagged proteins (see *Affinity Chromatography, Vol. 2: Tagged Proteins*)
- Enzyme ⟷ substrate analog, inhibitor, cofactor (this handbook)
- Lectin ⟷ polysaccharide, glycoprotein (this handbook)

AC is also used to remove specific contaminants, for example Benzamidine Sepharose™ 6 Fast Flow can remove serine proteases, such as thrombin and Factor Xa.

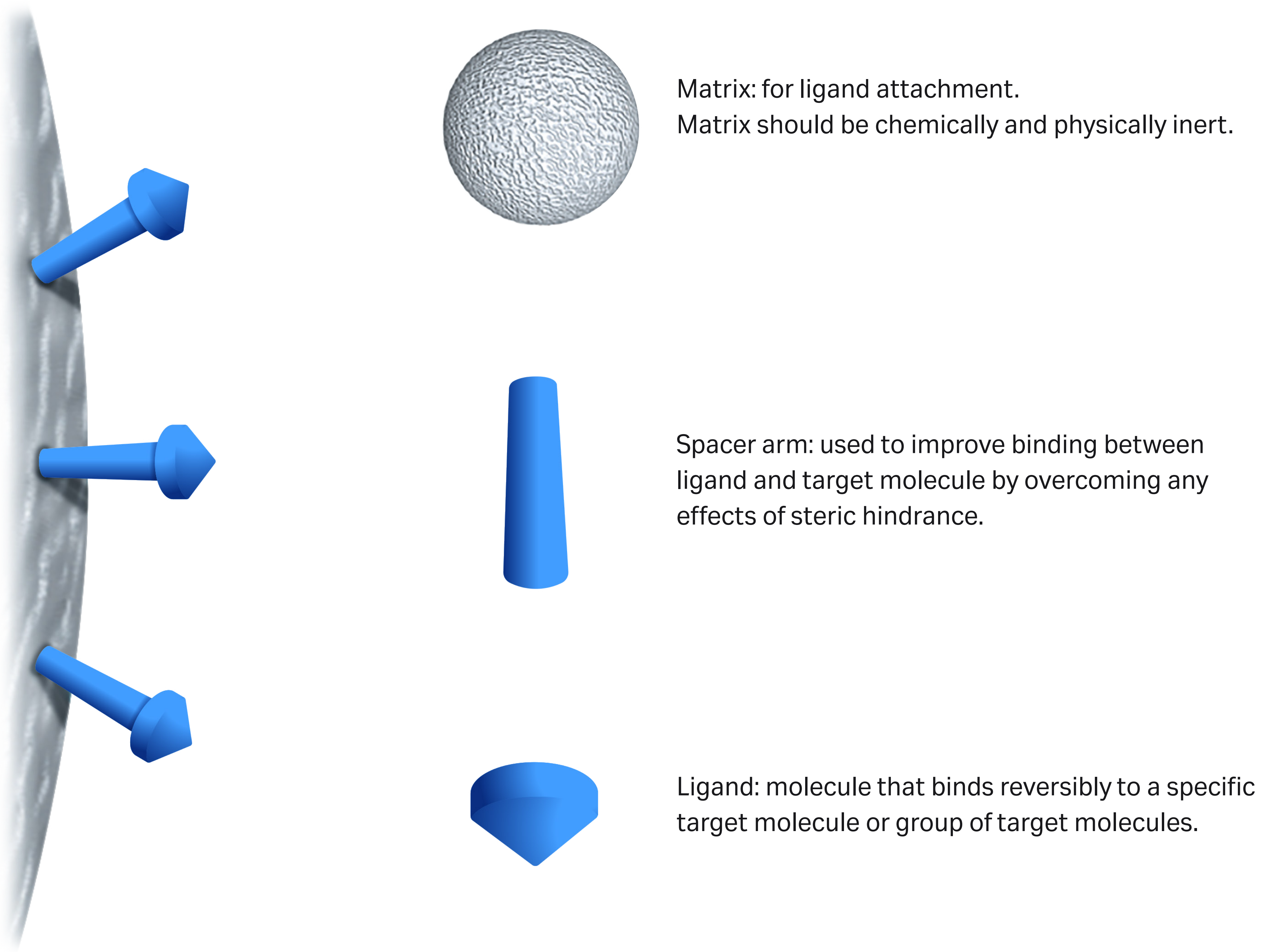
Components of an affinity chromatography medium

Matrix

The matrix is an inert support to which a ligand can be directly or indirectly coupled (Fig 1.2). The list below highlights many of the properties required for an efficient and effective chromatography matrix.

- Extremely low nonspecific adsorption, essential since AC relies on specific interactions
- Easily derivatized groups for covalent attachment of a ligand
- An open pore structure to ensure 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 (Fig 1.3), provides many of these properties.



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.

Fig 1.2. The three components of an AC medium.

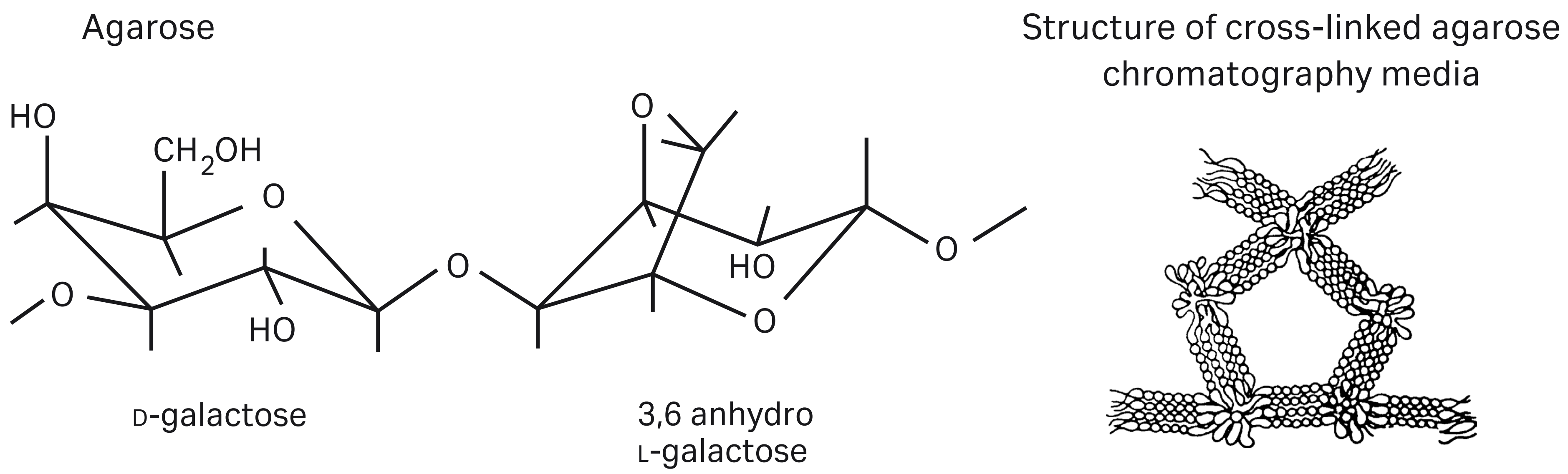


Fig 1.3. Partial structure of agarose chromatography media (Sepharose).

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 1.1). A more rigid agarose matrix is used for Capto™ chromatography media. The average particle sizes of Capto matrices used for AC media are either 75 or 90 µm.

In AC, the particle size and porosity are designed to maximize the surface area available for coupling a ligand and binding the target molecule. A small average 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.

Ligand

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

The selection of the ligand for AC 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. If the dissociation constant is outside the useful range, changing elution methods can help to promote successful AC.

If no information on the strength of the binding complex is available, a trial-and-error approach should be used.

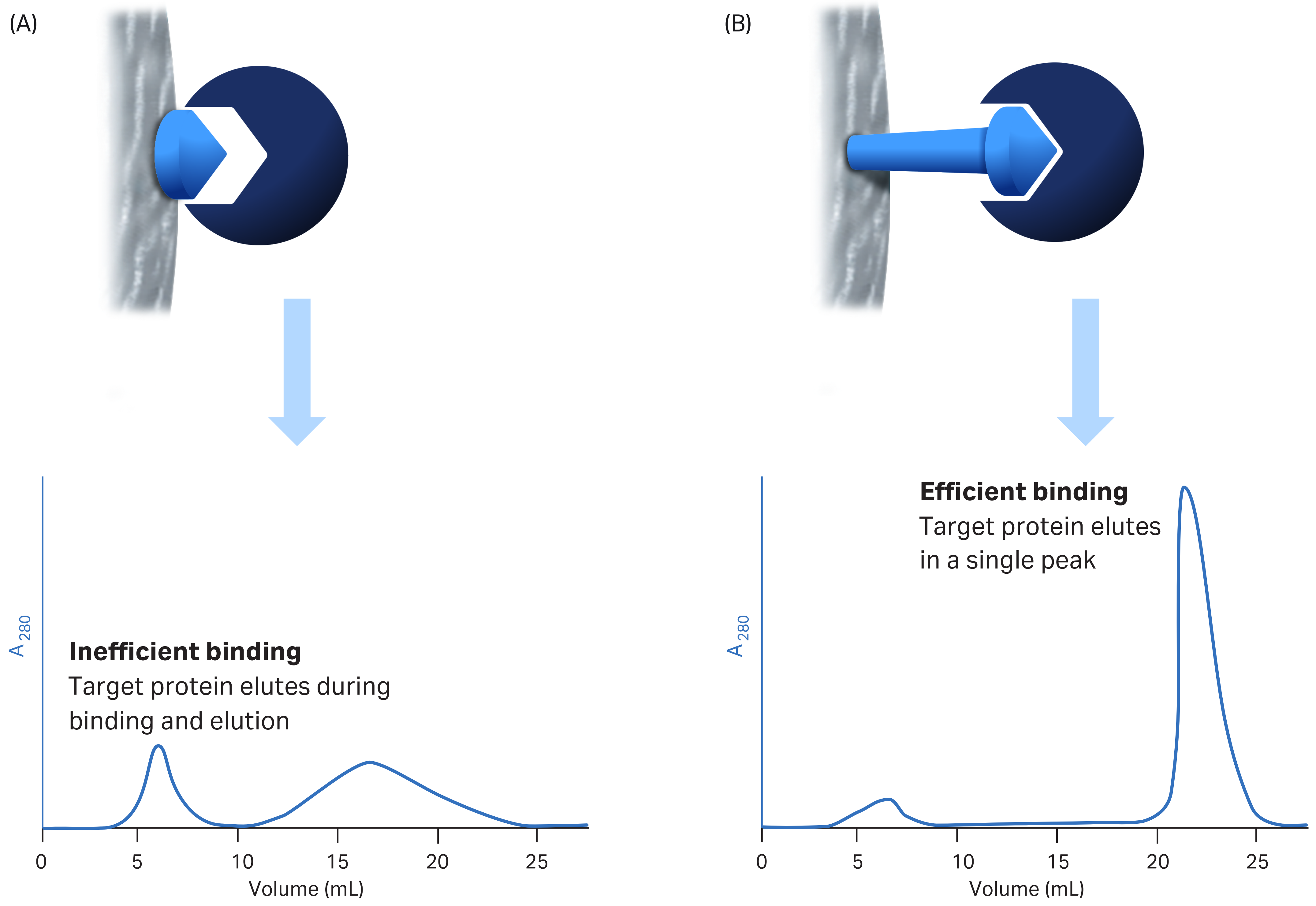
☞ For purification of specific molecules or groups of molecules, many ligands are available coupled to an appropriate matrix (see Chapter 3). Ligands can also be isolated and purified to prepare a specific AC medium for a specific target molecule. Coupling of ligands to preactivated matrices is described in Chapter 4.

Table 1.1. Sepharose and Capto matrices used with Cytiva affinity chromatography media.

Chromatography medium	Base matrix	Average particle size (µm)
Sepharose High Performance	6% highly cross-linked agarose	34
Sepharose 6 Fast Flow	6% highly cross-linked agarose	90
Sepharose 4 Fast Flow	4% highly cross-linked agarose	90
Sepharose 6B	6% agarose	90
Sepharose 4B	4% agarose	90
Capto	Highly cross-linked high-flow agarose	75 and 90

Spacer arms

The binding site of a target protein is often located deep within the molecule and an AC 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 1.4 (A). 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 nonspecific binding effects. Figure 1.4 (B) shows the improvement that can be seen in a purification as the spacer arm creates a more effective environment for binding.



Inefficient binding
Target protein elutes during binding and elution

Efficient binding
Target protein elutes in a single peak

Fig 1.4. The effect of spacer arms. (A) Ligand attached directly to the matrix. (B) Ligand attached to the matrix via a spacer arm.

02

Affinity chromatography in practice

Affinity chromatography in practice

This chapter provides guidance and advice that is generally applicable to any AC purification. The first steps towards a successful purification starts with a number of selections aiming for the most suitable chromatography medium, format, equipment, and purification method (Fig 2.1). The choices depend on factors such as the purpose of the purification, the purification scale, and the required purity and yield.

Selection of chromatography media

A suitable AC medium has a ligand which interacts reversibly with the target molecule or group of molecules. Media with ligands for purification of, for example, enzymes, coagulation factors, and proteases, are described in Chapters 3 and 5. The media can be used immediately for purification without any prepreparation, simply following the supplied purification protocol.

Preactivated chromatography media are useful when no ready to use media are available for the purpose. This requires a specific biomolecule (often an antibody) directed towards the target protein. The specific biomolecule is used as a ligand and covalently coupled to the preactivated media. The media can then be used for affinity purification of the target protein (see Chapters 4 and 5).

In addition to the ligand, the matrix of the chromatography medium affects the purification (see Chapter 1). The most suitable matrix can be selected according to the degree of resolution, binding capacity, and the scale desired for the separation. For example, performing gradient elution on Sepharose High Performance (34 µm) will result in high-resolution separations. Media with larger particles such as Sepharose Fast Flow and Capto have better pressure/flow properties and are suitable for small-scale purification as well as for scaling up.

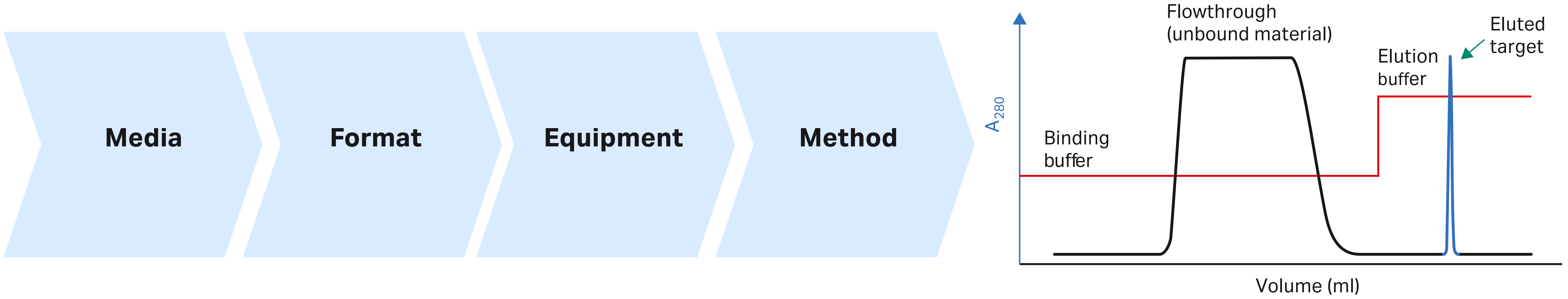


Fig 2.1. Successful AC purification requires making the right initial choices.

Selection of format

A number of prepacked formats are available from Cytiva to facilitate and speed up the affinity purification. Prepacked HiTrap™ (1 and 5 mL media, bed height 2.5 cm) and HiScreen™ (4.7 mL medium, bed height 10 cm) columns provide flexibility as they can be operated using a syringe, pump, or chromatography system. The columns are useful for fast method development before scaling up as well as for small-scale purification. The prepacked HiPrep™ column (20 mL) is suitable for preparative purification, and chromatography media can also be packed in XK, Tricorn™, or HiScale™ columns for larger scale purification.

Figure 2.2 shows the simple procedure to perform a typical affinity purification using prepacked HiTrap columns. The different method steps are discussed more in detail later in this chapter.

In addition, some AC media are available in other formats, such as small-scale SpinTrap™ columns and MultiTrap™ 96-well plates (Fig 2.3). Prepacked SpinTrap columns are used together with a microcentrifuge and can be an alternative to screening in MultiTrap 96-well plate format when fewer samples are to be screened.

Purification can also be performed in batch mode, where the loose chromatography medium is used directly in a container or test tube together with buffers and sample. This allows for increased binding time, for example, the sample can be incubated with the chromatography medium overnight. After binding, the chromatography medium can be poured into an empty gravity-flow column before wash and elution.

Avoid using magnetic stirrers when the medium is used in batch mode as they can damage the chromatography beads. Use mild rotation or end-over-end stirring.

Another example of batch mode purification is using magnetic beads in combination with a magnetic device. This approach is discussed in detail in Chapter 5.

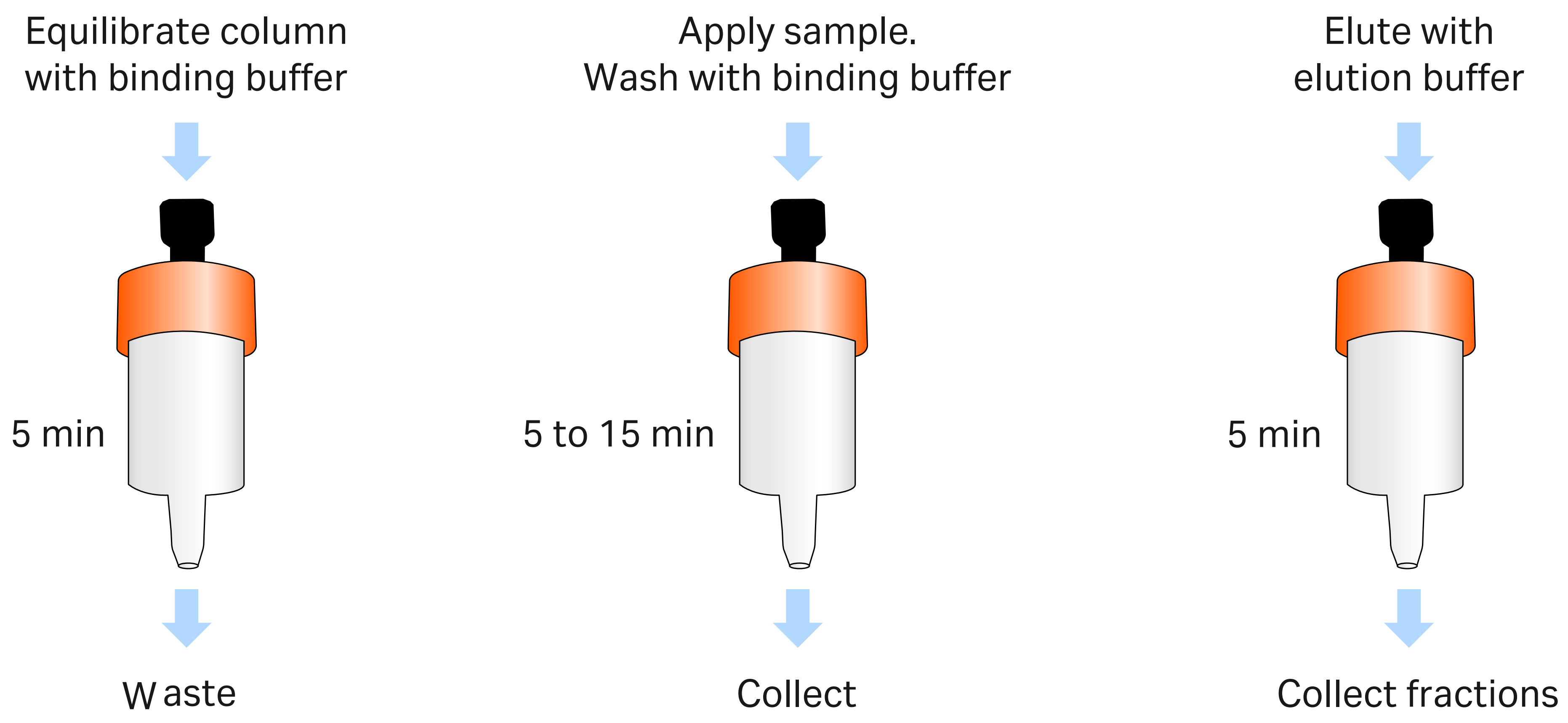


Fig 2.2. The purification procedure consists of equilibration, sample application, wash, and elution.

Fig 2.3. Examples of different prepacked formats. MultiTrap 96-well plates, SpinTrap columns, and HiTrap columns are designed for fast and convenient screening and small-scale AC purification.

Selection of equipment

The selection of equipment depends on the purpose of the purification. A simple stepwise purification can for example be performed using a HiTrap column and a syringe for the buffers and sample. More advanced methods require a chromatography system; Appendix 2 provides a guide for the selection of ÄKTA™ chromatography systems.

Selection of purification method

AC media are supplied with purification methods for the specific media. These methods are often sufficient for a successful purification, but in some cases additional optimization of the method might be required. A purification method consists of several different steps: equilibration, sample application, wash, elution and re-equilibration (see Fig 1.1, Chapter 1). As each step has an impact on the final results, the different steps are described in detail below.

Preparation of sample and buffers

Adjust the sample to the composition and pH of the binding buffer. This will promote efficient binding and can be done by performing a buffer exchange with a desalting column or simply by dilution in the binding buffer. Samples should also be clear and free from particulate matter in order to avoid clogging the column and reduce the need for stringent washing procedures. Appendix 1 contains an overview of sample preparation techniques.

Binding and elution buffers are specific for each AC medium since their influence on the interaction between the target molecule and the ligand affects the affinity-based separation. The instructions supplied with the AC medium contain suggested binding and elution buffers.

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

Flow rates

The optimal flow rate in AC depends on the dissociation rates of ligand/target molecule interactions and varies widely. For ready-to-use AC media, follow the supplied instructions and, if required, optimize:

- the flow rate to achieve efficient binding
- the flow rate for elution to maximize recovery

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

Equilibration

Equilibration of the AC medium with binding buffer is necessary since any remaining storage solution might disturb the binding of the target protein. Wash away the storage solution thoroughly according to the instructions. If the medium is supplied as a freeze-dried powder, reswell the medium in the correct buffer according to the instructions.

Sample application and wash

The column must be equilibrated in binding buffer before beginning sample application. The sample volume is not critical and does not affect the separation since AC is a binding technique. For interactions with weak affinity and/or slow equilibrium, a lower flow rate might be required; alternatively the purification can be performed in batch mode with increased time for binding.

Wash the column/medium thoroughly after sample application until all unbound material has been washed away, as determined by UV absorbance at 280 nm. This will improve the purity of the eluted target protein.

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

Elution

AC media from Cytiva are supplied with recommendations for the most suitable elution buffer to reverse the interaction between the ligand and target protein. Elution methods may be either selective or nonselective, as shown in Figure 2.4.

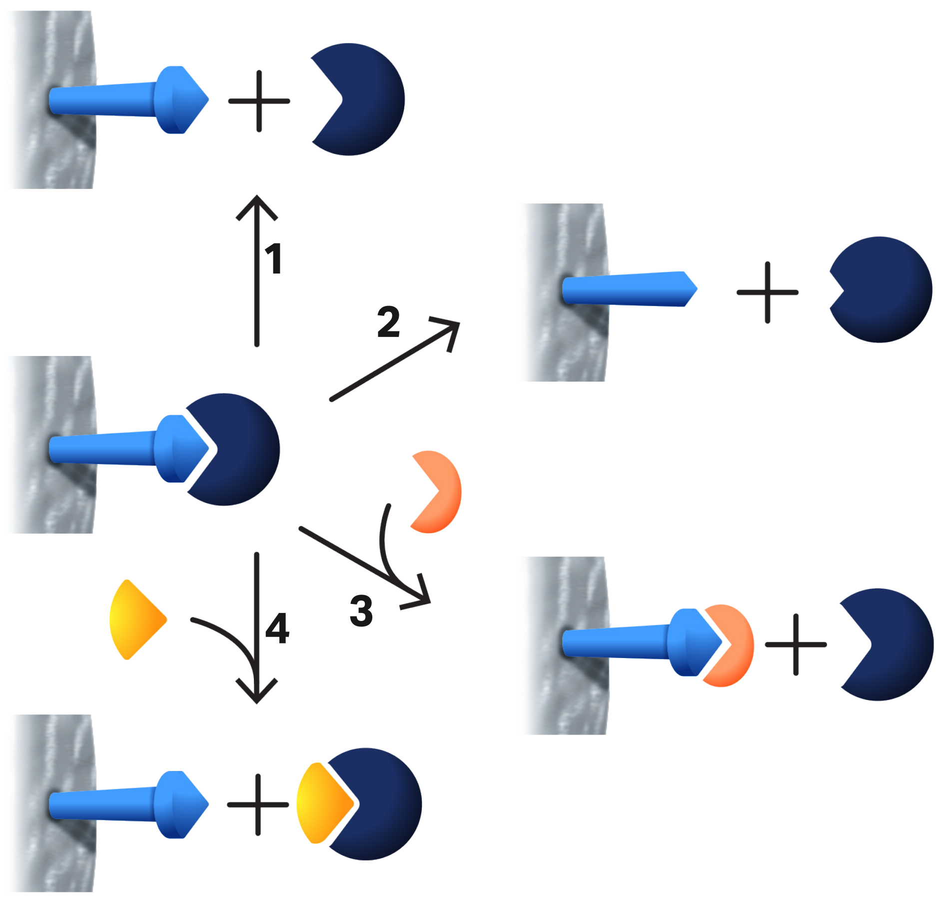
Ionic-strength elution

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.

pH elution

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

If low pH must be used, collect fractions into neutralization buffer such as 1 M Tris-HCl, pH 9.0 (60 to 200 µL/mL eluted fraction) to return the fraction to a neutral pH. The column should also be re-equilibrated to neutral pH immediately.



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 can 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 2.4. Elution methods in AC.

Competitive elution

Selective eluents are often used to separate substances on a group-specific chromatography 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 gradient or step elution (see below).

For elution, it is common to use a concentration 10-fold higher than that of the ligand.

Other elution methods

Substances that reduce the polarity of the buffer can facilitate elution without affecting protein activity, such as dioxane (up to 10%) and ethylene glycol (up to 50%).

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

☞ When substances are very tightly bound to the AC medium, it can 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.

Gradient and step elution

The figures below illustrate the principle of separations in which proteins are eluted using step elution or linear gradient elution (Fig 2.5).

Step elution can be used for less complex samples or after optimizing using gradient elution. Changing to a step elution speeds up separation time and reduces buffer consumption. Step elution can also be used for group separation in order to concentrate the proteins of interest and rapidly remove them from unwanted substances.

Gradient elution is often used when starting from an unknown sample (the components are bound to the column and eluted differentially to give a total protein profile) and for development of a purification method. The position of the eluted peaks can give information about the optimal binding and elution conditions to be used in step elution.
A chromatography system is essential when gradient elution is performed.

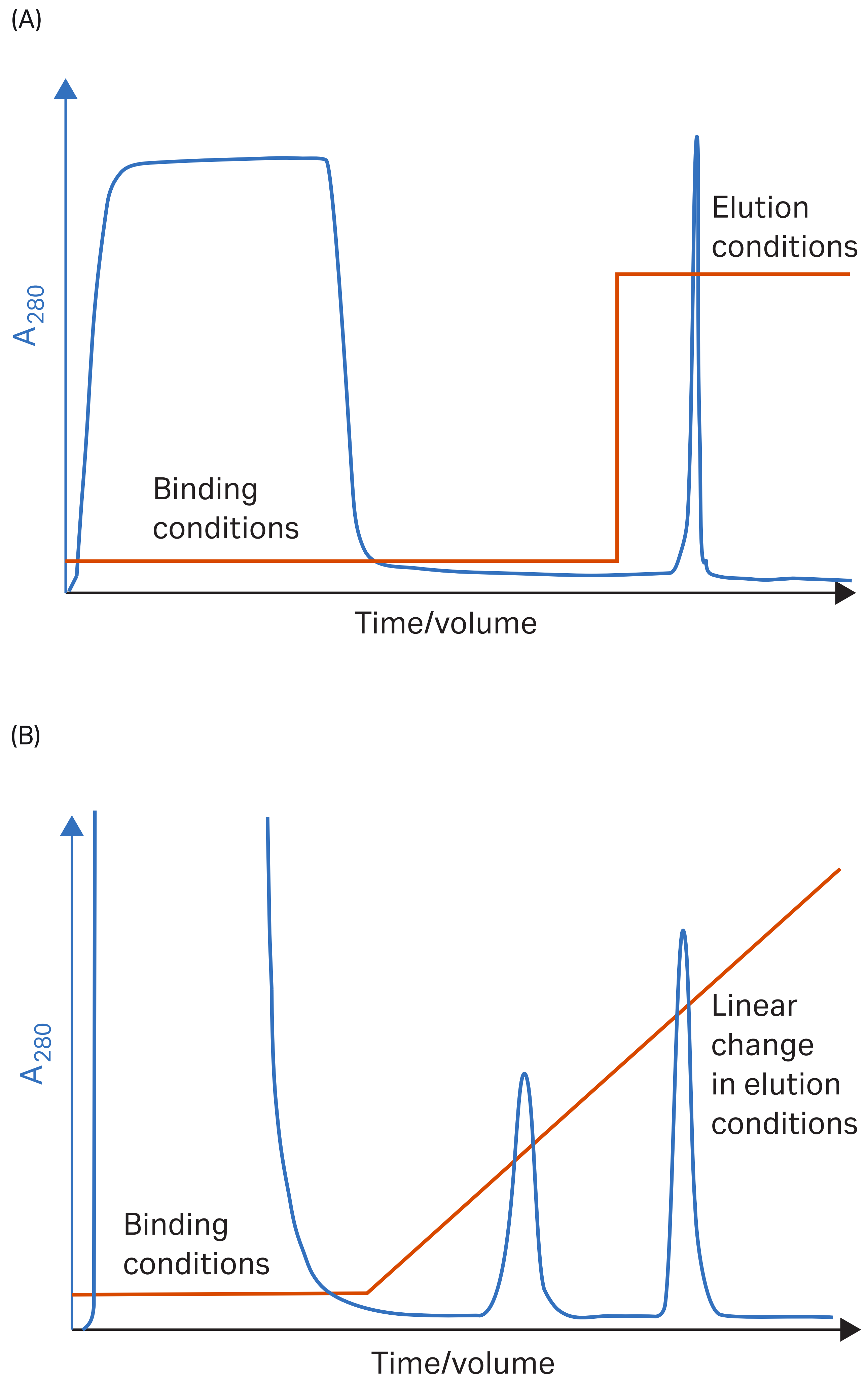


Fig 2.5. Typical conditions for (A) step and (B) gradient elution in AC.

Re-equilibration

After elution, the AC medium needs to be re-equilibrated before the next purification run. Depending on sample, it might also be necessary to perform additional cleaning, for example if pressure has increased or if color change is noted.

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

✋ If an AC medium is to be reused routinely, care must be taken to ensure that any contaminants from the applied sample can be removed by procedures that do not damage the ligand.

Analysis of results and further steps

The analysis of the eluted sample can indicate if the purification method needs to be optimized to increase the yield or achieve higher purity. Commonly used assays are outlined in Appendix 7.

AC offers high selectivity and is often the first and sometimes the only step required. The target molecule is concentrated into a small volume and purity levels are often above 95%. However, to achieve satisfactory sample homogeneity, a further polishing step, often size exclusion chromatography (SEC), might be required to remove any aggregates. SEC is 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, see the *Size Exclusion Chromatography Handbook*, 18102218 from Cytiva. 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 Appendix 1).

Troubleshooting

This section focuses on practical problems that can occur when running an AC column.

Situation	Cause	Remedy
Poor binding of the protein	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 *Buffer exchange and desalting* in Appendix 1)
	Solutions have wrong pH	Calibrate pH meter, prepare new solutions
	The column is not equilibrated sufficiently in the buffer	Repeat or prolong the equilibration step
	Column is overloaded with sample	Decrease the sample load
	Microbial growth has occurred in the column	Store in 20% ethanol when possible
Low yield	Protein is still attached to ligand	If using competitive elution, increase the concentration of the competitor in the elution buffer
	Protein has been degraded by proteases	Add protease inhibitors to the sample and buffers to prevent proteolytic digestion. Run sample through a medium such as Benzamidine Sepharose 4 Fast Flow (high sub) to remove serine proteases.
	Adsorption to filter during sample preparation	Use another type of filter
	Sample precipitates	Can 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
Low recovery of activity, but normal recovery of protein	Protein is 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 9.0 (60 to 200 µL per fraction) if elution is performed at low pH
	Enzyme separated from cofactor or similar	Test by pooling aliquots from the fractions and repeating the assay
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

Situation	Cause	Remedy
Reduced or poor flow through the column and/or too high back pressure	Presence of lipoproteins or protein aggregates	Remove lipoproteins and aggregates 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, adapter, or tubing	Remove and clean or use a new column
	Precipitated proteins	Clean the column using recommended methods or use a new column
	Bed is too compressed	Repack the column, if possible, or use a new column
	Microbial growth	Store in 20% ethanol when possible
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 degassed 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 in direct sunlight or by placement in close proximity to heating system. Repack column if possible (see Appendix 3).
	Buffers not properly degassed	Degas buffers thoroughly
Cracks in the bed	Large air leak in column	Check all connections for leaks. Repack the column if possible (see Appendix 3).

03

Purification of specific groups of molecules

Purification of specific groups of molecules

This chapter describes the affinity chromatography media and prepacked formats available from Cytiva for purification of specific groups of molecules, such as glycoproteins and coagulation factors. Advice on handling of the different formats is provided and purification protocols for each format are described. For purification of antibodies and tagged proteins, see the handbooks *Affinity Chromatography, Vol. 1: Antibodies*, 18103746 and *Vol. 2: Tagged Proteins*, 18114275, respectively. A group-specific AC 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 AC medium derives from the selectivity of the ligand and the use of selective elution conditions.

AC media can be used either for purification or removal of the target substance. In the case of removal, the depleted sample is collected during sample application and wash.

Purification or removal of albumin

Blue Sepharose High Performance, Blue Sepharose 6 Fast Flow, Capto Blue, Capto Blue (high sub)

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 AC media (Fig 3.1).

Capto Blue and Capto Blue (high sub) have a more rigid agarose base matrix compared with Blue Sepharose 6 Fast Flow, which results in improved pressure/flow properties, optimized pore structure, and high chemical stability to support cleaning-in-place (CIP) procedures.

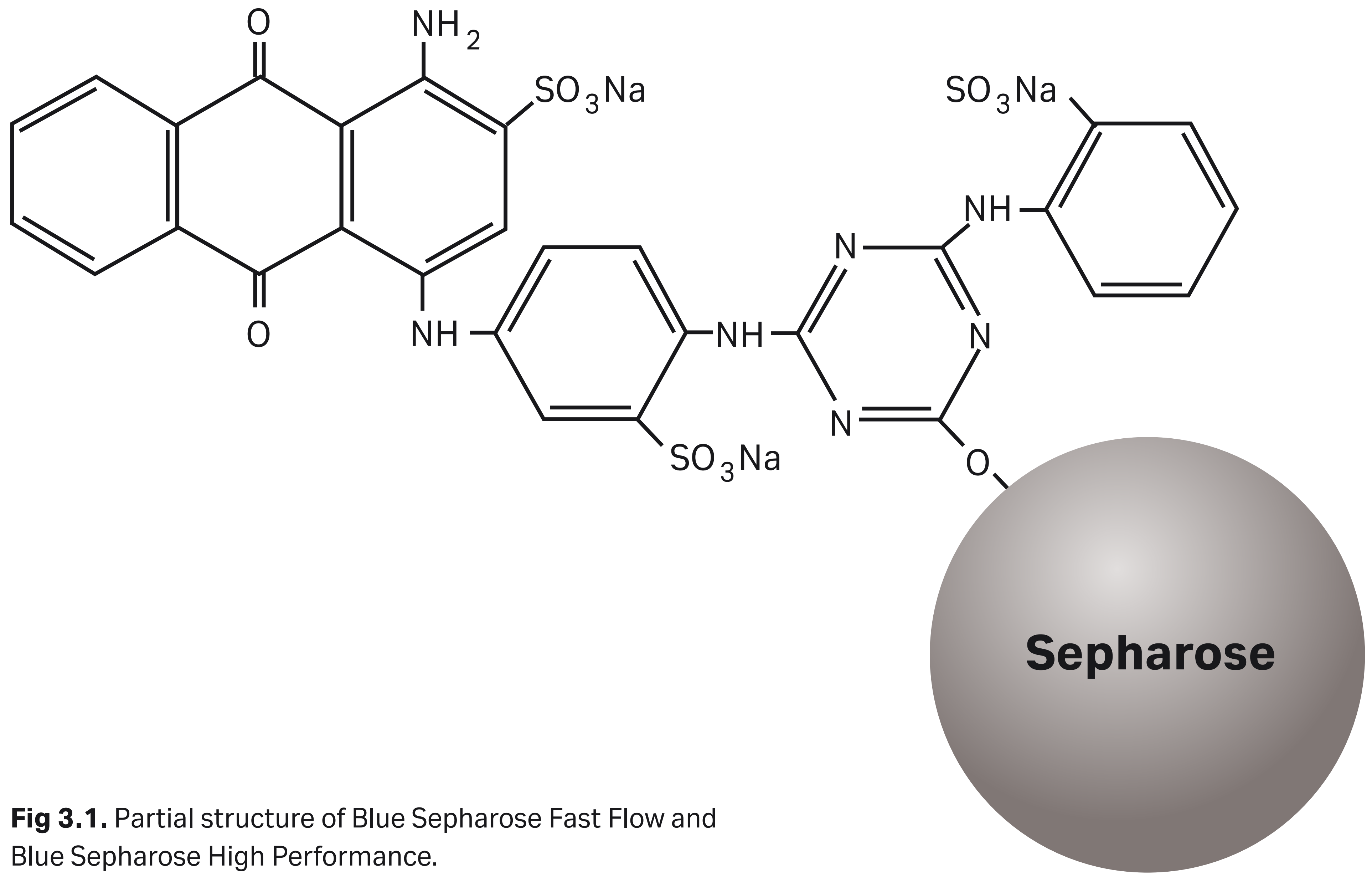


Fig 3.1. Partial structure of Blue Sepharose Fast Flow and Blue Sepharose High Performance.

☞ Use Blue Sepharose or Capto Blue to remove host albumin from mammalian expression systems, or when the sample is known to contain high levels of albumin that can mask the visualization of other protein peaks seen by UV absorption.

☞ Advice on the selection of techniques for the removal of albumin during antibody purification is given in the handbook *Affinity Chromatography, Vol. 1: Antibodies*, 18103746 from Cytiva.

☞ 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.

Chromatography media characteristics

Characteristics of Blue Sepharose and Capto Blue chromatography media are summarized in Table 3.1.

Table 3.1. Characteristics of Blue Sepharose and Capto Blue chromatography media

	Ligand density	Composition	pH stability[1]	Average particle size (μm)
Blue Sepharose High Performance	4 mg/mL	Cibacron Blue F3G-A coupled to Sepharose High Performance using the triazine method.	Short term: 3 to 13 Long term: 4 to 12	34
Blue Sepharose 6 Fast Flow	6.7 to 7.9 μmol/mL	Cibacron Blue F3G-A coupled to Sepharose 6 Fast Flow using the triazine method.	Short term: 3 to 13 Long term: 4 to 12	90
Capto Blue	13 μmol/mL	Cibacron Blue F3G-A coupled to Capto.	Short term: 3 to 13 Long term: 2 to 13.5	75
Capto Blue (high sub)	18 μmol/mL	Cibacron Blue F3G-A coupled to Capto.	Short term: 3 to 13 Long term: 2 to 13.5	75

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[1] Short term refers to the pH interval for regeneration, cleaning in place, and sanitization procedures. 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.

Purification options

Blue Sepharose and Capto Blue are available in chromatography media packs for packing into empty columns. The media are also available in prepacked columns for convenience. Purification options for the media and prepacked columns are shown in Table 3.2.

Table 3.2. Purification options for Blue Sepharose and Capto Blue chromatography media and prepacked columns

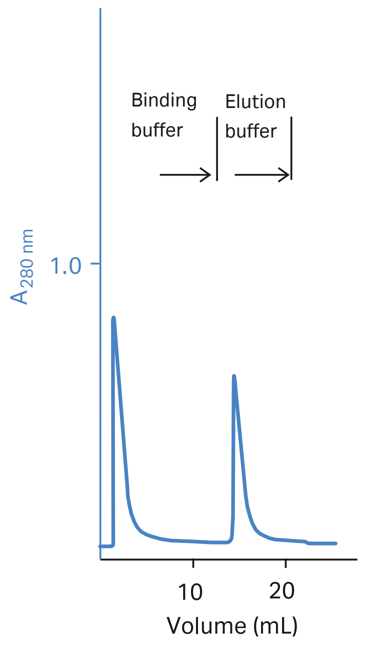
	Binding capacity	Maximum operating flow	Comments
Blue Sepharose 6 Fast Flow[1]	Human serum albumin (HSA), 18 mg/mL medium	> 750 cm/h[2]	Supplied as a suspension ready for column packing
HiTrap Blue HP, 1 mL	HSA, 20 mg/column	4 mL/min	Prepacked 1 mL column
HiTrap Blue HP, 5 mL	HSA, 100 mg/column	20 mL/min	Prepacked 5 mL column
HiScreen Blue FF	HSA, 85 mg/column	4.6 mL/min	Prepacked 4.7 mL column
Capto Blue	HSA, 24 mg/mL medium	At least 600 cm/h[1]	Supplied as suspension ready for packing
HiScreen Capto Blue	HSA, 118 mg/column	4.6 mL/min	Prepacked 4.7 mL column
Capto Blue (high sub)	HSA, 30 mg/mL medium	At least 600 cm/h[1]	Supplied as suspension ready for packing

[1] In a 1 m column with 20 cm bed height at 20°C using process buffers with the same viscosity as water.

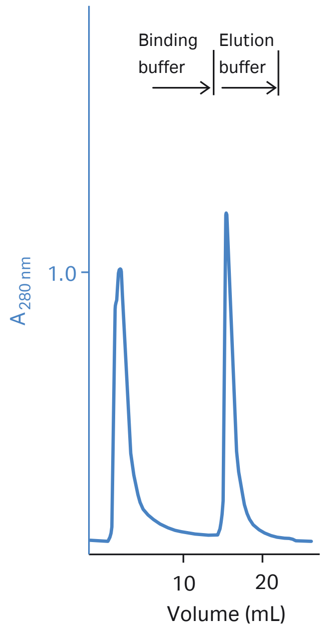
[2] See Appendix 4 to convert flow velocity (cm/h) to volumetric flow rate (mL/min) and vice versa. Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.

Column:: HiTrap Blue HP, 1 mL or 5 mL
Sample: Human serum buffer exchanged on a PD-10 Desalting column to binding buffer. Filtered on a 0.45 μm filter
Binding buffer: 50 mM potassium dihydrogen phosphate (KH_2PO_4), pH 7.0
Elution buffer: 50 mM KH_2PO_4, 1.5 M KCl, pH 7.0
Flow rate: 2 mL/min (1 mL column), 4 mL/min (5 mL column)

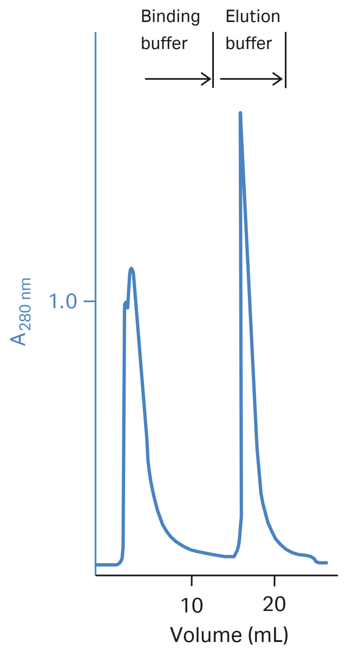
Configuration: 1 × 1 mL column
Sample vol.: 0.7 mL human serum
Yield: 16.7 mg HSA



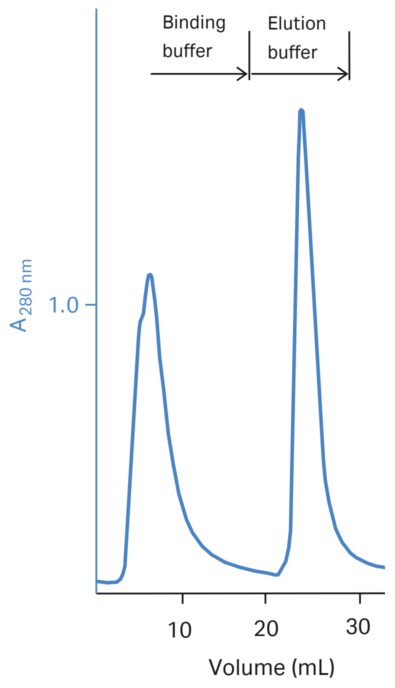
Configuration: 2 × 1 mL column
Sample vol. 1.4 mL human serum
Yield: 33.2 mg HSA



Configuration: 3 × 1 mL column
Sample vol.: 2.1 mL human serum
Yield: 52.0 mg HSA



Configuration: 1 × 5 mL column
Sample vol.: 3.5 mL human serum
Yield: 98.5 mg HSA



Configuration: 3 × 5 mL columns connected in series
Sample vol.: 10.5 mL human serum
Yield: 286.9 mg HSA

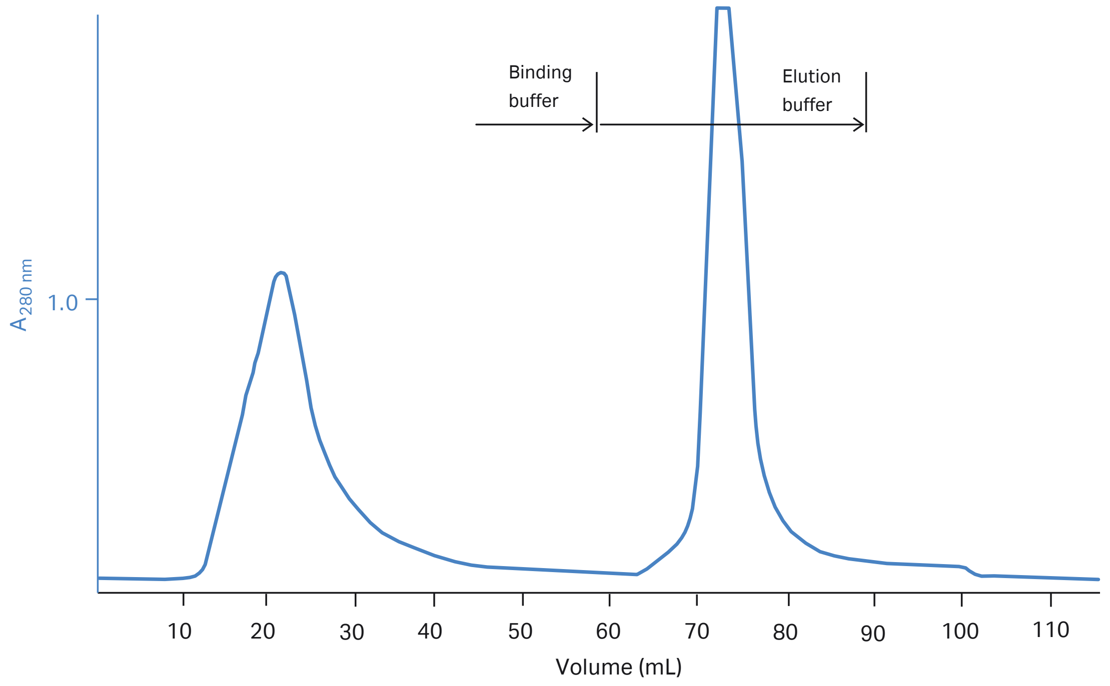
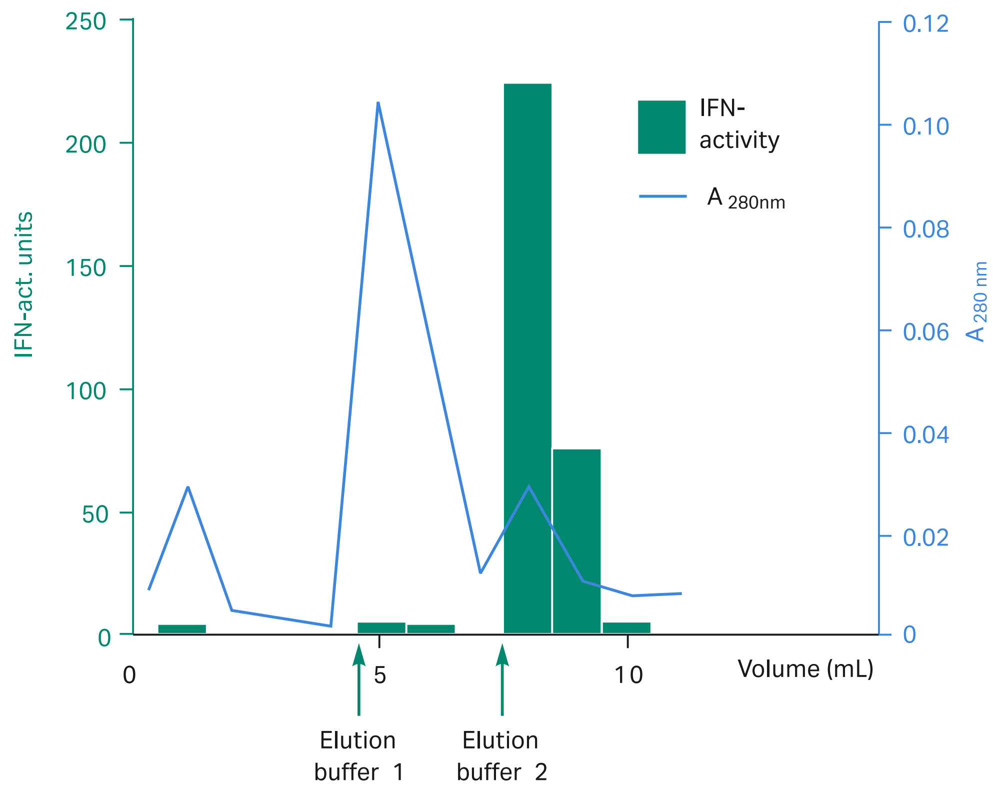


Fig. 3.2. Scaling up on HiTrap Blue HP gives predictable separations and quantitatively reproducible yields.

Purification examples

Figure 3.2 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 3.3 shows the use of Blue Sepharose 6 Fast Flow for the separation of HSA from interferon β.



Column: Blue Sepharose 6 Fast Flow (0.5 mL) in packed column
Sample: 0.5 mL of interferon β (1 000 000 U/mL) in 100 mM phosphate, pH 7.4, with 1 mg/mL of human serum albumin
Binding buffer: 20 mM phosphate, 150 mM NaCl, pH 7.2
Elution buffer 1: 20 mM phosphate, 2 M NaCl, pH 7.2
Elution buffer 2: 20 mM phosphate, 2 M NaCl, 50% ethylene glycol, pH 7.2
Flow rate: Gravity feed

Fig 3.3. 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 cofactor is preferable for the elution of specifically bound proteins.

Performing a separation

Binding buffer:	50 mM potassium dihydrogen phosphate (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 CV of binding buffer.
2. Adjust the sample to starting conditions and apply to the column, using a syringe or a pump.
3. Wash with 10 CV of binding buffer or until no material appears in the eluent (monitored by absorption at $A_{280\ nm}$).
4. Elute with 5 CV of elution buffer (step elution) or with 0% to 100% elution buffer in binding buffer (gradient elution).

Cleaning

Wash with 5 CV of high pH (100 mM Tris-HCl, 500 mM NaCl, pH 8.5) followed by low pH (100 mM sodium acetate, 500 mM NaCl, pH 4.5). Repeat four to five times. Re-equilibrate immediately with binding buffer.

Remove precipitated proteins with 4 CV of 100 mM NaOH at a low flow rate, followed by washing with 3 to 4 CV of 70% ethanol or 2 M potassium thiocyanate. Alternatively, wash with 2 CV of 6 M guanidine hydrochloride. Re-equilibrate immediately with binding buffer.

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

Chemical stability

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

Storage

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

Purification or removal of albumin and IgG

Albumin and IgG Depletion Sepharose High Performance

Albumin and IgG are the most abundant proteins in plasma which tend to obscure the signals of less abundant proteins, preventing accurate detection. The high abundance of albumin and IgG also interferes with the detection of other proteins by preventing a sufficient amount of less abundant proteins from being included in the analysis. By depleting samples of albumin and IgG, the quality and depth of the analysis can be greatly enhanced. Depletion of the two removes more than 60% of the total protein content in human plasma, allowing proteins normally obscured by albumin and IgG to be visualized.

Albumin and IgG Depletion Sepharose High Performance is available prepacked in in HiTrap and SpinTrap formats for removal of human serum albumin (HSA) and IgG. Both column types are prepacked with a mixture of antiHSA Sepharose High Performance and Protein G Sepharose High Performance. The ligand of antiHSA Sepharose High Performance is based on a single domain antibody fragment with high specificity and capacity for HSA. The ligand of Protein G Sepharose High Performance is derived from the IgG binding regions of Protein G, a cell surface protein of *Streptococcus* bacteria. The protein G ligand binds human IgG_1, IgG_2, IgG_3, and IgG_4.

The primary use of the products is small-scale preparation of protein samples prior to downstream analyses such as 1-D or 2-D gel electrophoresis and mass spectrometry (MS).

☞ A lower sample volume should be used when applying plasma containing albumin and IgG above the normal levels of human plasma (40 mg albumin/mL and 15 mg IgG/mL).

Chromatography medium characteristics

Table 3.3 shows the characteristics of the chromatography medium.

Table 3.3. Characteristics of Alumin and IgG Depletion Sepharose High Performance medium

Product	Ligand	Composition	pH stability[1]	Average particle size (µm)
Albumin and IgG Depletion Sepharose High Performance	Recombinant protein G fragment and recombinant protein binding HSA	Ligand coupled to Sepharose High Performance	Short term: 2 to 9 Long term: 3 to 9	34

[1] Short term refers to the pH interval for regeneration, cleaning-in-place, and sanitization procedures. 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.

Purification options

The purification options for HiTrap Albumin and IgG Depletion and Albumin and IgG Depletion SpinTrap column are shown in Table 3.4.

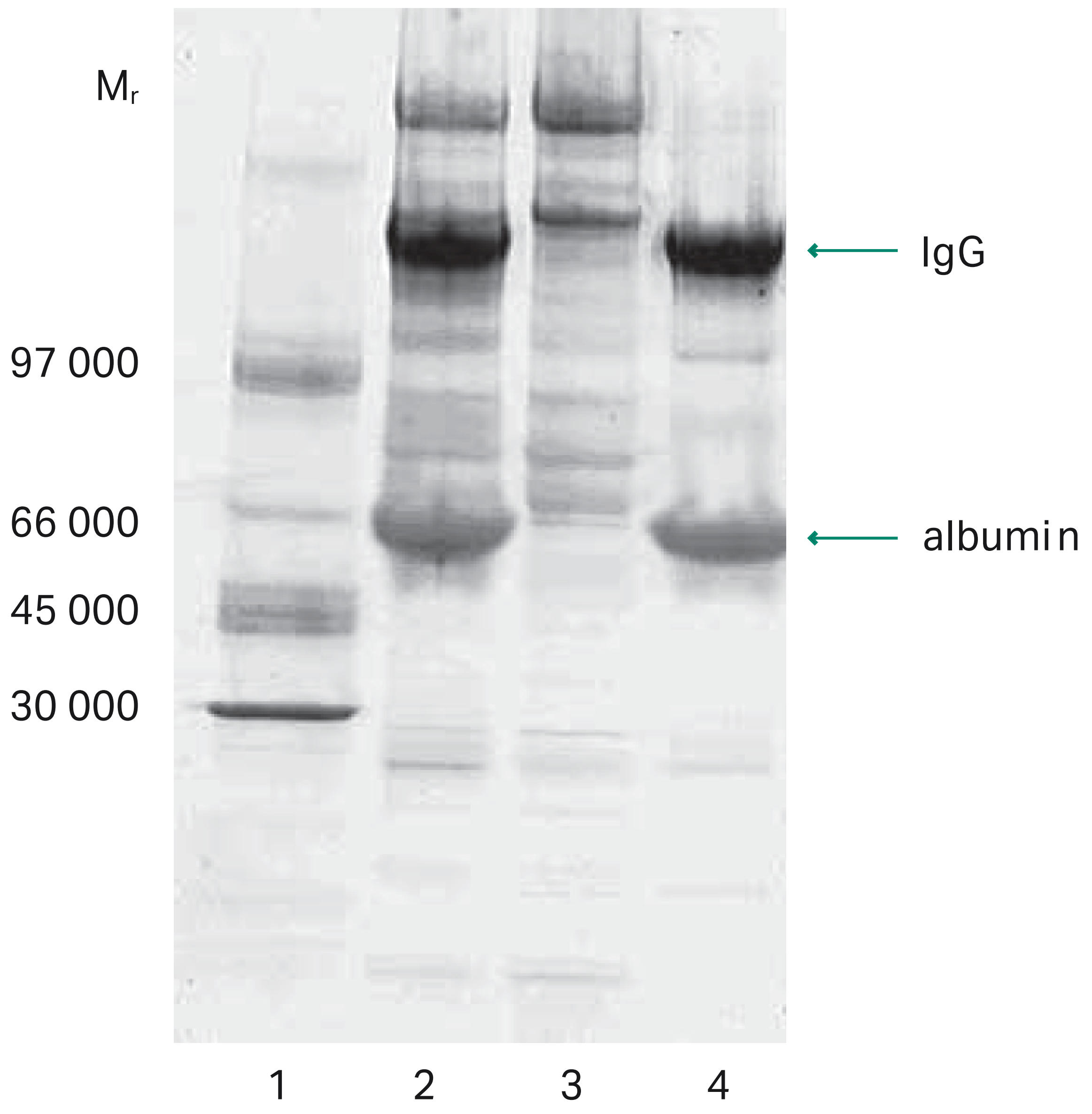
Table 3.4. Purification options: HiTrap Albumin and IgG Depletion as well as Albumin and IgG Depletion SpinTrap

	Binding capacity	Maximum operating flow rate (mL/min)	Comments
HiTrap Albumin and IgG Depletion	Human plasma, ~ 150 µL[1]	4	Prepacked 1 mL column.
Albumin and IgG Depletion SpinTrap	Human plasma, ~ 50 µL[1]	Not applicable	To be used with a benchtop centrifuge.

[1] Human plasma containing ~ 40 mg albumin/mL and ~ 15 mg IgG/mL. Results according to ELISA: > 95% albumin depletion and > 90% IgG depletion.

Purification examples

HiTrap Albumin and IgG Depletion can be used for depletion of human plasma without dilution of the sample before loading. A volume of 150 µL human plasma was applied to the 1 mL column, and the unbound fraction containing the depleted sample was collected. The depletion of albumin and IgG is shown by SDS-PAGE analysis (Fig 3.4). The depletion level was also determined by ELISA, and the result for the unbound fraction was 99% albumin depletion and 98% IgG depletion.



Lanes
1. LMW marker
2. Human plasma
3. Depleted fraction
4. Bound fraction containing albumin and IgG

Fig 3.4. Deep Purple stained SDS-PAGE analysis (nonreducing conditions) of fractions from the depletion of human plasma using HiTrap Albumin and IgG Depletion.

Performing a separation

Binding buffer:	20 mM sodium phosphate, 150 mM NaCl, pH 7.4
Elution buffer:	100 mM glycine-HCl, pH 2.7

☞ Sample preparation: Dilution of the human plasma is not required. Filter the human plasma through a 0.22 or 0.45 µm filter shortly before applying it to the column.

HiTrap Albumin and IgG Depletion

☞ A flow rate of 1 mL/min is recommended for the entire depletion procedure.

1. Fill the pump tubing with binding buffer. Remove the stopper and the snap-off end from the column and connect it to the pump tubing 'drop-to-drop' to avoid introducing air into the system.
2. Wash the column with 5 mL binding buffer to remove the 20% ethanol storage solution.
3. Equilibrate with 10 mL of binding buffer.
4. Apply 150 µL filtrated human plasma and wash with at least 5 mL binding buffer until the absorbance reaches a steady baseline. Collect the sample flowthrough during sample application and wash. The flowthrough contains the depleted sample.
5. Optional: Elute and collect the bound proteins (albumin and IgG) with 10 mL of elution buffer.

Note: Step 5 should be performed if the column is to be reused or if the bound albumin and IgG fraction is to be analyzed.

For the manual depletion procedure (without using a pump), the syringe is connected to the column by the provided Luer connector. Be sure to use a flow rate of approximately 1 mL/min.

Note that too high a flow rate will damage the packing of the chromatography medium and result in high back pressure.

Albumin & IgG Depletion SpinTrap

1. Remove storage solution
   - A. Invert and shake the SpinTrap column repeatedly to resuspend the medium.
   - B. Twist off the bottom cap from the SpinTrap column and loosen the top cap one-quarter of a turn.
   - C. Place the column in a 2 mL microcentrifuge tube and centrifuge for 30 s at 70 to 100 × g. Discard the collected liquid.
   - D. Remove and discard the top cap.
2. Column equilibration
   - A. Add 400 µL binding buffer and centrifuge for 30 s at 800 × g. Discard the collected liquid.
   - B. Add 400 µL binding buffer a second time and centrifuge for 30 s at 800 × g. Discard the collected liquid.
3. Sample application and incubation
   - A. Place the column in a new 2 mL tube.
   - B. Dilute the 50 µL plasma sample with binding buffer to a final volume of 100 µL and apply to the column.
   - C. Incubate for 5 min without mixing.
4. Collection of depleted sample
   - A. Centrifuge for 30 s at 800 × g. Collect the eluate.
   - B. Add 100 µL binding buffer and centrifuge for 30 s at 800 × g. Collect the eluate.
   - C. Add 100 µL binding buffer a second time and centrifuge for 30 s at 800 × g. Collect the eluate.

Note: All eluates can be collected in the same 2 mL tube.

5. Optional: elution of albumin and IgG
   - A. Bound albumin and IgG can be eluted by 100 mM glycine-HCl, pH 2.7.

Storage

Store at 4°C to 8°C in 20% ethanol.

Purification or removal of biotin and biotinylated substances

Streptavidin Sepharose High Performance

Biotin and biotinylated substances bind to streptavidin, a molecule isolated from *Streptomyces avidinii*. The binding of streptavidin to biotin is one of the strongest known noncovalent biological interactions. Hence, denaturing conditions are generally required for the efficient elution of biotinylated biomolecules. Alternatively, biotinylated biomolecules bound to streptavidin chromatography media can be used to capture interacting target substances such as proteins. Impurities are removed by washing, and the enriched target protein is eluted using relatively mild elution conditions.

Chromatography medium characteristics

Characteristics of Streptavidin Sepharose High Performance AC medium are given in Table 3.5

Table 3.5. Characteristics of Streptavidin Sepharose High Performance chromatography medium

	Composition	pH stability[1]	Average particle size (µm)
Streptavidin Sepharose High Performance	Streptavidin is coupled to Sepharose High Performance using a N-hydroxysuccinimide coupling method	Short term: 2 to 10.5 Long term: 4 to 9	34

[1] Short term refers to the pH interval for regeneration, cleaning in place and sanitization procedures. 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.

Purification options

Streptavidin Sepharose High Performance is available in chromatography media packs and is prepacked in HiTrap 1 mL columns, MultiTrap 96-well plates, and SpinTrap minicolumns (Table 3.6). These different formats can be used for protein purification and enrichment, where a biotinylated antibody (or other biotinylated molecule) is attached to the Streptavidin and the protein of interest is enriched through the affinity interaction with the antibody/target molecule.

Streptavidin Mag Sepharose magnetic beads are also available for small-scale immunoprecipitation and purification of biotinylated molecules, see Chapter 5.

Table 3.6. Purification options for Streptavidin High Performance chromatography media and prepacked columns

	Binding capacity	Maximum operating flow	Comments
HiTrap Streptavidin HP, 1 mL	Biotin, > 300 nmol/column Biotinylated BSA, 6 mg/mL medium	4 mL/min	Prepacked 1 mL column
Streptavidin Sepharose High Performance	Biotin, > 300 nmol/medium Biotinylated BSA, 6 mg/mL medium	150 cm/h[1]	Supplied as a suspension ready for column packing
Streptavidin HP MultiTrap	Biotin, > 15 nmol/well Biotinylated BSA, 0.3 mg/well	Not applicable	96-well filter plate
Streptavidin HP SpinTrap	Biotin, > 30 nmol/column Biotinylated BSA, 0.6 mg/column	Not applicable	To be used with a benchtop centrifuge

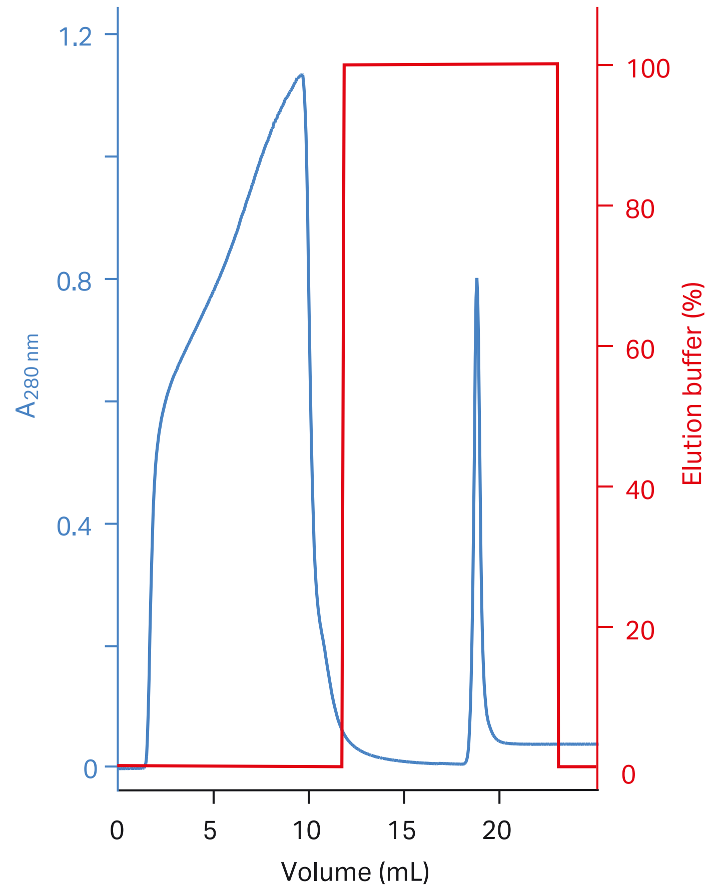
[1] See Appendix 4 to convert flow velocity (cm/h) to volumetric flow rate (mL/min). Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.

Purification example

An alternative to labeling the biomolecule, for example the antibody, with biotin is to use 2-iminobiotin that binds to streptavidin above pH 9.5 and can be eluted at pH 4.0 (Fig 3.5).

Enrichment of a particular protein is often desired to increase its signal in subsequent analysis steps. In this example Streptavidin HP SpinTrap was used for enrichment of human transferrin from *E. coli* sample. The concentration of the protein of interest was 0.15% of the total *E. coli* protein content, which approximately corresponds to the concentration of a medium-abundance protein. Capture of transferrin was achieved using a biotinylated antibody (polyclonal rabbit antihuman transferrin immobilized on the medium).

Analysis by SDS-PAGE of the collected fractions from the runs revealed a significant enrichment of transferrin (Fig 3.6). Recovery of the start material was 60% to 70% with the majority of the protein eluted in the first elution step. The enrichment of transferrin relative to the start material was approximately 100-fold with Streptavidin HP SpinTrap.



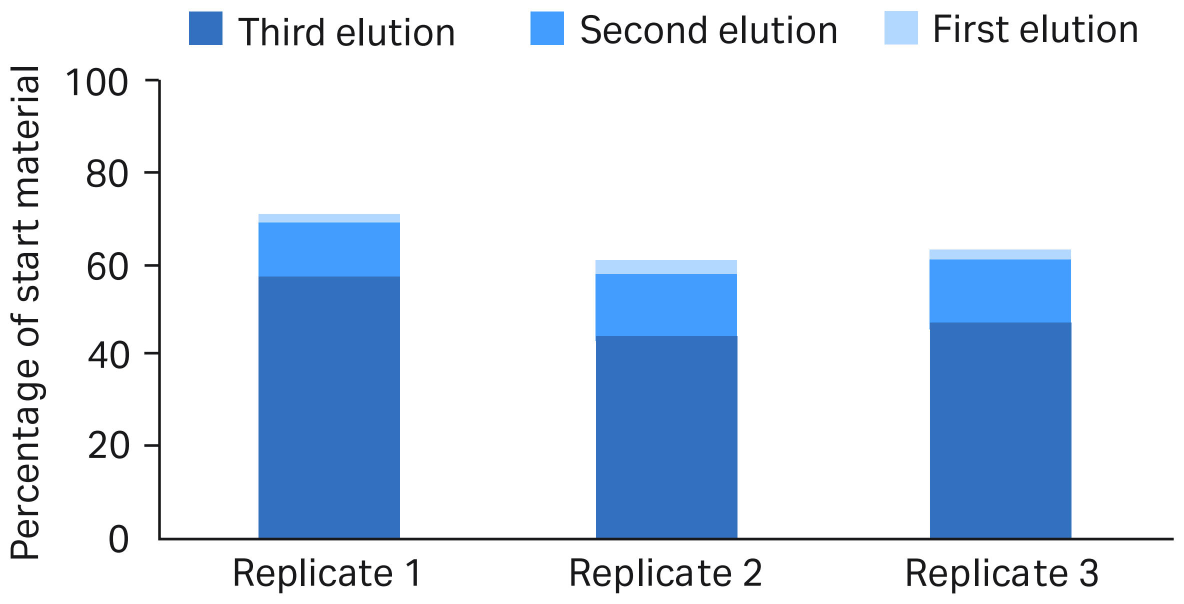
Column:	HiTrap Streptavidin HP, 1 mL
Sample:	9.0 mL of a mixture of BSA and iminobiotinylated BSA, filtered through a 0.45 µm filter
Binding buffer:	50 mM ammonium carbonate buffer, 500 mM NaCl, pH 10.0
Elution buffer:	50 mM ammonium acetate buffer, 500 mM NaCl, pH 4.0
Flow rate:	1 mL/min (0.3 mL/min during sample application)
System:	ÄKTA system

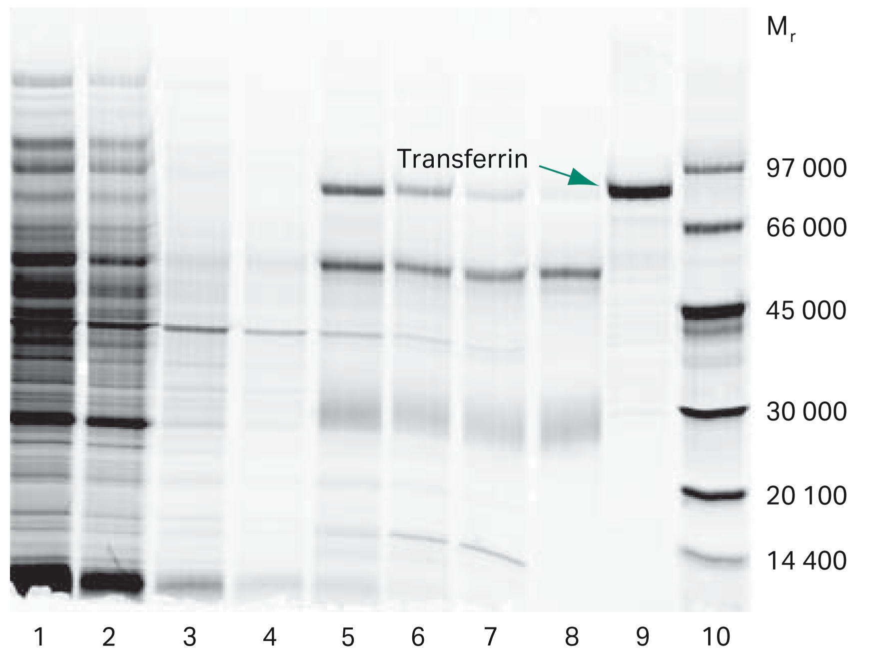
Fig 3.5. Purification of iminobiotinylated BSA on HiTrap Streptavidin HP, 1 mL.

(A)

Column:	Streptavidin HP SpinTrap
Sample:	5 mg/mL *E. coli* protein containing 7.5 µg/mL human transferrin
Sample volume:	200 µL
Binding buffer:	Tris buffered saline (TBS: 50 mM Tris, 150 mM NaCl, pH 7.5)
Wash buffer:	TBS, 2 M urea, pH 7.5
Elution buffer:	100 mM glycine/HCl, 2 M urea, pH 3.0
Antibody:	Polyclonal rabbit antihuman transferrin (biotinylated)

(B)





Lanes

1. Flowthrough (diluted 1:30)
2. First wash (diluted 1:10)
3. Third wash
4. Fifth wash
5. First elution
6. Second elution
7. Third elution
8. Antibody
9. Transferrin, M_r 77 000
10. LMW markers

Fig 3.6. Enrichment of transferrin from *E. coli* cell lysate. (A) Analysis by SDS-PAGE (wash steps 2 and 4 have been omitted from the gel). The gel was post-stained with Deep Purple Total Protein Stain and scanned. (B) All three elution steps were analyzed using ImageQuant™ TL software. Recovery (percentage of start material) of three replicates is shown.

Performing a separation

HiTrap Streptavidin HP

The following protocol describes AC using a HiTrap Streptavidin HP 1 mL column by syringe, using a pump, or a chromatography system.

Biotinylated substances

Binding buffer: 20 mM sodium phosphate, 150 mM NaCl, pH 7.5

Elution buffer: 8 M guanidine-HCl, pH 1.5

Iminobiotinylated substances

Binding buffer: 50 mM ammonium carbonate, 500 mM NaCl, pH 10.0

Elution buffer: 50 mM ammonium acetate, 500 mM NaCl, pH 4.0

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

---

[1] *Since elution conditions can be quite harsh, collect fractions into neutralization buffer (100 to 200 µL 1 M Tris-HCl, pH 9.0 per mL of fraction), so that the final pH of the fractions will be approximately neutral or perform a rapid buffer exchange on a desalting column (see Buffer exchange and desalting, Appendix 1).*

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

Antigen purification

Antigens can be purified from biotinylated biomolecule (often antibody)-antigen complexes bound to streptavidin. The following method is adapted for HiTrap Streptavidin HP.

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

Elution buffer: 100 mM 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 CV 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 CV of solubilization buffer or until no material is found in eluent (monitored by UV absorption at $A_{280\ nm}$).
8. Elute with 5 to 10 CV of elution buffer[1].

---

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

Streptavidin HP MultiTrap 96-well plate

The protocol is designed for enrichment of target proteins by using immobilized antibodies. Centrifuge the MultiTrap 96-well plates at 700 × g or use vacuum. If vacuum is used, apply -0.15 bar until the wells are empty, then slowly increase the vacuum to -0.3 bar (do not apply more vacuum than -0.5 bar). Turn off the vacuum after approximately 5 s.

Mix briefly before removal of liquid in the equilibration, wash, and elution steps to increase the efficiency of the step. Incubating on a plate shaker is recommended. Remember to change or empty the collection plate between steps.

Binding buffer:	TBS (50 mM Tris, 150 mM NaCl, pH 7.5)
Washing buffer:	TBS with 2 M urea, pH 7.5
Elution buffer:	100 mM glycine with 2 M urea, pH 3.0
Blocking buffer:	2 mM biotin in TBS

1. Remove storage solution
    A. Suspend the medium by gently shaking the plate upside down.
    B. Remove top and bottom seals and place plate on the collection plate.
    C. Remove the storage solution by centrifugation for 1 min at 700 × g.
2. Equilibration for immobilization (perform this step three times)
    A. Add 400 µL binding buffer per well, mix briefly and centrifuge for 1 min at 700 × g to equilibrate the medium.
3. Binding of biotinylated antibody
    A. Immediately after equilibration, add 200 µL of the biotinylated antibody solution per well (0.1 to 1.0 mg/mL in binding buffer).
    B. Incubate on shaker for 20 min.
    C. Centrifuge for 1 min at 700 × g to remove unbound antibody.
4. Blocking (perform this step twice)
    A. Add 400 µL blocking buffer per well and incubate on shaker for 5 min to block free biotin binding sites.
    B. Centrifuge for 1 min at 700 × g.
5. Washing (perform this step three times)
    A. Add 400 µL binding buffer per well and mix briefly.
    B. Centrifuge for 1 min at 700 × g.
6. Binding of target protein
    A. Add 200 µL clarified sample in binding buffer per well and incubate on shaker for 60 min.
    B. Centrifuge for 1 min at 700 × g to wash out unbound protein. Collect flowthrough.
7. Washing
    A. Add 400 µL binding/wash buffer per well and mix briefly.
    B. Centrifuge for 1 min at 700 × g. Perform this step five times in total. (Collect and save washes in case troubleshooting is needed).
8. Elution (perform this step three times)
    A. Add 200 µL of desired elution buffer and shake for 1 min.
    B. Centrifuge for 1 min at 700 × g.
    C. Collect the eluates in separate collection plates.

Streptavidin HP SpinTrap columns

This protocol is designed for enrichment of target proteins by using immobilized antibodies. In each step, place the SpinTrap column in a fresh 2 mL microcentrifuge tube for liquid collection. Lids and bottom caps of Streptavidin HP SpinTrap are used during the incubation and elution but not during equilibration and washing. Before centrifugation, remove the bottom cap and slightly open the screw cap lid (twist the cap lid ~ 90° counterclockwise).

Binding buffer:	TBS (50 mM Tris, 150 mM NaCl, pH 7.5)
Washing buffer:	TBS with 2 M urea, pH 7.5
Elution buffer:	100 mM glycine with 2 M urea, pH 2.9
Blocking buffer:	2 mM biotin in TBS

Storage

Wash chromatography media and HiTrap columns with 20% ethanol (use approximately 5 CV for packed media) and store at 4°C to 8°C. Streptavidin MultiTrap and Streptavidin SpinTrap are for single-use only.

1. Remove storage solution
   - A. Break off the bottom cap from the spin column. Save the bottom cap.
   - B. Remove the storage solution by centrifugation for 1 min at 150 × g.
2. Equilibrate for immobilization (perform this step three times)
   - A. Add 400 µL binding buffer and centrifuge for 1 min at 150 × g to equilibrate the medium.
   - B. Remove the binding buffer.
3. Binding biotinylated antibody
   - A. Immediately after the equilibration, add 200 µL of the biotinylated antibody (0.1 to 1.0 mg/mL).
   - B. Fully suspend the medium by manual inversion and incubate with slow, end-overend mixing for 20 min at room temperature.
   - C. Centrifuge for 1 min at 150 × g to remove unbound antibody.
4. Blocking (perform this step twice)
   - A. Add 400 µL blocking buffer.
   - B. Mix by manual inversion and incubate with end-over-end mixing for 5 min to block free biotin binding sites.
   - C. Centrifuge for 1 min at 150 × g.
5. Washing (perform this step three times)
   - A. Add 400 µL binding buffer and centrifuge for 1 min at 150 × g.
6. Binding of target protein
   - A. Add 200 µL clarified sample in binding buffer.
   - B. Mix by manual inversion and incubate with slow, end-over-end mixing for 60 min at room temperature.
   - C. Centrifuge for 1 min at 150 × g to wash out unbound protein. Collect flowthrough.
7. Washing (perform this step five times)
   - A. Add 400 µL wash buffer and centrifuge for 1 min at 150 × g.
   - B. During optimization/troubleshooting: collect flowthrough.
8. Elution (perform this step three times)
   - A. Add 200 µL of desired elution buffer and mix by inversion.
   - B. Centrifuge for 1 min at 1000 × g.
   - C. Collect the eluates in individual tubes.

Purification or removal of 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 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 can be enhanced if the enzyme substrate is present and enzyme-substrate-calmodulin-Ca^{2+} complexes are particularly stable.

Chromatography medium characteristics

The characteristics of Calmodulin Sepharose 4B chromatography medium are shown in Table 3.7.

Table 3.7. Characteristics of Calmodulin Sepharose 4B chromatography medium

	Ligand density (mg/mL)	Composition	pH stability[1]	Average particle size (µm)
Calmodulin Sepharose 4B	0.9 to 1.3	Bovine testicular calmodulin coupled to Sepharose 4B by the CNBr method	Short term: 4 to 9 Long term: 4 to 9	90

[1] Short term refers to the pH interval for regeneration, cleaning-in-place, and sanitization procedures. 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.

Purification options

Calmodulin Sepharose 4B chromatography medium is available in chromatography media packs for packing in columns, Table 3.8.

Table 3.8. Purification options for Calmodulin Sepharose 4B

	Binding capacity	Maximum operating flow velocity (cm/h[1])	Comments
Calmodulin Sepharose 4B	No data available	75	Supplied as a suspension ready for column packing

[1] See Appendix 4 to convert flow velocity (cm/h) to volumetric flow rate (mL/min). 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, 50 to 200 mM NaCl, 2 mM $CaCl_2$, pH 7.5
Elution buffer: 50 mM Tris-HCl, 50 to 200 mM NaCl, 2 mM EGTA, pH 7.5

1. Pack the column (see Appendix 3) and wash with at least 10 CV of binding buffer to remove preservative.
2. Equilibrate the column with 10 CV 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 to 10 CV of binding buffer or until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
5. Elute with 5 CV 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 *Purification or removal of serine proteases* in this chapter).

☞ Remove free calmodulin from the sample by HIC in the presence of Ca^{2+} on HiTrap Phenyl FF (high sub) or by IEX on HiTrap Q FF.

☞ Since some nonspecific ionic interactions can occur, a low salt concentration (50 to 200 mM NaCl) is recommended to promote binding to the ligand while eliminating any nonspecific 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 can also be displaced by a high salt concentration, 1 M NaCl.

Cleaning

Alternative 1

Wash with 3 CV of 50 mM Tris-HCl, 1 M NaCl, 2 mM EGTA, pH 7.5 and reequilibrate immediately with 5 to 10 CV of binding buffer.

Alternative 2

Wash with 3 CV of 100 mM ammonium carbonate buffer, 2 mM EGTA, pH 8.6 followed by 3 CV of 1 M NaCl, 2 mM $CaCl_2$. Continue washing with 3 CV of 100 mM sodium acetate buffer, 2 mM $CaCl_2$, pH 4.4 followed by 3 CV of binding buffer.

Remove severe contamination by washing with nonionic detergent such as 0.1% Tween™ 20 at 37°C for 1 min.

Chemical stability

Stable in all commonly used aqueous solutions.

Storage

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

Purification or removal of coagulation factors

VIISelect, VIIISelect, IXSelect, Heparin Sepharose High Performance, Heparin Sepharose 6 Fast Flow, Capto Heparin

Blood coagulation factors form an extremely important group of proteins for research, medical and clinical applications.

Different coagulation factors involved in bleeding disorders are selectively purified using VIISelect, VIIISelect, and IXSelect. Hemophilia type A is caused by a deficiency or defect in factor VIII (FVIII) while hemophilia type B is known as factor IX (FIX) deficiency. Factor VII (FVII) is used for hemophilia patients with FVIII or FIX deficiencies who have developed inhibitors against the replacement coagulation factors.

In addition, coagulation factors obtained from plasma or expressed as recombinant proteins in various cell types can be purified by Heparin Sepharose and Capto Heparin chromatography media. For details about Heparin Sepharose High Performance, Heparin Sepharose 6 Fast Flow and Capto Heparin, see *Purification or removal of DNA-binding proteins* in this chapter. The protocols are also applicable for coagulation factors.

Chromatography media characteristics

The characteristics of VIISelect, VIIISelect, and IXSelect chromatography media for purification of coagulation factors are shown in Table 3.9.

Table 3.9. Characteristics of VIISelect, VIIISelect, and IXSelect chromatography media

Product	Ligand	Composition	pH stability[1]	Average particle size (μm)
VIISelect	Recombinant protein (M_r 14 080) produced in *Saccharomyces cerevisiae*	Ligand coupled to Capto	Short term: 2 to 12 Long term: 3 to 10	75
VIIISelect	Recombinant protein (M_r 13 000) produced in *Saccharomyces cerevisiae*	Ligand coupled to Capto	Short term: 2 to 12 Long term: 3 to 10	75
IXSelect	Single-chain antibody fragment (M_r 13 151) directed against FIX and produced in *Saccharomyces cerevisiae*	Ligand coupled to Capto	Short term: 2 to 12 Long term: 3 to 10	75

[1] Short term refers to the pH interval for regeneration, cleaning in place, and sanitization procedures. 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.

Purification options

A wide range of chromatography media packs and prepacked columns for purification of coagulation factors is available (Table 3.10).

Table 3.10. Chromatography media and prepacked columns for purification of coagulation factors

Product	Binding capacity	Maximum operating flow	Comments
VIISelect	FVII, ~ 8 mg/mL medium	At least 600 cm/h in a 1 m diameter column with 20 cm bed height[1]	Supplied as suspension ready for column packing
VIIISelect	Typically 20 000 IU/mL medium	Up to 300 cm/h at 30 cm bed height[1]	Supplied as suspension ready for column packing
IXSelect	FIX, ~ 6 mg/mL medium	At least 600 cm/h in a 1 m diameter column, with 20 cm bed height[1]	Supplied as suspension ready for column packing
HiTrap IXSelect, 1 mL	FIX, ~ 6 mg/column	4 mL/min	Prepacked 1 mL column
HiTrap IXSelect, 5 mL	FIX, ~ 30 mg/column	20 mL/min	Prepacked 5 mL column
HiScreen IXSelect	FIX, ~ 28 mg/column	4.6 mL/min	Prepacked 4.7 mL column

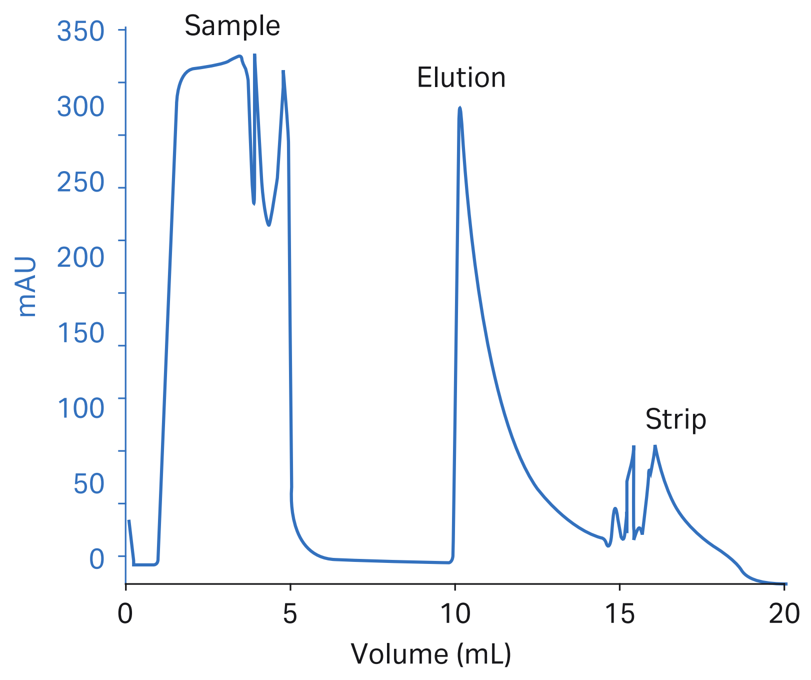
[1] 20°C using buffers with the same viscosity as water at < 0.3 MPa (3 bar, 43.5 psi).

Purification of FVII

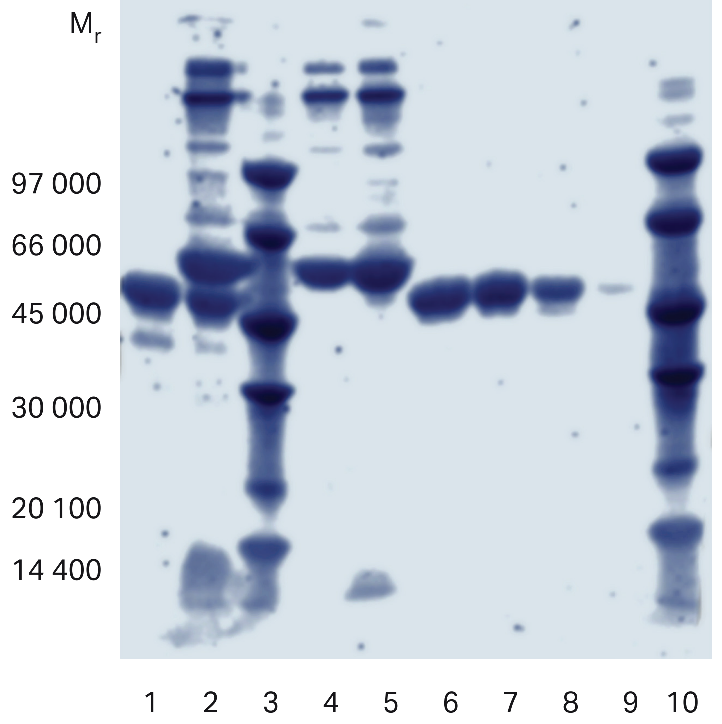
A commercially available drug approved for infusion therapy was spiked in human plasma, and FVII was purified (Fig 3.7A). Gel electrophoresis was run on SDS-PAGE gradient gels, 8% to 16%, under nonreducing conditions. Figure 3.7B shows the high purity of FVII in the eluted fractions obtained using VIISelect.

(A)

Column: Tricorn 5/20 packed with 0.45 mL of VIISelect
Sample: Registered pharmaceutical FVII drug diluted with solution for injection and spiked in human plasma
Sample load: 7 mg/mL of chromatography VIISelect (below the maximum capacity)
Binding/equilibration buffer: 10 CV of 50 mM Tris, 150 mM NaCl, pH 7.5
Elution buffer: 12 CV of 50 mM Tris, 1.5 M NaCl, 50% (v/v) propylene glycol, pH 7.5
Wash: 12 CV binding/equilibration buffer
Flow rate (flow velocity): Sample load, 0.2 mL/min (61 cm/h); wash and elution, 0.5 mL/min (153 cm/h)



(B)



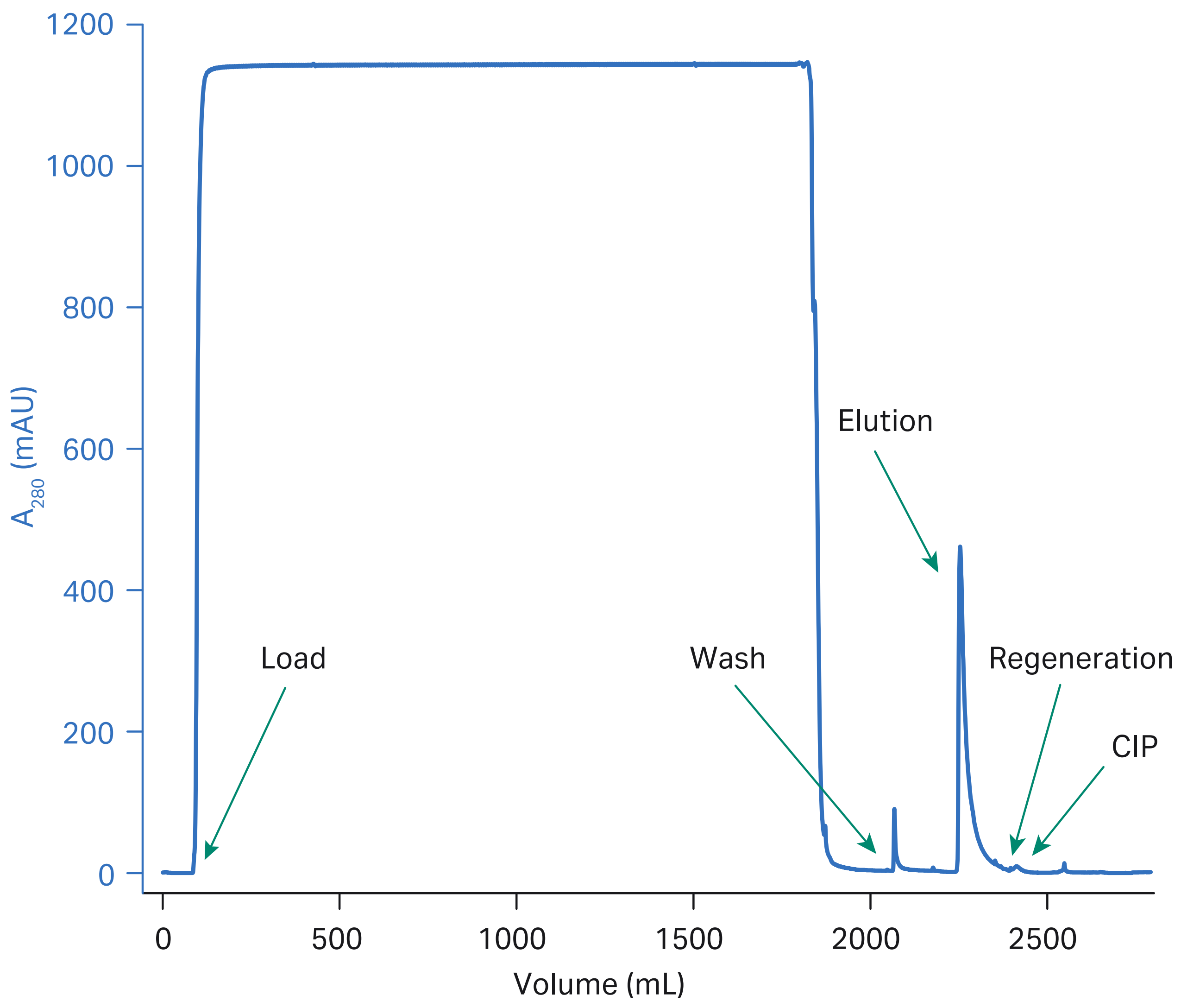
Lanes
1. Commercial FVII
2. Commercial FVII spiked in plasma
3. LMW markers
4. Flowthrough fraction
5. Wash after sample loading
6. Elution fraction 5
7. Elution fraction 6
8. Elution fraction 1 to 12 (pooled)
9. Strip after elution
10. LMW markers

Fig 3.7. (A) UV280 absorbance curve for purification of FVII from spiked plasma using VIISelect for the initial capture step. (B) SDS-PAGE of the FVII drug before and after purification on VIISelect. The gel was stained with Deep Purple total protein stain and scanned in a Typhoon™ scanner.

Capture of FIX

Capture of FIX from a Chinese hamster ovary (CHO) cell lysate was performed using IXSelect chromatography medium (Fig 3.8A). Fractions from the FIX capture step were analyzed by SDS-PAGE using a FIX reference preparation as standard (Fig 3.8B). The identity of the target protein was confirmed by Western blot analysis (Fig 3.8C).

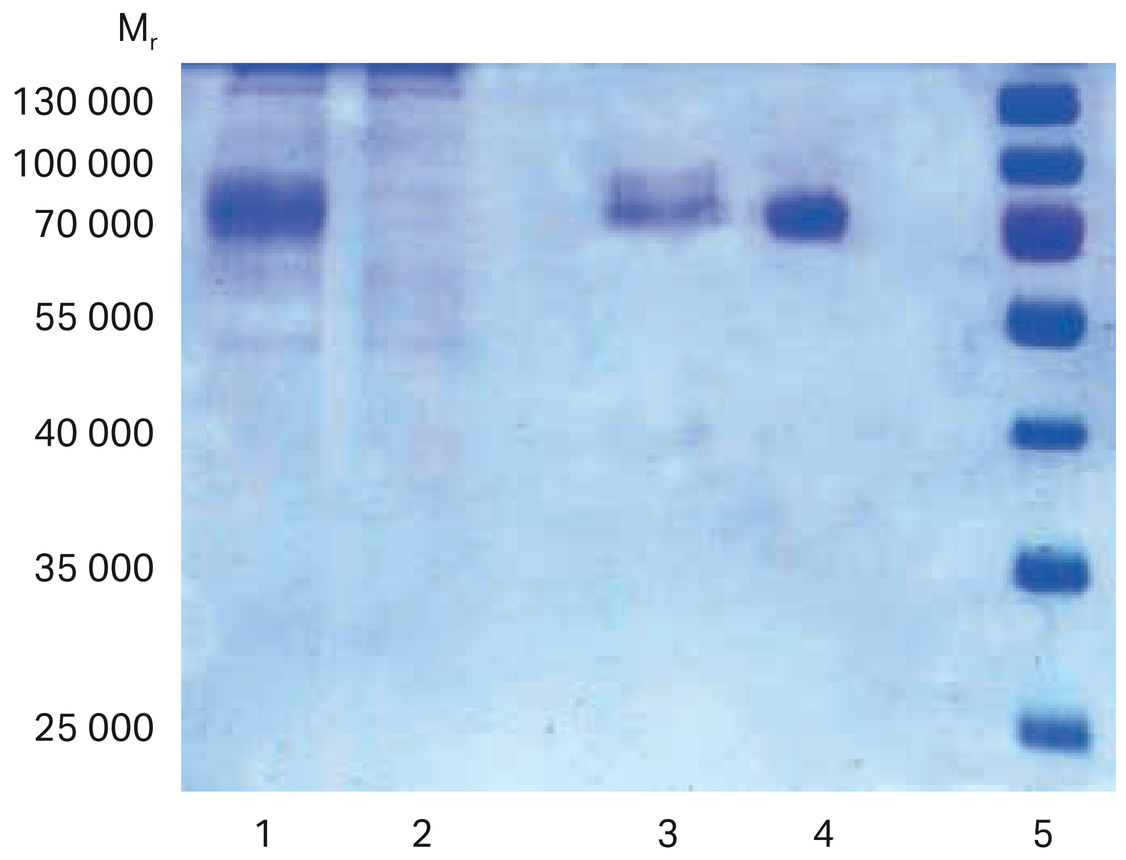
(A)



Column:	HiScale 16/20 packed with 13 mL of IXSelect medium
Sample:	FIX-containing CHO cell lysate
Equilibration:	6 CV of 20 mM Tris-HCl, 150 mM NaCl, pH 7.4
Sample load:	1500 mL sample feed loaded at 100 cm/h
Wash:	10 CV of equilibration buffer 10 CV of 20 mM Tris-HCl, 500 mM NaCl, 0.01% Tween 80, pH 7.4 3 CV of equilibration buffer
Elution:	20 mM Tris-HCl, 2 M $MgCl_2$, pH 7.4
Regeneration:	100 mM glycine buffer
CIP:	10 mM NaOH

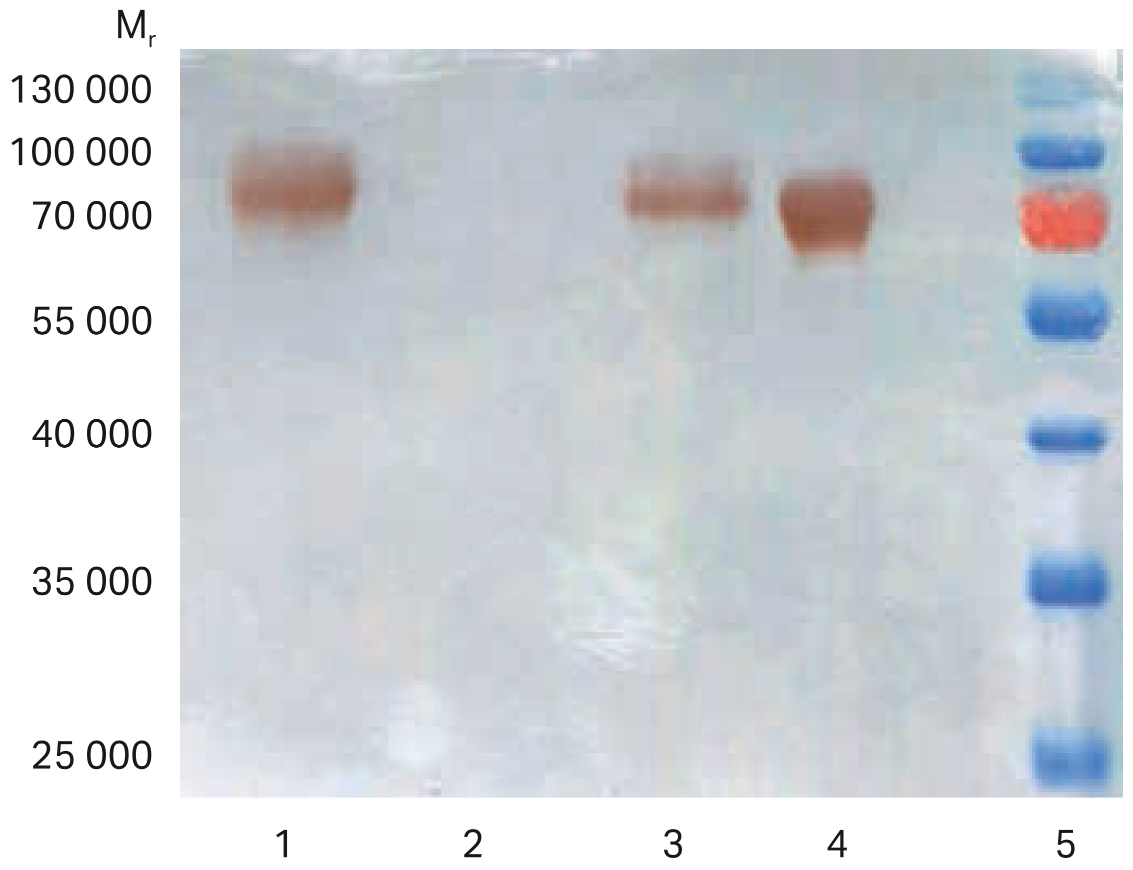
Fig 3.8. (A) FIX capture using IXSelect. (B) SDS-PAGE analysis of fractions from FIX capture step. (C) Western blot analysis of fractions from FIX capture step. Data from customer evaluation.

(B)



Lanes
1. Cell culture medium
2. Flowthrough
3. Elution peak
4. FIX standard
5. LMW markers

(C)



Lanes
1. Cell culture supernatant
2. Flowthrough
3. Elution peak
4. FIX standard
5. LMW markers

Purification of antithrombin III from bovine plasma

Figure 3.9 shows the result from purification of antithrombin III from bovine plasma using HiTrap Heparin HP. The antithrombin III eluted in the second peak.

Storage

Store at 4°C to 8°C in 20% ethanol.

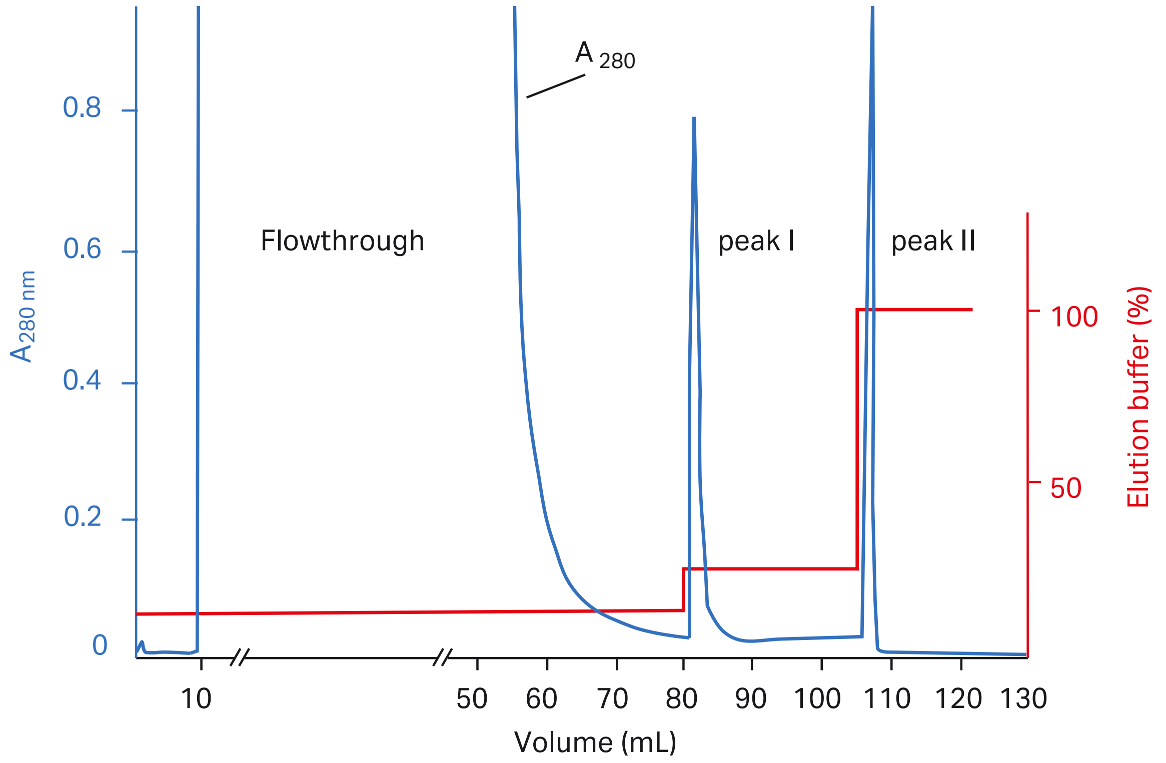
Performing a separation

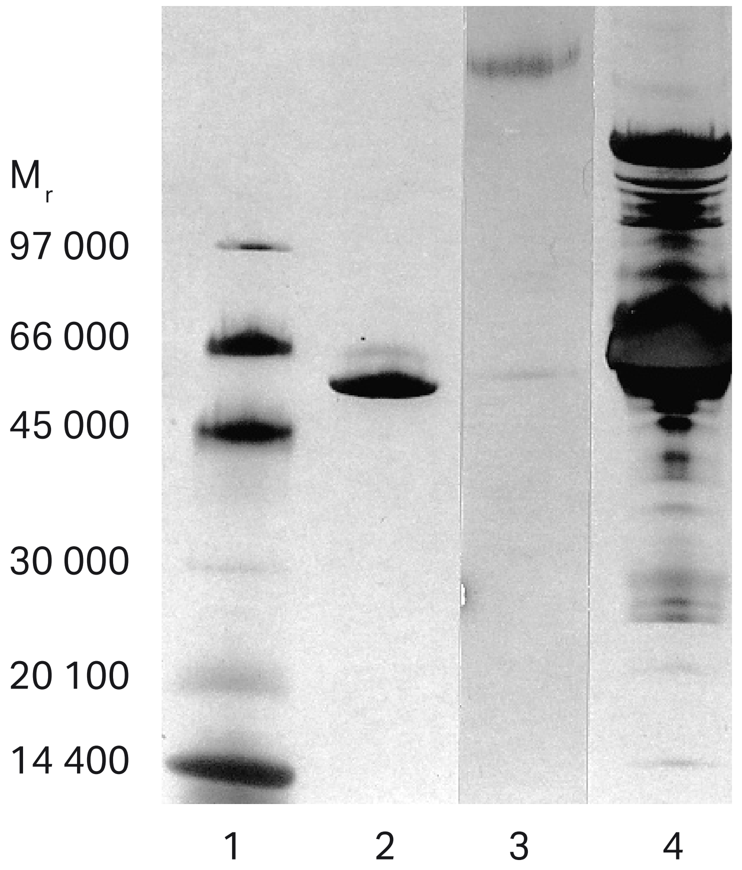
VIISelect

Binding buffer:	50 mM Tris-HCl, 150 mM NaCl, pH 7.5
Elution buffer:	50 mM Tris, 1.5 M NaCl, 50% (v/v) propylene glycol, pH 7.5
Regeneration buffer:	100 mM glycine

1. Equilibrate with 6 to 10 CV of binding buffer.
2. Load the sample.
3. Wash with 10 to 12 CV of binding buffer.
4. Elute with 6 to 12 CV elution buffer.
5. Regenerate the column with regeneration buffer.

Column:	HiTrap Heparin HP, 1 mL
Sample:	30 mL bovine plasma diluted with 15 mL 100 mM Tris, 10 mM citrate, 225 mM NaCl, pH 7.4
Binding buffer:	100 mM Tris, 10 mM citric acid, 225 mM NaCl, pH 7.4
Elution buffer:	100 mM Tris, 10 mM citric acid, 2 M NaCl, pH 7.4, 2.9 mg antithrombin-III was eluted in peak II
Flow rate:	1.0 mL/min
Electrophoresis:	SDS-PAGE, PhastSystem™ electrophoresis unit, PhastGel™ Gradient 8–25 SDS gel, 1 µL sample, silver stained





Lanes

1. LMW-SDS Marker Kit, reduced
2. Peak II, reduced, diluted two-fold
3. Peak I, reduced, diluted two-fold
4. Unbound material, reduced, diluted 15-fold

Fig 3.9. Purification of antithrombin III from bovine plasma on HiTrap Heparin HP, 1 mL.

VIIISelect

Binding buffer:	10 mM histidine, 20 mM calcium chloride, 300 mM sodium chloride, 0.02% Tween 80, pH 7.0
Wash buffer 1:	20 mM histidine, 20 mM calcium chloride, 300 mM sodium chloride, 0.02% Tween 80, pH 6.5
Wash buffer 2:	20 mM histidine, 20 mM calcium chloride, 1 M sodium chloride, 0.02% Tween 80, pH 6.5
Elution buffer:	20 mM histidine, 20 mM calcium chloride, 1.5 M sodium chloride, 0.02% Tween 80 dissolved in 50% ethylene glycol at pH 6.5

1. Equilibrate with 10 CV of binding buffer.
2. Load the sample in loading buffer.
3. Wash with 5 CV of wash buffer 1.
4. Wash with 5 CV of wash buffer 2.
5. Elute with 5 to 10 CV of elution buffer.

IXSelect

Binding buffer:	20 mM Tris-HCl, 150 mM NaCl, pH 7.4
Wash buffer:	20 mM Tris-HCl, 500 mM NaCl, 0.01% Tween 80, pH 7.4
Elution buffer:	20 mM Tris-HCl, 2 M $MgCl_2$, pH 7.4
Regeneration buffer:	100 mM glycine, 100 mM NaCl, pH 2.0

1. Equilibrate with 6 to 10 CV binding buffer.
2. Load the sample.
3. Wash with 10 CV of binding buffer.
4. Wash with 10 CV washing buffer.
5. Wash with 3 CV of binding buffer.
6. Elute with 5 to 10 CV elution buffer.
7. Regenerate the column with regeneration buffer.

Cleaning

The following are suggestions for solutions to be used during cleaning. Prolonged exposure to pH < 2.0 and pH > 12.0 should be avoided. The required cleaning is strongly dependent on the sample used, number of runs, conditions of the chromatography media etc. and has to be designed for each application.

- PAB (120 mM phosphoric acid, 167 mM acetic acid, 2.2% benzyl alcohol). Store in dark.
- 10 mM sodium hydroxide
- 100 mM citric acid

Purification or removal of DNA-binding proteins

Heparin Sepharose High Performance, Heparin Sepharose 6 Fast Flow, Capto Heparin

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 tagged proteins to enable more specific purification but their ability to bind DNA also enables group specific affinity purification using heparin as a ligand. Heparin is a highly sufonated 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 estrogen and androgen receptors
- Lipoproteins

The structure of heparin is shown in Figure 3.10. 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.

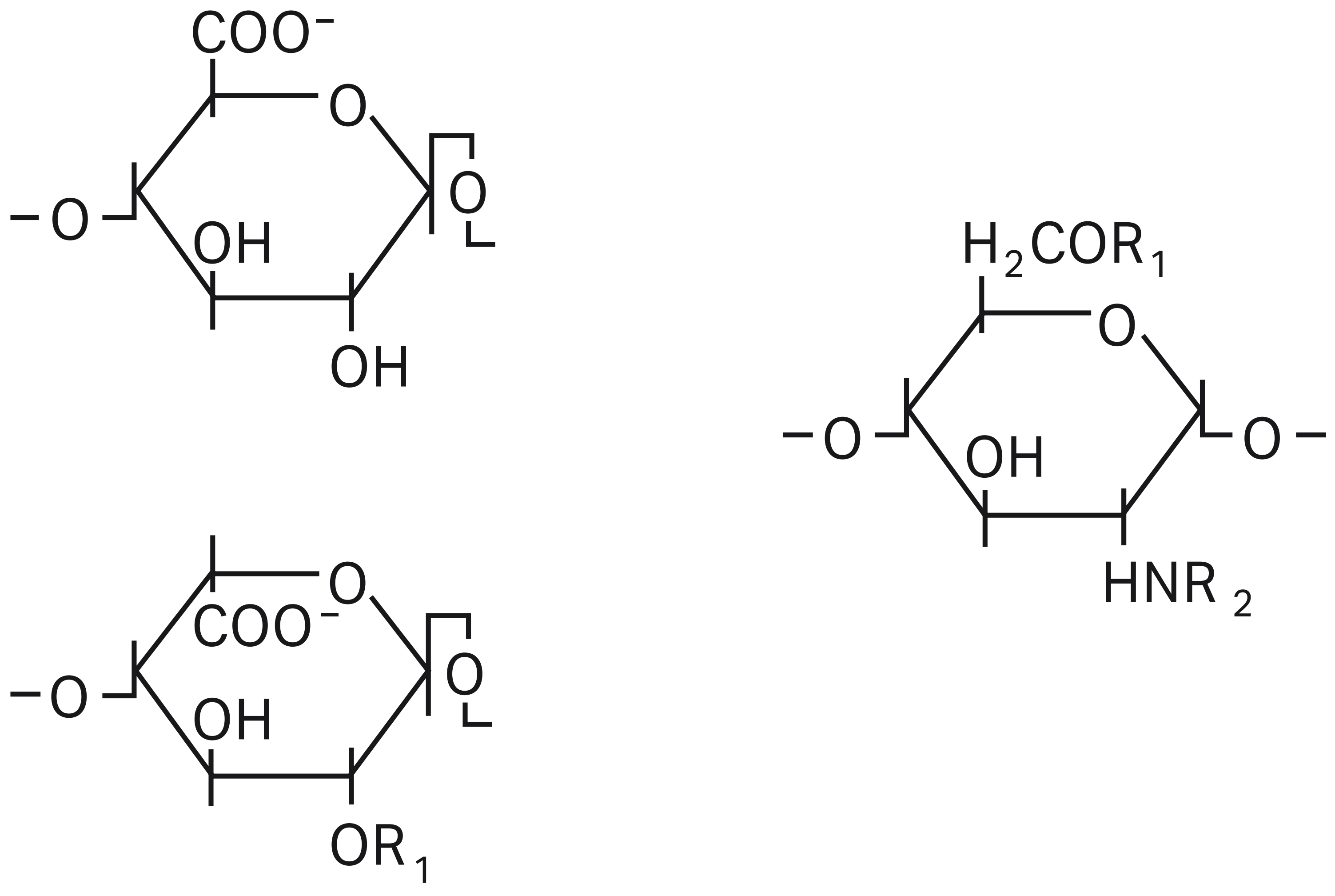


Fig 3.10. Structure of a heparin polysaccharide consisting of alternating hexuronic acid (A) and D-glucosamine residues (B). The hexuronic acid can either be D-glucuronic acid (top) or its C-5 epimer, L-iduronic acid (bottom). R_1 = -H or $-SO_3^-$, R_2 = $-SO_3^-$ or $-COCH_3$.

Chromatography media characteristics

Characteristics of chromatography media for purification or removal of DNA-binding proteins are shown in Table 3.11.

Table 3.11. Characteristics of Heparin Sepharose and Capto Heparin chromatography media

	Ligand density (mg/mL)	Composition	pH stability[1]	Average particle size (µm)
Heparin Sepharose High Performance	10	Heparin coupled to Sepharose High Performance by reductive amination to give a stable attachment even in alkaline conditions	Short term: 5 to 10 Long term: 5 to 10	34
Heparin Sepharose 6 Fast Flow	5.0	Heparin coupled to Sepharose 6 Fast Flow by reductive amination to give a stable attachment even in alkaline conditions	Short term: 4 to 13 Long term: 4 to 12	90
Capto Heparin	1.8	Heparin coupled to Capto	Short term: 4 to 13 Long term: 4 to 12	90

[1] Short term refers to the pH interval for regeneration, cleaning in place, and sanitization procedures. 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.

Purification options

Purification options for Heparin Sepharose 6 Fast Flow chromatography medium and prepacked columns as well as Capto Heparin are shown in Table 3.12.

Table 3.12. Purification options for purification of DNA-binding proteins

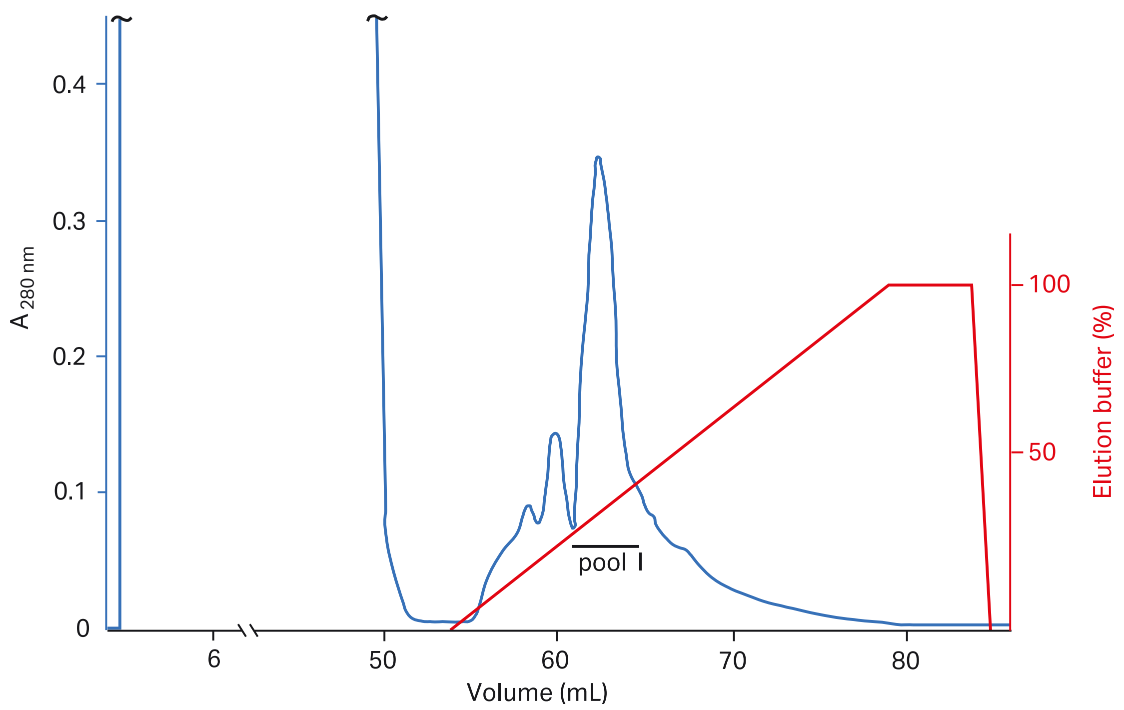
	Binding capacity	Maximum operating flow	Comments
HiTrap Heparin HP, 1 mL	Bovine antithrombin III, 3 mg/column	4 mL/min	Prepacked 1 mL column
HiTrap Heparin HP, 5 mL	Bovine antithrombin III, 15 mg/column	20 mL/min	Prepacked 5 mL column
HiPrep Heparin FF 16/10	Bovine antithrombin III, 40 mg/column	10 mL/min	Prepacked 20 mL column
Heparin Sepharose 6 Fast Flow	Bovine antithrombin III, 2 mg/mL medium	400 cm/h[1]	Supplied as suspension ready for column packing.
Capto Heparin	Antithrombin III, 1.4 mg/mL medium	700 cm/h[2]	Supplied as suspension ready for column packing

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

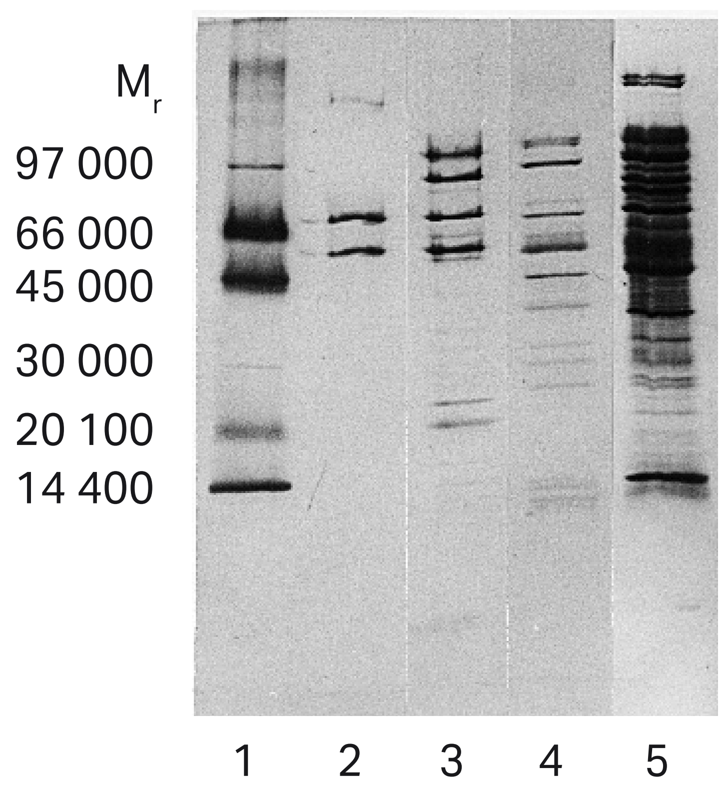
[2] 1 m diameter column, 20 cm bed height.

Purification examples

Figures 3.11 to 3.13 show examples of conditions used for the purification of different DNA binding proteins.



Column:	HiTrap Heparin HP, 1 mL
Sample:	49 mL *E. coli* lysate (= 1 g cells) after passage through a 5 mL DEAE Sepharose Fast Flow packed in column
Binding buffer:	20 mM Tris-HCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 2% glycerol, pH 8.0
Elution buffer:	Binding buffer + 1 M NaCl
Elution conditions:	25 mL elution buffer, linear gradient 0% to 100%
Flow rate:	1.0 mL/min

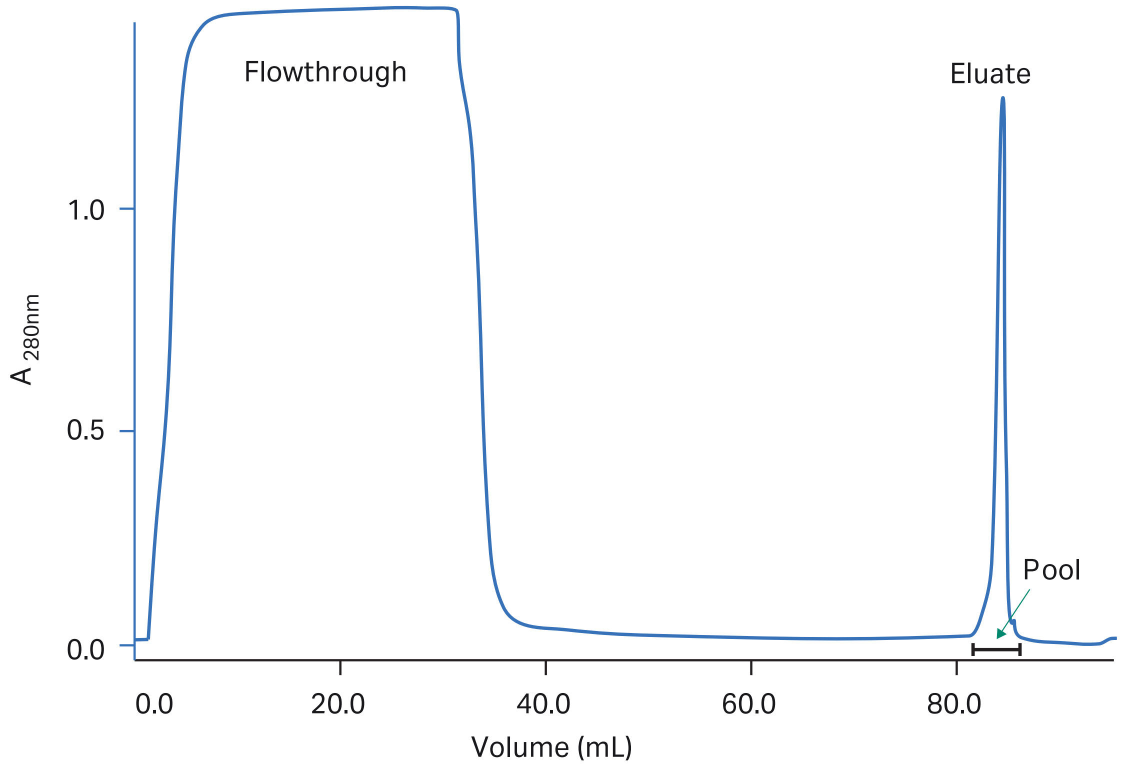


Lanes

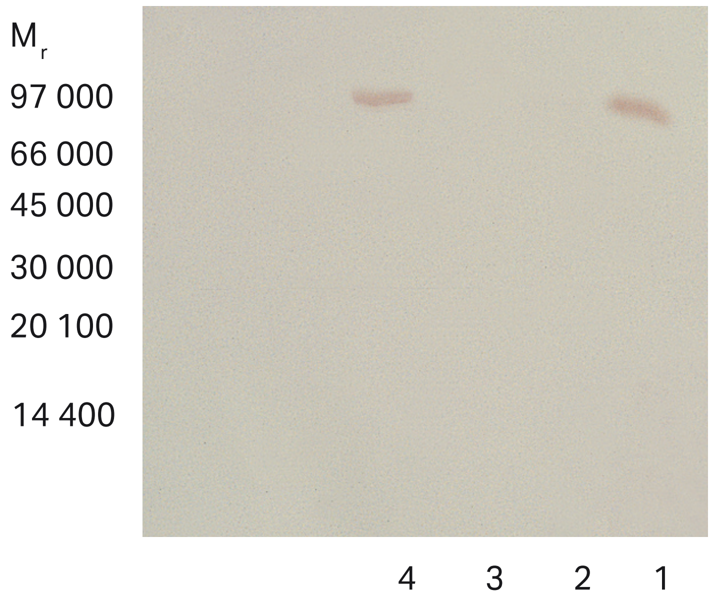
SDS-PAGE, Gradient 8–25, 1 mL sample, silver stained.

1. LMW-SDS Marker Kit, reduced
2. Reverse transciptase, reduced
3. Pool I from HiTrap Heparin HP, 1 mL reduced
4. Unbound material from DEAE Sepharose Fast Flow, reduced
5. Cell lysate, reduced

Fig 3.11. Partial purification of recombinant HIV-reverse transcriptase on HiTrap Heparin HP.



Column:	HiTrap Heparin HP, 5 mL
Sample:	30 mL extract containing Oct-1, filtered (0.45 µm) and transferred to binding buffer using a PD-10 desalting column
Binding buffer:	20 mM Tris-HCl, 5% (v/v) glycerol, 0.1 mM EDTA, 10 mM 2-mercaptoethanol 0.1 mM Pefabloc™, 500 mM 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 8.0
Flow rate (flow velocity):	1 mL/min (30 cm/h)

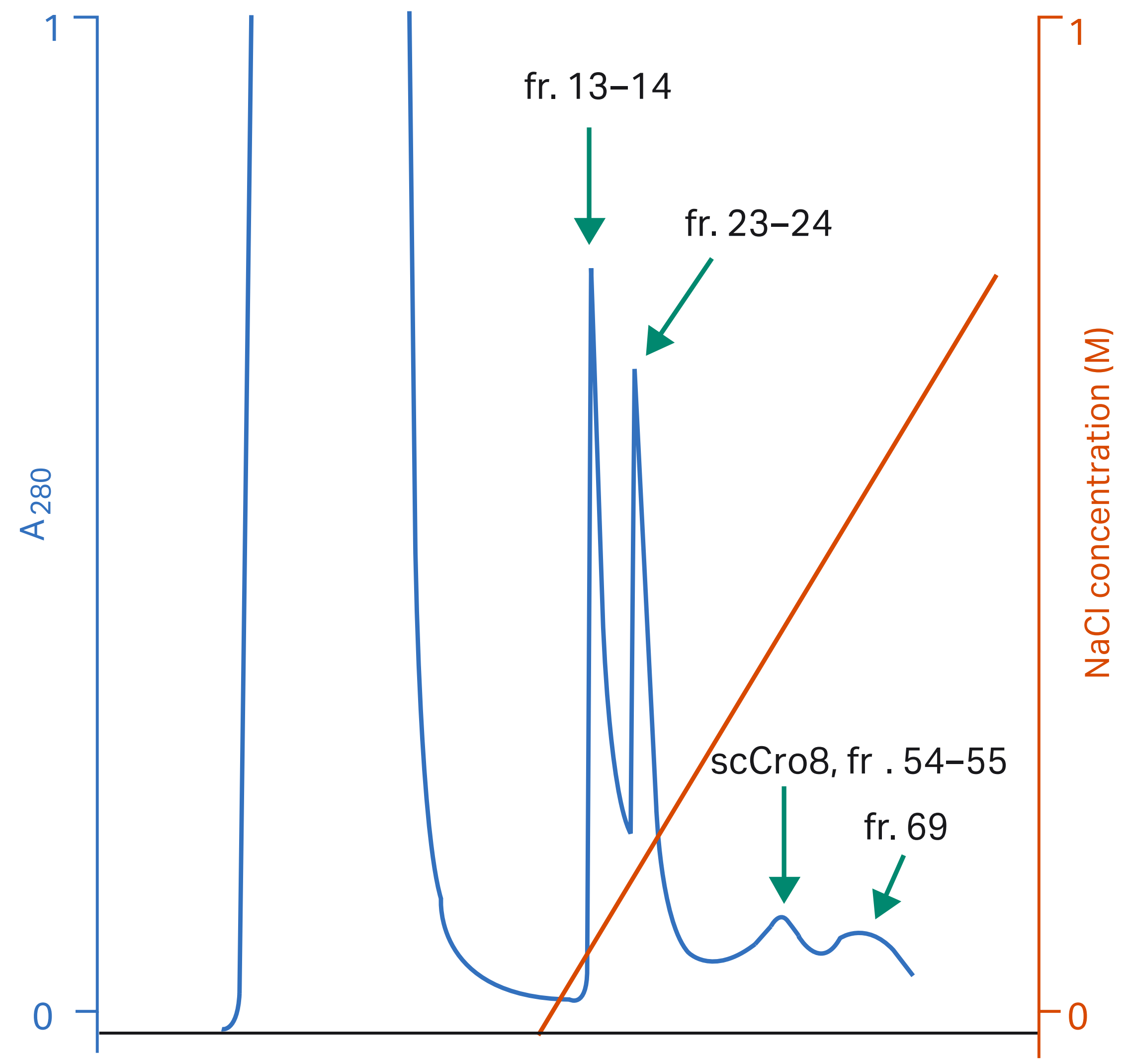


Lanes

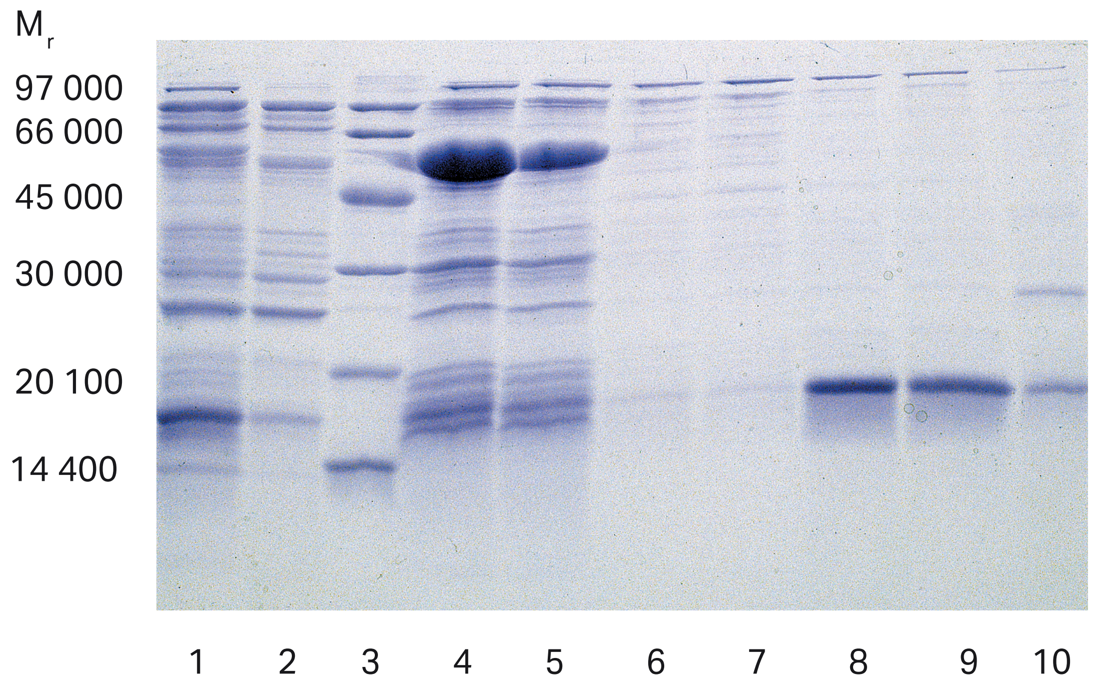
Western blot of the electrophoresis gel using rabbit anti-Oct human-1 and alkaline phosphatase

1. Starting material
2. Flowthrough
3. LMW-SDS Marker Kit
4. Eluate pool

Fig 3.12. 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.



Column:	HiPrep Heparin FF 16/10
Sample:	2000 mL partially purified sample from DEAE Sepharose CL-4B flowthrough, pH 7.0
Binding buffer:	50 mM sodium phosphate, pH 7.5
Elution buffer:	50 mM sodium phosphate, 1 M sodium chloride, pH 7.5
Flow rate (flow velocity):	1.5 mL/min (45 cm/h)



Lanes

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

1. Pool from HiPrep 26/10 Desalting
2. Flowthrough pool from DEAE Sepharose CL-4B
3. LMW-SDS Marker Kit

4–10. Eluted fractions from HiPrep Heparin FF 16/10

4. Fraction 13
5. Fraction 14
6. Fraction 23
7. Fraction 24
8. Fraction 54
9. Fraction 55
10. Fraction 69

Fig 3.13. scCro8 purification on HiPrep Heparin FF 16/10.

Performing a separation

Heparin Sepharose 6 Fast Flow, Heparin Sepharose High Performance

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

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

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

If used for purification or removal of coagulation factors:

☞ Since the heparin acts as an affinity ligand for coagulation factors, it is advisable to include a minimum concentration of 150 mM NaCl in the binding buffer.

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

Cleaning

Remove ionically bound proteins by washing with 0.5 CV of 2 M NaCl for 10 to 15 min.

Remove precipitated or denatured proteins by washing with 4 CV of 100 mM NaOH for 1 to 2 h; or 2 CV of 6 M guanidine hydrochloride for 30 to 60 min; or 2 CV of 8 M urea for 30 to 60 min.

Remove hydrophobically bound proteins by washing with 4 CV of 0.1% to 0.5% Tween 20 for 1 to 2 h.

Chemical stability

100 mM NaOH (1 w at 20°C), 50 mM sodium acetate, pH 4.0, 4 M NaCl, 8 M urea, 6 M guanidine hydrochloride.

Storage

Wash chromatography media and columns with 50 mM sodium acetate containing 20% ethanol (use approximately 5 CV for packed media) and store at 4°C to 8°C.

Capto Heparin

Binding buffer:	100 mM Tris-HCl, 10 mM trisodium citrate, 225 mM NaCl, pH 7.4
Wash buffer:	100 mM Tris-HCl, 10 mM trisodium citrate, 330 mM NaCl, pH 7.4
Elution buffer:	100 mM Tris-HCl, 10 mM trisodium citrate, 2 M NaCl, pH 7.4

1. Equilibrate with 5 CV of binding buffer.
2. Apply the sample.
3. Wash step 1: wash with 40 CV of binding buffer.
4. Wash step 2: wash with 15 CV of wash buffer.
5. Elute with 9.5 CV of elution buffer

☞ A flow rate of 0.5 mL/min is recommended for a 1 mL column.

Cleaning

Substances such as denatured proteins that do not elute during regeneration can be removed by cleaning-in-place (CIP) procedures. A recommended CIP procedure for Capto Heparin is 4 CV of 100 mM NaOH with a contact time of 1 to 2 h.

Storage

Store unused chromatography media at 4°C to 30°C in 20% ethanol and 50 mM of sodium acetate.

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.

Chromatography medium characteristics

The characteristics of Gelatin Sepharose 4B chromatography medium are shown in Table 3.13.

Table 3.13. Characteristics of Gelatin Sepharose 4B chromatography medium

	Ligand density (mg/mL)	Composition	pH stability[1]	Average particle size (µm)
Gelatin Sepharose 4B	4.5 to 8.0	Gelatin coupled to Sepharose 4B using the CNBr method	Short term: 3 to 10 Long term: 3 to 10	90

[1] Short term refers to the pH interval for regeneration, cleaning in place, and sanitization procedures. 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.

Purification option

Gelatin Sepharose 4B is available in chromatography media packs for packing into columns of your choice (Table 3.14).

Table 3.14. Purification option for Gelatin Sepharose 4B

	Binding capacity/mL medium	Maximum operating flow (cm/h)[1]	Comments
Gelatin Sepharose 4B	Human plasma fibronectin, 1 mg	75	Supplied as suspension ready for column packing

[1] See Appendix 4 to convert flow velocity (cm/h) to volumetric flow rate (mL/min). 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:	50 mM sodium acetate, 1 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 three times with 2 to 3 CV of buffer, alternating between high pH (100 mM Tris-HCl, 500 mM NaCl, pH 8.5) and low pH (100 mM sodium acetate, 500 mM NaCl, pH 4.5). Re-equilbrate immediately with 3 to 5 CV of binding buffer. Remove denatured proteins or lipids by washing the column with 0.1% Tween 20 at 37°C for 1 min. Re-equilibrate immediately with 5 CV of binding buffer.

Chemical stability

Stable in all commonly used aqueous buffers.

Storage

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

Purification or removal of glycoproteins and polysaccharides

Con A Sepharose 4B, Lentil Lectin Sepharose 4B, Capto Lentil 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.

Chromatography media screening

To select the optimum lectin for purification, it may be necessary to screen different chromatography media. The ligands, Concanavalin A (Con A) and Lentil Lectin provide a spectrum of parameters for the separation of glycoproteins. Table 3.15 gives their specificity.

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, for example, during development
- Isolation of cell surface glycoproteins from detergent-solubilized membranes
- Separation of membrane vesicles into "inside out" and "right side out" fractions

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 hemagglutinin 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.

Table 3.15. 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 a(1,6) to N-acetyl-glucosamine, (αMan > αGlc > GlcNAc)

Chromatography media characteristics

Characteristics of Con A and Lentil Lectin chromatography media are shown in Table 3.16.

Table 3.16. Characteristics of Con A and Lentil Lectin chromatography media

	Ligand density (mg/mL)	Composition	pH stability[1]	Average particle size (μm)
Con A Sepharose 4B	10 to 16	Con A coupled to Sepharose 4B by CNBr method	Short term: 4 to 9 Long term: 4 to 9	90
Lentil Lectin Sepharose 4B	2.5	Lentil lectin coupled to Sepharose 4B by CNBr method	Short term: 3 to 10 Long term: 3 to 10	90
Capto Lentil Lectin	3	Lentil lectin coupled to Capto matrix by NHS-method	Short term: 3 to 10 Long term: 3 to 10	75

[1] Short term refers to the pH interval for regeneration, cleaning in place, and sanitization procedures. 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.

Purification options

Purification options for Con A and Lentil Lectin chromatography media and prepacked columns are shown in Table 3.17.

Table 3.17. Con A and Lentil Lectin chromatography media and prepacked columns

	Binding capacity/mL medium	Maximum operating flow	Comments
Con A Sepharose 4B	Porcine thyroglobulin, 20 to 45 mg	75 cm/h[2]	Supplied as suspension ready for column packing[1]
Lentil Lectin Sepharose 4B	Porcine thyroglobulin, 16 to 35 mg	75 cm/h[1]	Supplied as suspension ready for column packing
Capto Lentil Lectin	Porcine thyroglobulin ~ 15 mg	100 to 300 cm/h[2]	Supplied as suspension ready for column packing
HiTrap Con A 4B, 1 mL	Porcine thyroglobulin, 20 to 45 mg	4 mL/min	Prepacked 1 mL column
HiTrap Con A 4B, 5 mL	100 to 225 mg Porcine thyroglobulin	20 mL/min	Prepacked 5 mL column
HiTrap Capto Lentil Lectin, 1 mL	Porcine thyroglobulin ~ 15 mg	2 mL/min	Prepacked 1 mL column
HiTrap Capto Lentil Lectin, 5 mL	Porcine thyroglobulin ~ 75 mg	10 mL/min	Prepacked 5 mL column
HiScreen Capto Lentil Lectin	Porcine thyroglobulin ~ 70 mg	2.3 mL/min	Prepacked 4.7 mL column

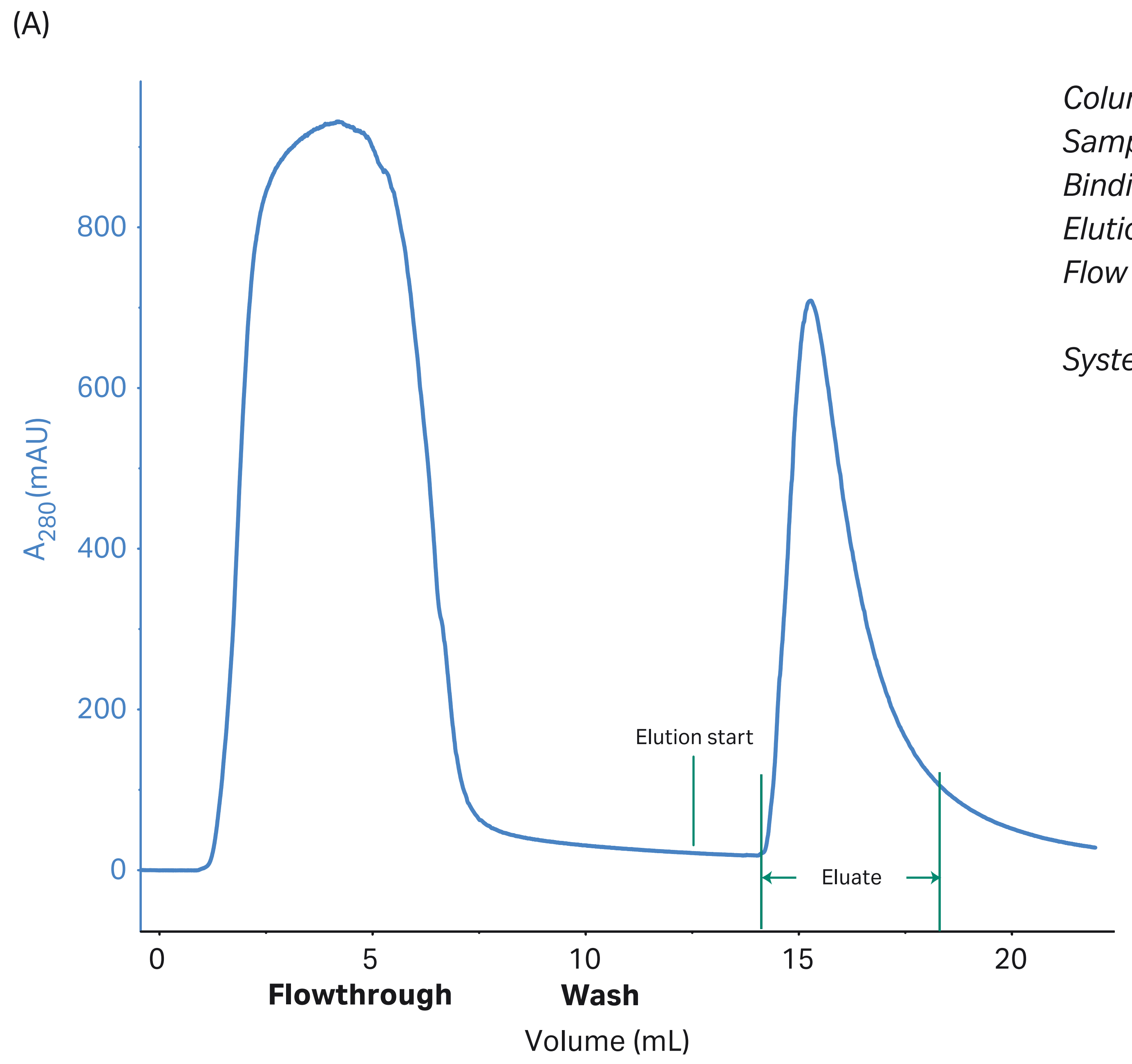
[1] See Appendix 4 to convert flow velocity (cm/h) to volumetric flow rate (mL/min). Maximum operating flow is calculated from measurement in a packed column with a bed height of 10 cm and i.d. of 5 cm.

[2] Supplied in acetate buffer solution (100 mM, pH 6.0) containing 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20% ethanol.

Purification example

Enrichment of glycoproteins from human plasma

Glycoproteins from human plasma were enriched on a HiTrap Con A 4B 1 mL column (Fig 3.15A). Analysis by Coomassie stained SDS-PAGE (nonreducing conditions), showed that unfractionated plasma (start material), flowthrough, and wash fractions all had band corresponding to molecular weight of 67 000 (Fig 3.15B). This corresponds to high-abundance, nonglycosylated serum albumin, which was removed from the sample and not detected in the eluate containing the glycoproteins.



Column:	HiTrap Con A 4B, 1 mL
Sample:	0.5 mL human plasma diluted to 5 mL with binding buffer
Binding buffer:	20 mM Tris, 500 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4
Elution buffer:	20 mM Tris, 500 mM NaCl, 300 mM methyl-D-glucoside, pH 7.4
Flow rates:	0.2 mL/min (sample load) 0.1 mL/min with pause 4 min (elution)
System:	ÄKTA system

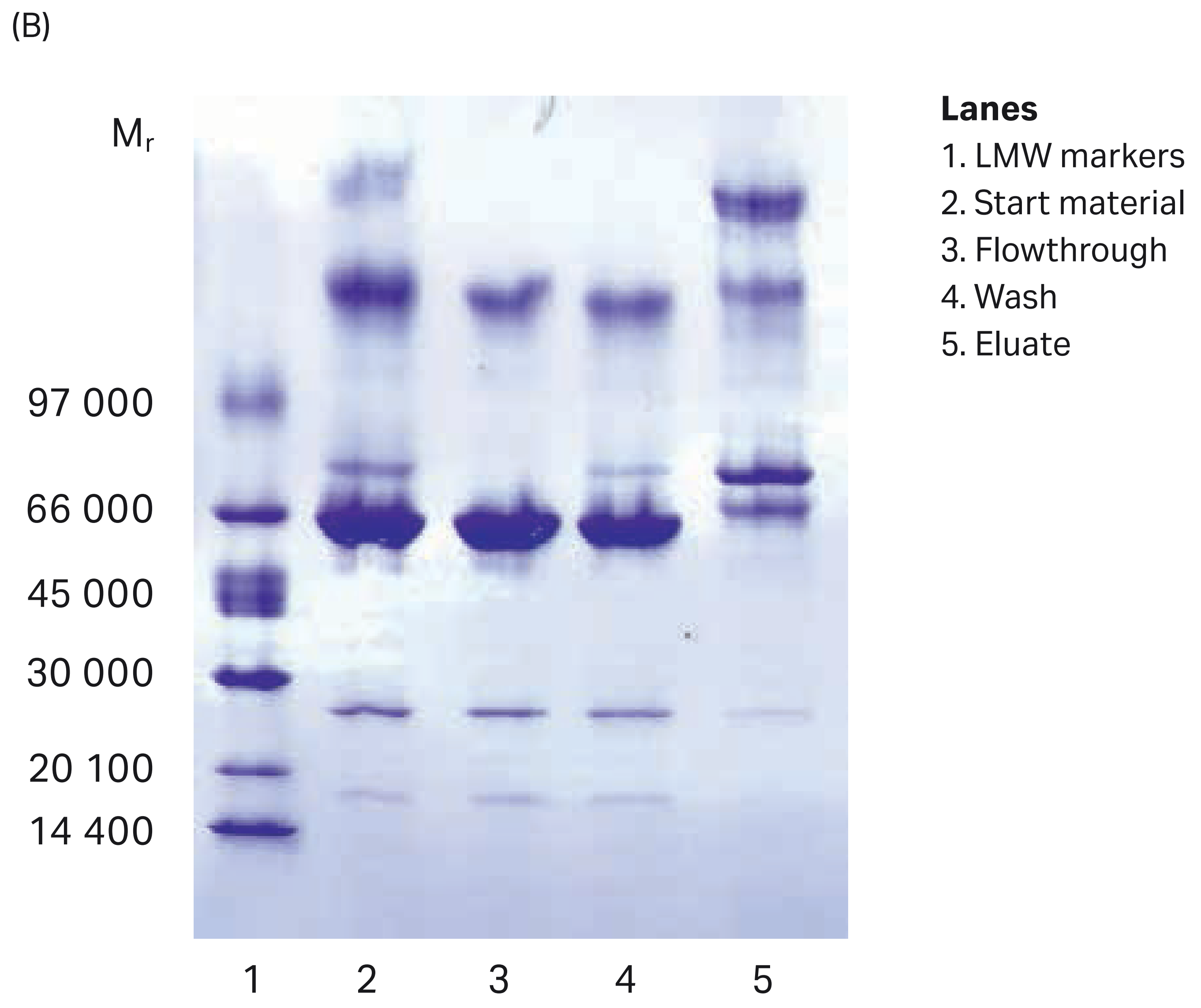


Fig 3.15. (A) Chromatographic enrichment of glycoproteins from human plasma using HiTrap Con A 4B, 1 mL. (B) SDS-PAGE analysis with Coomassie stained ExcelGel™ 8–18 Gradient gel (nonreducing conditions) of fractions from enrichment of glycoproteins from human plasma using HiTrap Con A 4B, 1 mL.

Performing a separation

Con A Sepharose 4B

Binding buffer:	20 mM Tris-HCl, 500 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4
Elution buffer:	100 to 500 mM methyl-α-D-glucopyranoside (methyl-α-D-glucoside) or methyl-α-D-mannopyranoside (methyl-α-D-mannoside), 20 mM Tris-HCl, 500 mM NaCl, pH 7.4

1. Pack the column (see Appendix 3) and wash with at least 10 CV of binding buffer to remove preservative.
2. Equilibrate the column with 10 CV of binding buffer.
3. Apply the sample, using a low flow velocity from 15 cm/h, during sample application (flow velocity is the most significant factor to obtain maximum binding).
4. Wash with 5 to 10 CV of binding buffer or until no material appears in the eluent (monitored by UV absorption at A_{280} nm).
5. Elute with 5 CV 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 lentil lectin, the Lentil Lectin Sepharose 4B chromatography medium provides a suitable alternative to improve recovery.

☞ For complex samples containing glycoproteins with different affinities for the lectin, a continuous gradient or step elution can improve resolution. Recovery can sometimes be improved by pausing the flow for a few 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, manganese ions (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 CV of 500 mM NaCl, 20 mM Tris-HCl, pH 8.5, followed by 500 mM NaCl, 20 mM acetate, pH 4.5. Repeat three times before re-equilibrating with binding buffer.

Remove strongly bound substances by:

- washing with 100 mM borate, pH 6.5 at a low flow rate
- washing with 20% ethanol or up to 50% ethylene glycol
- washing with 0.1% Tween 20 at 37°C for 1 min

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

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 Mn^{2+} from the lectin or dissociate Con A, resulting in loss of activity.

Storage

Wash chromatography media and columns with 20% ethanol in 100 mM acetate, 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, pH 6.0 (use approximately 5 CV for packed media) and store at 4°C to 8°C.

Lentil Lectin Sepharose

Binding buffer: 20 mM Tris-HCl, 500 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4

Elution buffer: 100 to 500 mM methyl-α-D-glucopyranoside (methyl-α-D-glucoside), 20 mM Tris-HCl, 500 mM NaCl, pH 7.4

Buffers for soluble glycoproteins:

Binding buffer: 20 mM Tris-HCl, 500 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4

Elution buffer: 300 mM methyl-α-D-mannopyranoside (methyl-α-D-mannoside), 20 mM Tris-HCl, 500 mM NaCl, pH 7.4

Buffers for detergent-solubilized proteins:

Equilibrate column with the buffer 20 mM Tris-HCl, 500 mM 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, 500 mM NaCl, 0.5% sodium deoxycholate, pH 8.3

Elution buffer: 300 mM methyl-α-D-mannopyranoside, 20 mM Tris-HCl, 500 mM NaCl, 0.5% sodium deoxycholate, pH 8.3

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

☞ Below pH 5.0, 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 multistep elution can improve resolution. Recovery can sometimes be improved by pausing the flow for a few minutes during elution

☞ Elute tightly bound substances by lowering pH, but not below pH 3.0. In some cases, strongly bound substances can be eluted with detergent, for example 1.0% deoxycholate.

Cleaning

Wash with 10 CV of 500 mM NaCl, 20 mM Tris-HCl, pH 8.5, followed by 500 mM NaCl, 20 mM acetate, pH 4.5. Repeat three times before re-equilibrating with binding buffer.

Remove strongly bound substances by:

- washing with 100 mM borate, pH 6.5 at a low flow rate
- washing with 20% ethanol or up to 50% ethylene glycol
- washing with 0.1% Tween 20 at 37°C for 1 min

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

Chemical stability

To avoid loss of activity of the coupled lectin, avoid solutions having a pH below 3.0 or above 10.0, buffers that contain metal chelating agents such as EDTA, high concentrations of guanidine hydrochloride, or high concentrations of urea.

Storage

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

Purification or removal of granulocyte-colony stimulating factor

GCSFSelect

Granulocyte-colony stimulating factor (G-CSF) is a hormone that stimulates production of white blood cells in bone marrow. GSCFSelect is specifically designed for purification of recombinant G-CSF and is based on a highly rigid agarose base matrix that allows for high flow rates at large production scales. For a highly selective purification step, the affinity ligand is based on a single-chain antibody fragment directed against G-CSF. To facilitate binding of the target molecule, the ligand is attached to the base matrix through a hydrophilic spacer arm (Fig 3.16). The ligand is produced in a yeast expression system, where fermentation, subsequent purification, and formulation are performed in the absence of animal-derived components. The rigid agarose base matrix of GCSFSelect allows for processing of large sample volumes.

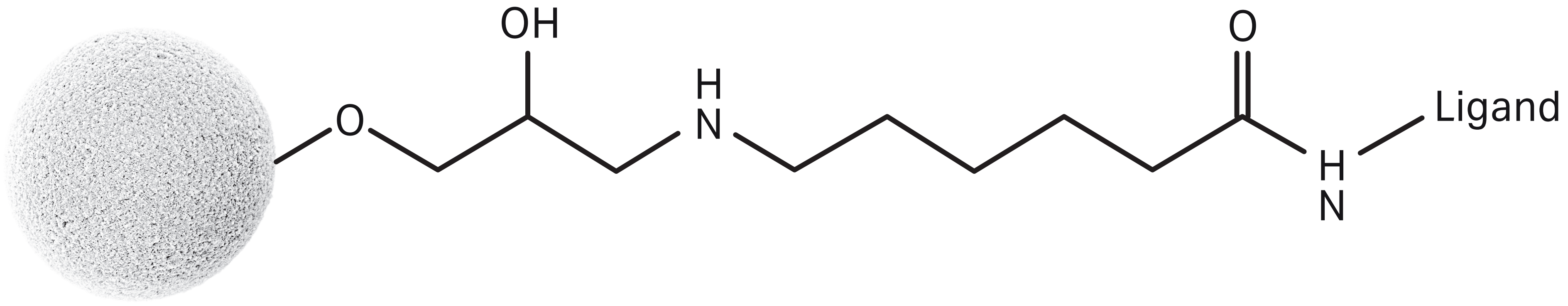


Fig 3.16. Structure of GCSFSelect.

Chromatography medium characteristics

Characteristics of GCSFSelect chromatography medium are shown in Table 3.18.

Table 3.18. Characteristics of GCSFSelect chromatography medium

Product	Ligand	Composition	pH stability[1]	Average particle size (µm)
GCSFSelect	Single-chain antibody fragment (M_r 14 400) directed against G-CSF and produced in *Saccharomyces cerevisiae*	Ligand coupled to Capto via stable amide bonds	Short term: 2 to 12 Long term: 3 to 10	75

[1] Short term refers to the pH interval for regeneration, cleaning in place, and sanitization procedures. 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.

Purification options

GCSFSelect is available in chromatography media packs for packing in columns and in prepacked HiTrap columns, see Table 3.19.

Table 3.19. Purification options for GCSFSelect chromatography medium and prepacked columns

	Binding capacity	Maximum operating flow	Comments
GCSFSelect	G-CSF, 3.9 mg/mL medium	600 cm/h[1]	Media suspension ready for column packing
HiTrap GCSFSelect, 1 mL	G-CSF, 3.9 mg/column	4 mL/min	Prepacked 1 mL column
HiTrap GCSFSelect, 5 mL	G-CSF, 19.5 mg/column	20 mL/min	Prepacked 5 mL column

[1] Flow velocity measured in a 1 m diameter column, 20 cm bed height at 20°C; buffers used had same viscosity as water at < 0.3 MPa (3 bar, 43.5 psi).

Purification examples

Figure 3.17A shows an example of a purification of G-CSF using GCSFSelect. The sample was *E. coli* lysate spiked with recombinant G-CSF and elution was performed using bis-Tris buffer containing 0.08% Tween and 1 M $MgCl_2$ (pH 7.0). Fractions from the purification were further analyzed by SDS-PAGE (Fig 3.17B). The single eluted peak with high purity demonstrates the high selectivity for G-CSF of the GCSFSelect medium.

Performing a separation

Binding buffer:	PBS (10 mM sodium phosphate, 140 mM NaCl), pH 7.4
Elution buffer:	20 mM bis-Tris, 0.08% Tween 20, 1 M $MgCl_2$, pH 7.0

1. Equilibrate with 10 CV of binding buffer.
2. Load the sample.
3. Wash with binding buffer until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
4. Elute with 5 to 10 CV of elution buffer.

Cleaning

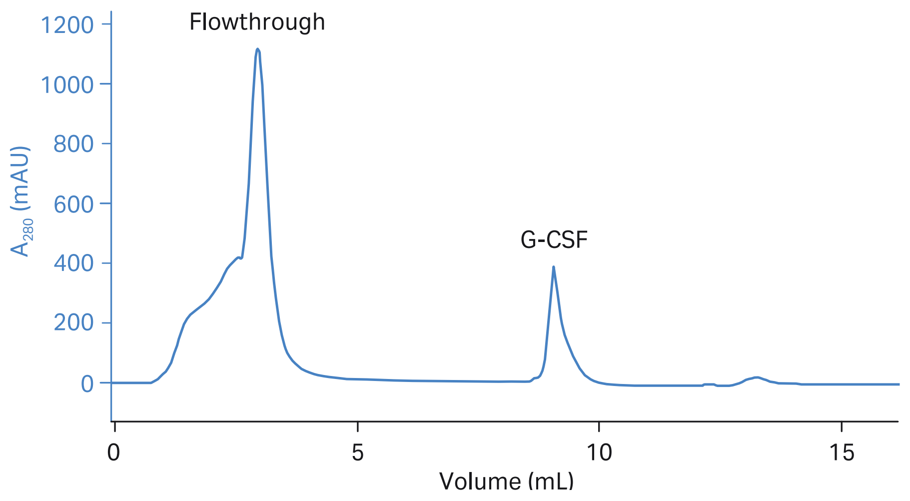
Solutions such as PAB (120 mM phosphoric acid, 167 mM acetic acid, 2.2% benzyl alcohol) in cleaning protocols are suggested. Cleaning and sanitization protocols should be designed for each process as the efficiency of the protocol is strongly associated with the sample and other related operating conditions.

Storage

Store at 4°C to 8°C in 20% ethanol.

(A)

Column:	Tricorn 5/20 packed with 1 mL of GCSFSelect
Sample:	1.65 mL homogenized and clarified *E. coli* lysate spiked with 2.5 mg/mL G-CSF registered drug
Binding buffer:	PBS, pH 7.4
Elution buffer:	20 mM bis-Tris, 0.08% Tween 20, 1 M $MgCl_2$, pH 7.0
CIP:	100 mM glycine, pH 2.0
System:	ÄKTA system



(B)

Lanes

1. G-CSF registered drug (M_r 19 900)
2. *E. coli* lysate spiked with G-CSF registered drug
3. Flowthrough
4. Wash
5. Eluate
6. LMW markers

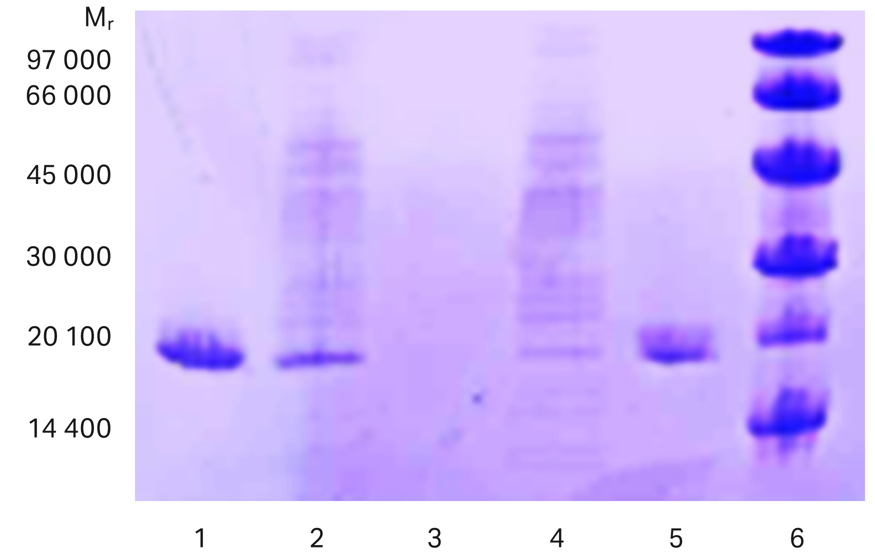


Fig 3.17. (A) Purification of G-CSF from a G-CSF spiked *E. coli* lysate. (B) SDS-PAGE of the different fractions collected during purification of G-CSF using GCSFSelect.

Purification or removal of NAD^+-dependent dehydrogenases and ATP-dependent kinases

Blue Sepharose 6 Fast Flow, Capto Blue, Capto Blue (high sub)

NAD^+-dependent dehydrogenases and ATP-dependent kinases are members of a 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, 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. For details about Blue Sepharose 6 Fast Flow, Capto Blue, and Capto Blue (high sub), see *Purification or removal of albumin* in this chapter.

Performing a separation

Blue Sepharose 6 Fast Flow, Capto Blue, Capto Blue (high sub)

The information supplied in *Purification or removal of albumin* earlier in this chapter is applicable also to the purification of enzymes with an affinity for NAD^+, but note the following:

☞ For elution, use low concentrations of the free cofactor, NAD^+ or $NADP^+$ (1 to 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.

Purification or removal of NADP+-dependent dehydrogenases and other enzymes with affinity for NADP+

2′5′ ADP Sepharose 4B

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 N6 of the purine ring. The derivatized ADP is then coupled to Sepharose 4B via the aminohexane spacer. Figure 3.18 shows the partial structure of 2′5′ ADP Sepharose 4B.

Chromatography medium characteristics

Characteristics of 2′5′ ADP Sepharose 4B are shown in Table 3.21.

Table 3.21. Characteristics of 2′5′ ADP Sepharose 4B chromatography medium

	Ligand density (µmol/mL)	Composition	pH stability[1]	Average particle size (µm)
2′5′ ADP Sepharose 4B	2	N6-(6-aminohexyl) adenosine 2′5′ bisphosphate coupled to Sepharose 4B by CNBr method[2]	Short term: 4 to 10 Long term: 4 to 10	90

[1] Short term refers to the pH interval for regeneration, cleaning in place, and sanitization procedures. 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.

[2] Coupling via the N^6 position of the NADP+-analog, adenosine 2′5′ bisphosphate, gives a ligand that is stereochemically acceptable to most NADP+-dependent enzymes.

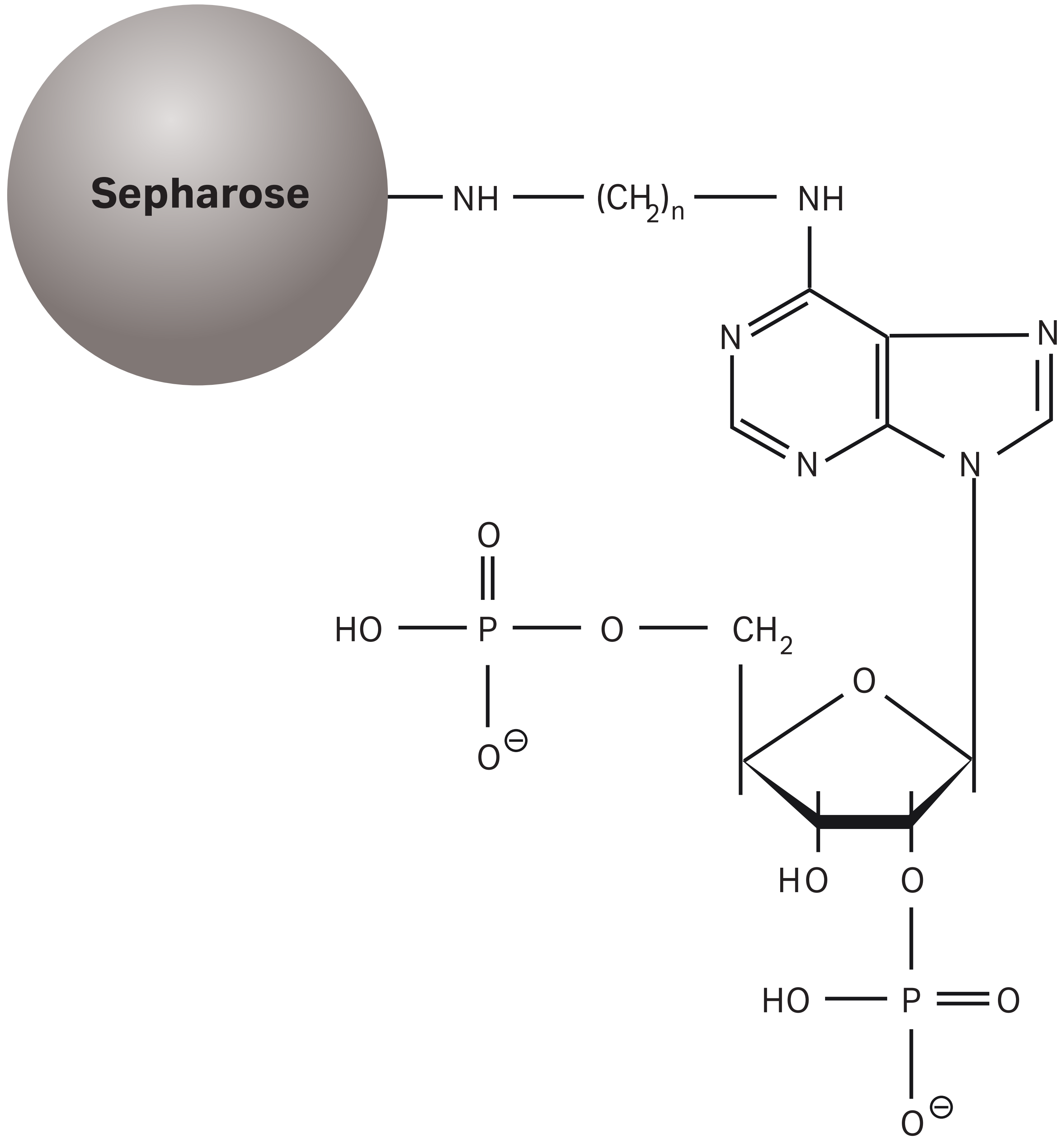


Fig 3.18. Partial structure of 2′5′ ADP Sepharose 4B.

Purification options

2'5' ADP Sepharose 4B is available in chromatography media packs for packing in columns, see Table 3.22.

Table 3.22. Purification options for 2'5' ADP Sepharose 4B

	Binding capacity/mL medium	Maximum operating flow velocity (cm/h)[1]	Comments
2'5' ADP Sepharose 4B	Glucose-6-phosphate, dehydrogenase, 0.4 mg (100 mM Tris-HCl, 5 mM EDTA, 1 mM 2-mercaptoethanol buffer, pH 7.6)	75	Supplied as a freeze-dried powder, rehydration required

[1] See Appendix 4 to convert flow velocity (cm/h) to volumetric flow rate (mL/min). 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 3.19 shows a linear gradient elution used for the initial separation of $NADP^+$-dependent enzymes from a crude extract of *Candida utilis*.

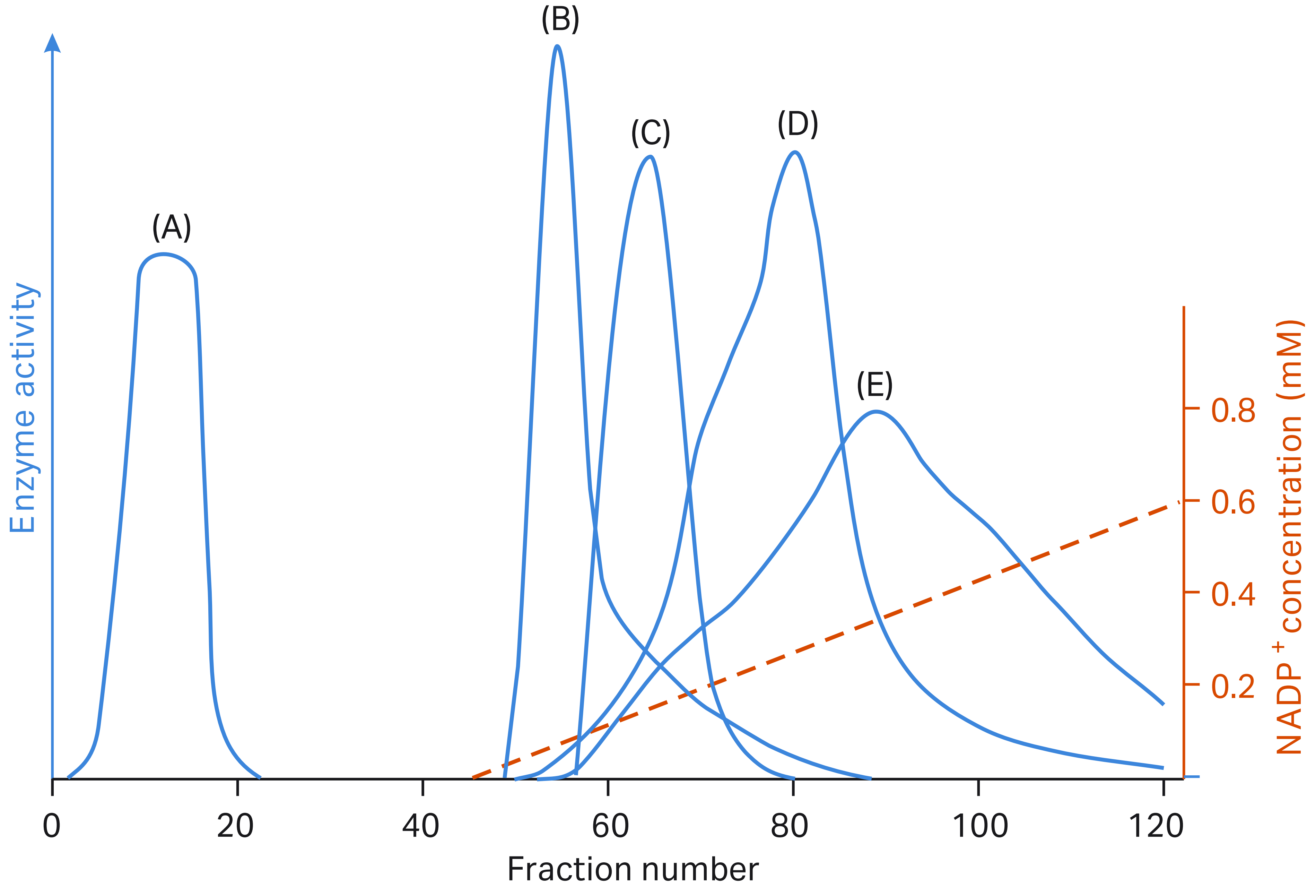


Fig 3.19. Gradient elution with 0 to 0.6 mM $NADP^+$. (A) noninteracting protein, (B) glucose-6-phosphate dehydrogenase, (C) glutamate dehydrogenase, (D) glutathione reductase, (E) 6-phosphogluconate dehydrogenase.

Performing a separation

Swell the required amount of powder for 15 min. in 100 mM 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, 150 mM NaCl, pH 7.3

☞ If the protein of interest binds to the medium via ionic forces, it might 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 three times with 2 to 3 CV of buffers, alternating between high pH (100 mM Tris-HCl, 500 mM NaCl, pH 8.5) and low pH (100 mM sodium acetate, 500 mM NaCl, pH 4.5). Re-equilibrate immediately with 3 to 5 CV of binding buffer.

Remove denatured proteins or lipids by washing the column with 2 CV of detergent, for example, 0.1% Tween 20 for 1 min. Re-equilibrate immediately with 5 CV of binding buffer.

Chemical stability

Stable in 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 > 10.0 can cause loss of phosphate groups.

Storage

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

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

Purification or removal of proteins and peptides with exposed amino acids: His, Cys, Trp, and/or with affinity for metal ions

Chelating Sepharose High Performance, Chelating Sepharose Fast Flow, Capto Chelating

Proteins and peptides that have an affinity for metal ions can be separated using immobilized metal-ion affinity chromatography, IMAC. The metals are immobilized onto a chromatographic medium by chelation. Certain amino acids, for example, histidine and cysteine, form complexes with the chelated metals around neutral pH (pH 6.0 to 8.0) and it is primarily the histidine-content of a protein which is responsible for its binding to a chelated metal.

IMAC is excellent for purifying recombinant $(his)_6$-tagged proteins (see the handbook *Affinity Chromatography, Vol. 2: Tagged Proteins*, 18114275) as well as many natural proteins. Chelating Sepharose, the medium used for IMAC purification of proteins with exposed His, Cys, and Trp, 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+}, or Co^{2+}. The binding reaction with the target protein is pH dependent and bound sample is eluted by reducing the pH and increasing the ionic strength of the buffer or by including imidazole in the buffer. The structure of the ligand, iminodiacetic acid, is shown in Figure 3.20.

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

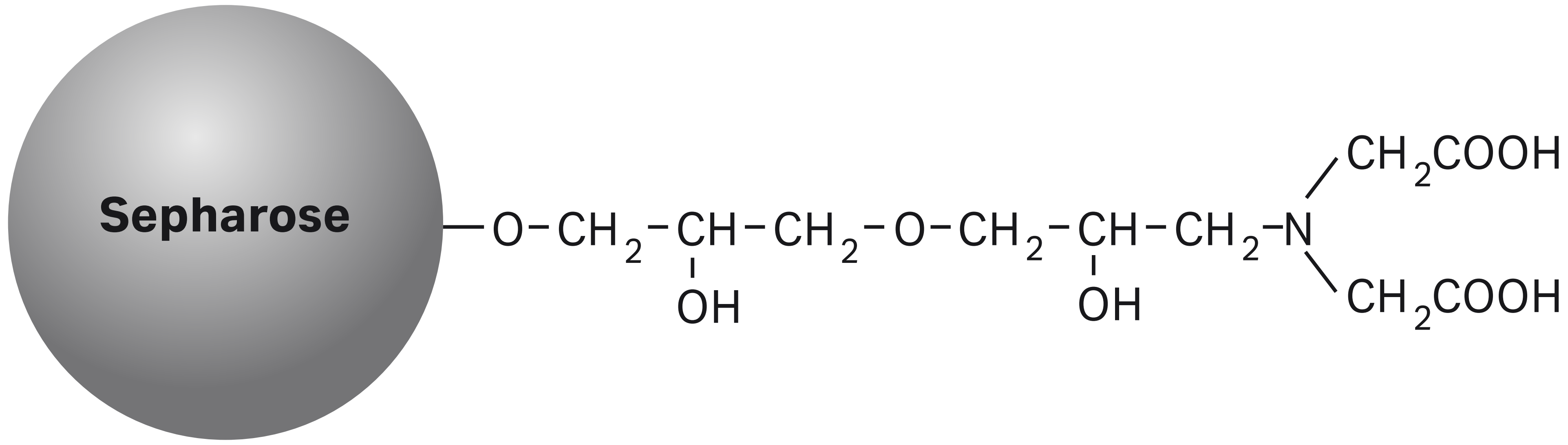


Fig 3.20. Partial structure of Chelating Sepharose High Performance and Chelating Sepharose Fast Flow.

Chromatography media characteristics

Characteristics of Chelating Sepharose and Capto Chelating chromatography media are given in Table 3.23.

Table 3.23. Characteristics of Chelating Sepharose and Capto Chelating chromatography media

	Composition	Metal ion capacity	pH stability[1]	Average particle size (µm)
Chelating Sepharose High Performance	Iminodiacetic acid coupled to Sepharose High Performance via an ether bond	23 µmol Cu^{2+}/mL	Short term: 2 to 14 Long term: 3 to 13	34
Chelating Sepharose Fast Flow	Iminodiacetic acid coupled to Sepharose Fast Flow via a spacer arm using epoxy coupling	22 to 30 µmol Zn^{2+}/mL	Short term: 2 to 14 Long term: 3 to 13	90
Capto Chelating	Iminodiacetic acid coupled to Capto	22 to 33 µmol Cu^{2+}/mL medium	Short term: 2 to 14 Long term: 3 to 12	75

[1] Short term refers to the pH interval for regeneration, cleaning in place, and sanitization procedures. 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.

Purification options

Options for purification of proteins and peptides with exposed amino acid groups are shown in Table 3.24.

Table 3.24. Purification options for Chelating Sepharose and Capto Chelating chromatography media packs and prepacked columns

	Binding capacity	Maximum operating flow	Comments
HiTrap Chelating HP, 1 mL	12 mg/column	4 mL/min	Prepacked 1 mL column
HiTrap Chelating HP, 5 mL	60 mg/column	20 mL/min	Prepacked 5 mL column
Chelating Sepharose Fast Flow	12 mg/mL medium	400 cm/h[1]	Supplied as suspension ready for column packing
HiTrap Capto Chelating, 1 mL	30 mg green fluorescent protein (GFP)-his/column	3.8 mL/min	Prepacked 1 mL column
HiTrap Capto Chelating, 5 mL	150 mg GFP-his/column	20 mL/min	Prepacked 5 mL column
HiScreen Capto Chelating	130 mg GFP-his/column	4.6 mL/min	Prepacked 4.7 mL column
Capto Chelating	30 mg GFP-his/mL medium	600 cm/h	Supplied as suspension ready for column packing

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

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 metal-ion solution equivalent to 60% of the packed column volume during charging 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 metal-ion solution equivalent to 85% of the packed column volume to charge the column.
- Ni^{2+} is commonly used for his-tagged 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, for example, $ZnCl_2$, $NiSO_4$, 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 (50 mM), sodium phosphate (20 to 50 mM) and Tris-HCl (20 to 50 mM) 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., 500 mM to 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 to 500 mM), ammonium chloride (0 to 150 mM), 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 stepwise reduction in pH can be used for nonspecific elution of bound material. A range of pH 7.0 to 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 (50 mM) 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 *Buffer exchange and desalting*, Appendix 1).

☞ 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.

☞ Although metal-ion 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.

Performing a separation

This protocol can be used as a base from which to develop purification methods for proteins and peptides with affinity for metal ion

Metal-ion solution:	100 mM $CuSO_4$
Binding buffer:	20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4

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 bubbles are expelled.

1. Wash the column with at least 2 CV of distilled water.
2. Load 0.5 CV of the 100 mM copper solution onto the column.
3. Wash with 5 CV of distilled water.
4. Equilibrate the column with 10 CV of binding buffer.
5. Apply sample at a flow rate of 1 to 4 mL/min (1 mL column) or 5 mL/min (5 mL column). Collect the flowthrough fraction. A pump is more suitable for application of sample volumes greater than 15 mL.
6. Wash with 10 CV of binding buffer. Collect wash fraction.
7. Elute with 5 CV of elution buffer. Collect eluted fractions in small fractions such as 1 mL to avoid dilution of the eluate.
8. Wash with 10 CV 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-tagged protein is to be purified.

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

Scaling up

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 CV of 20 mM sodium phosphate, 500 mM NaCl, 50 mM EDTA, pH 7.4.

Remove precipitated proteins by filling the column with 1 M NaOH and incubate for 2 h. Wash out dissolved proteins with 5 CV of water and a buffer at pH 7.0 until the pH of the flowthrough reaches pH 7.0.

Alternatively wash with a nonionic detergent such as 0.1% Tween 20 at 37°C for 1 min.

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

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

Before long-term storage, remove metal ions by washing with 5 CV of 20 mM sodium phosphate, 500 mM NaCl, 50 mM EDTA, pH 7.4.

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

Purification or removal of serine proteases, such as thrombin and trypsin, and zymogens

Benzamidine Sepharose 4 Fast Flow (low 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 AC 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 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 3.21 shows the partial structure of Benzamidine Sepharose 4 Fast Flow and Table 3.25 gives examples of different serine proteases. Benzamidine Sepharose 4 Fast Flow (high sub) is a medium designed for high capacity. It has a trypsin binding capacity greater than 35 mg/mL medium. For some applications, Benzamidine Sepharose 4 Fast Flow (high sub) induces strong interactions between ligand and target molecules, which may lead to reduced purity and recovery. Benzamidine Sepharose 4 Fast Flow (low sub) is designed to balance good capacity with high purity and recovery.

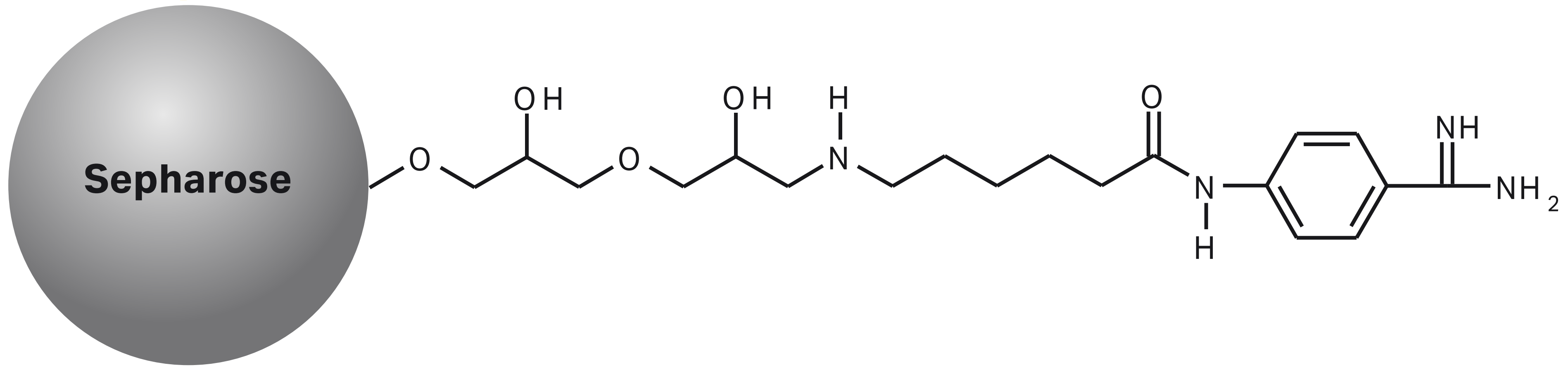


Fig 3.21. Partial structure of Benzamidine Sepharose 4 Fast Flow.

Table 3.25. Examples of different serine proteases

	Source	M_r	pI
Thrombin	Bovine pancreas	37 000	10.5
Trypsin	Human plasma chain A Human plasma chain B	5700 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)

Chromatography media characteristics

Characteristics of Benzamidine Sepharose chromatography media are shown in Table 3.26.

Table 3.26. Characteristics of Benzamidine Sepharose 4 Fast Flow (low sub) and (high sub) chromatography media

	Ligand density (µmol/mL)	Composition	pH stability[1]	Average particle size (µm)
Benzamidine Sepharose 4 Fast Flow (high sub)	≥ 12	Amide coupling of p-aminobenzamidine ligand via a 14 atom spacer to Sepharose 4 Fast Flow (high sub)	Short term: 1 to 9 Long term: 2 to 8	90
Benzamidine Sepharose 4 Fast Flow (low sub)	6	Amide coupling of p-aminobenzamidine ligand via a 14 atom spacer to Sepharose 4 Fast Flow (high sub)	Short term: 1 to 9 Long term: 2 to 8	90

[1] Short term refers to the pH interval for regeneration, cleaning in place, and sanitization procedures. 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.

Purification options

Benzamidine Sepharose 4 Fast Flow low and high sub chromatography media and prepacked columns are described in Table 3.27.

Table 3.27. Purification options for Benzamidine Sepharose 4 Fast Flow chromatography media and prepacked columns

	Binding capacity	Maximum operating flow	Comments
HiTrap Benzamidine FF (high sub)	Trypsin, > 35 mg/column Trypsin, > 175 mg/column	4 mL/min 20 mL/min	Prepacked: 1 mL column[2] Prepacked: 5 mL column[2]
Benzamidine Sepharose 4 Fast Flow (high sub)	Trypsin, > 35 mg/mL medium	300 cm/h[1]	Supplied as suspension ready for column packing[2]
Benzamidine Sepharose 4 Fast Flow (low sub)	Trypsin, 25 mg/mL medium	300 cm/h[1]	Supplied as suspension ready for column packing[2]

[1] See Appendix 4 to convert flow velocity (cm/h) to volumetric flow rate (mL/min). 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] Supplied in 50 mM acetate, pH 4.0 containing 20% ethanol.

Purification examples

Figure 3.23 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.

Column:	HiTrap Benzamidine FF (high sub), 1 mL
Sample:	1 mL human plasma filtered through a 0.45 µm filter
Binding buffer:	20 mM Tris-HCl, 500 mM NaCl, pH 7.4
Elution buffer:	50 mM glycine, pH 3.0
	0% to 100% elution buffer in one step
Flow rate:	1.0 mL/min
Protease activity:	S-2288 from Chromogenix, Heamochrom Diagnostica AB
	A_{405} measurement. The activity is presented as the proteolytic activity/mg protein
System:	ÄKTA system

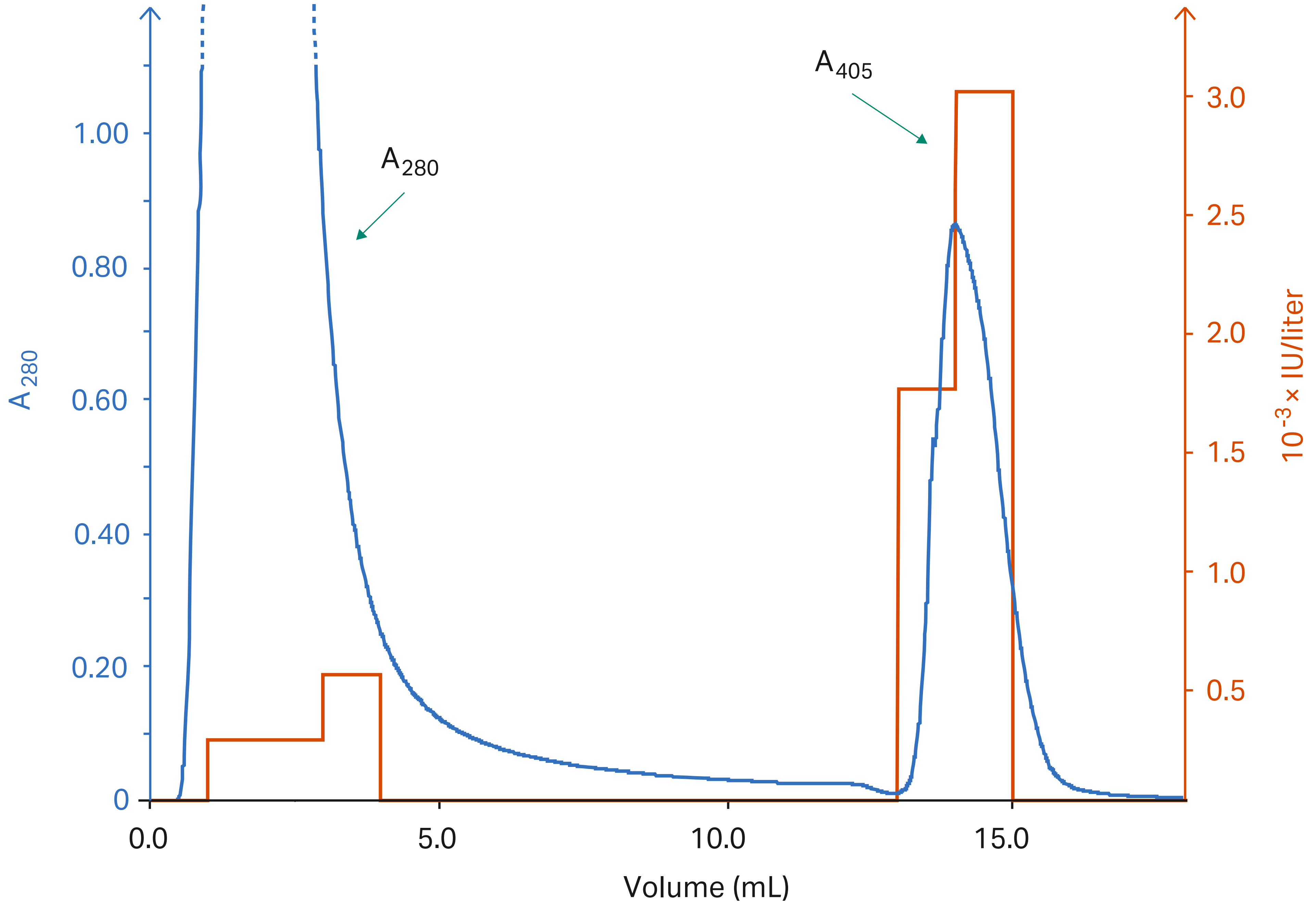
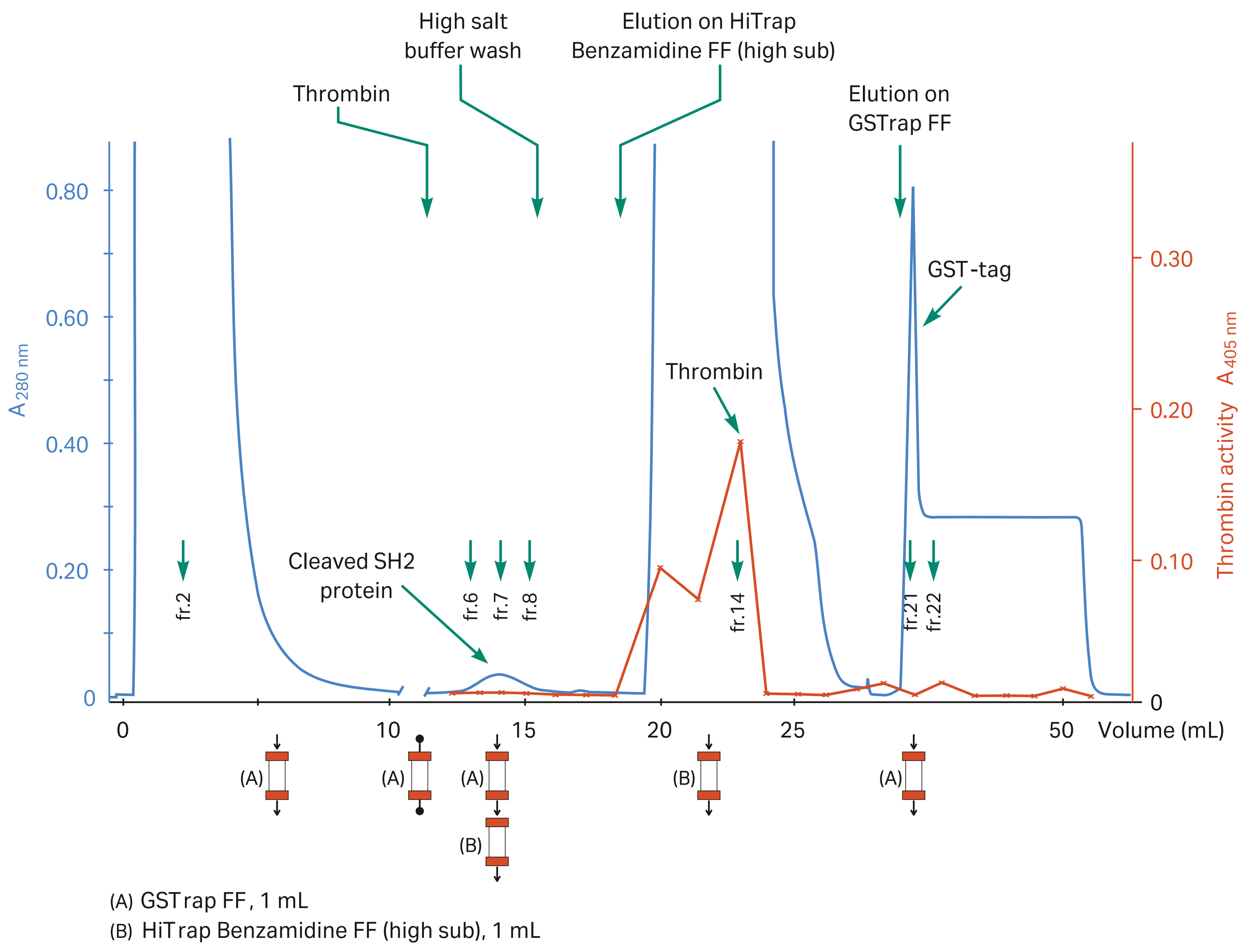


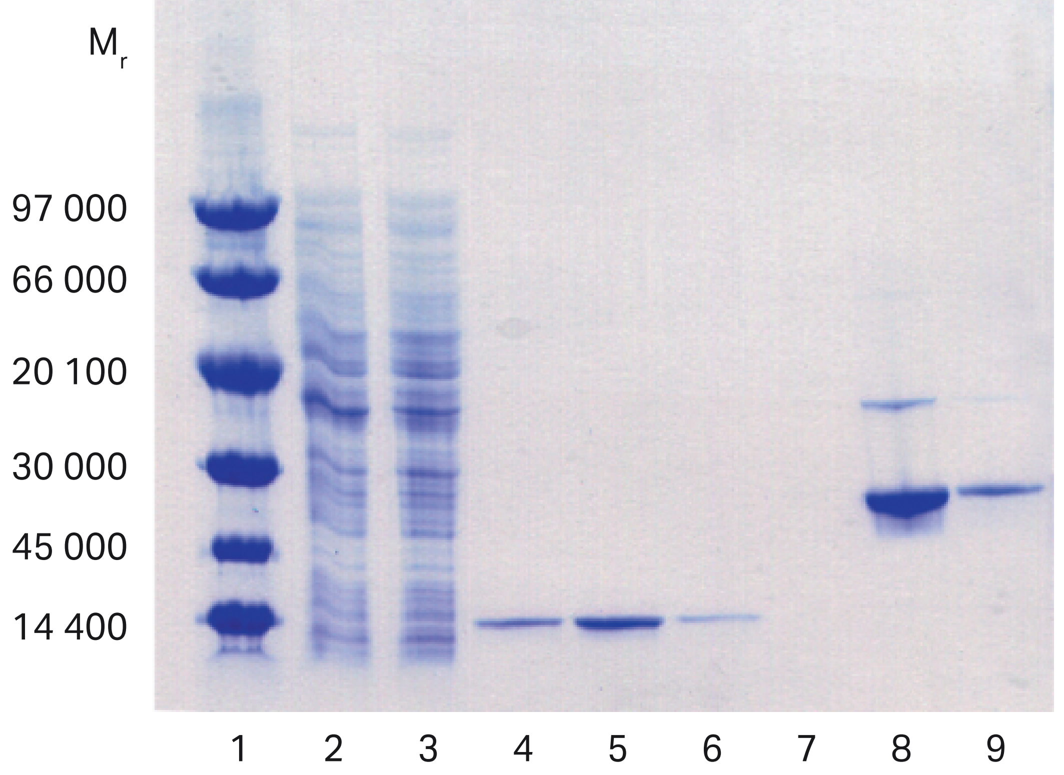
Fig 3.22. Removal of trypsin-like serine proteases from human plasma using HiTrap Benzamidine FF (high sub), 1 mL.

Figure 3.23 shows the effectiveness of using a GSTrap™ FF column with a HiTrap Benzamidine FF (high sub) for purification of a GST-tagged protein, followed by cleavage of the GST tag via the thrombin cleavage site and subsequent removal of the thrombin enzyme. The GST-tagged protein binds to the GSTrap FF column as other proteins wash through the column. Thrombin is applied to the column and incubated for 2 h.

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.



Columns:	GSTrap FF, 1 mL and HiTrap Benzamidine FF (high sub), 1 mL
Sample:	2 mL clarified *E. coli* homogenate expressing a M_r 37 000 SH2-GST-tagged protein with a thrombin cleavage site
Binding buffer:	20 mM sodium phosphate, 150 mM NaCl, pH 7.5
High salt wash buffer:	20 mM sodium phosphate, 1 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 rate:	0.5 mL/min
Protease treatment:	20 units/mL thrombin (Cytiva) for 2 h at room temperature
Thrombin activity:	S-2238 (Chromogenix, Haemochrom Diagnostica AB) was used as a substrate and its absorbance at 405 nm was measured
System:	ÄKTA system



Lanes

ExcelGel SDS Gradient 8–18, Coomassie Blue staining

1. LMW-SDS Marker Kit
2. Clarified *E. coli* homogenate expressing SH2-GST-tagged protein
3. Flowthrough from GSTrap FF (Fraction 2)
4. SH2 GST-tag cleaved, washed off with binding buffer through both columns (Fraction 6)
5. as above (Fraction 7)
6. as above (Fraction 8)
7. Elution of thrombin, HiTrap Benzamidine FF (high sub)
8. Elution of GST-tag and some noncleaved SH2-GST, GSTrap FF (Fraction 21)
9. as above (Fraction 22)

Fig 3.23. On-column cleavage of a GST-tagged protein and removal of thrombin after on-column cleavage, using GSTrap FF and HiTrap Benzamidine FF (high sub).

Performing a separation

Binding buffer: 50 mM Tris-HCl, 500 mM NaCl, pH 7.4

Elution buffer alternatives:

- pH elution: 50 mM glycine-HCl, pH 3.0 or 10 mM HCl, 50 mM 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 CV of binding buffer.
2. Apply the sample.
3. Wash with 5 to 10 CV of binding buffer or until no material appears in the eluent (monitored by UV absorption at $A_{280\text{ nm}}$).
4. Elute with 5 to 10 CV of elution buffer. Collect fractions in neutralization buffer if low pH elution is used[1]. The purified fractions can be buffer exchanged using desalting columns (see *Buffer exchange and desalting*, Appendix 1).

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[1] Since elution conditions are quite harsh, collect fractions into neutralization buffer (60 to 200 µL of 1 M Tris-HCl, pH 9.0 per milliliter of fraction), so that the final pH of the fractions will be approximately neutral.

Since Benzamidine Sepharose 4 Fast Flow has some ionic binding characteristics, the use of 500 mM NaCl and pH elution between 7.4 and 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 to 5 CV of 100 mM Tris-HCl, 500 mM NaCl, pH 8.5 followed with 3 to 5 CV of 100 mM sodium acetate, 500 mM NaCl, pH 4.5 and re-equilibrate immediately with 3 to 5 CV of binding buffer.

Remove severe contamination by washing with nonionic detergent such as 0.1% Tween 20 at 37°C for 1 min.

Chemical stability

All commonly used aqueous buffers.

Storage

Wash chromatography media and columns with 20% ethanol in 50 mM sodium acetate, pH 4.0 (use approximately 5 CV for packed media) and store at 4°C to 8°C.

Purification or removal of viruses including adeno-associated virus

Capto DeVirS, AVB Sepharose High Performance

Capto DeVirS is a chromatography medium for capture and intermediate purification of virus. The dextran sulfate ligand (Fig 3.24) has affinity for several virus types, which makes Capto DeVirS suitable for different virus applications, for example vaccine manufacturing processes. The matrix of Capto DeVirS is based on highly cross-linked high-flow agarose that is highly rigid and offers outstanding pressure/flow properties, enabling rapid processing of large sample volumes.

AVB Sepharose High Performance is designed for the purification of adeno-associated virus (AAV) of subclasses 1, 2, 3, and 5. Adeno associated viruses are of increasing interest as potential vectors for gene therapy. The ligand of AVB Sepharose High Performance is a recombinant protein, M_r 14 000, attached to a highly cross-linked 6% agarose matrix via a long, hydrophilic spacer arm to make it easily available for binding of the virus (Fig 3.25).

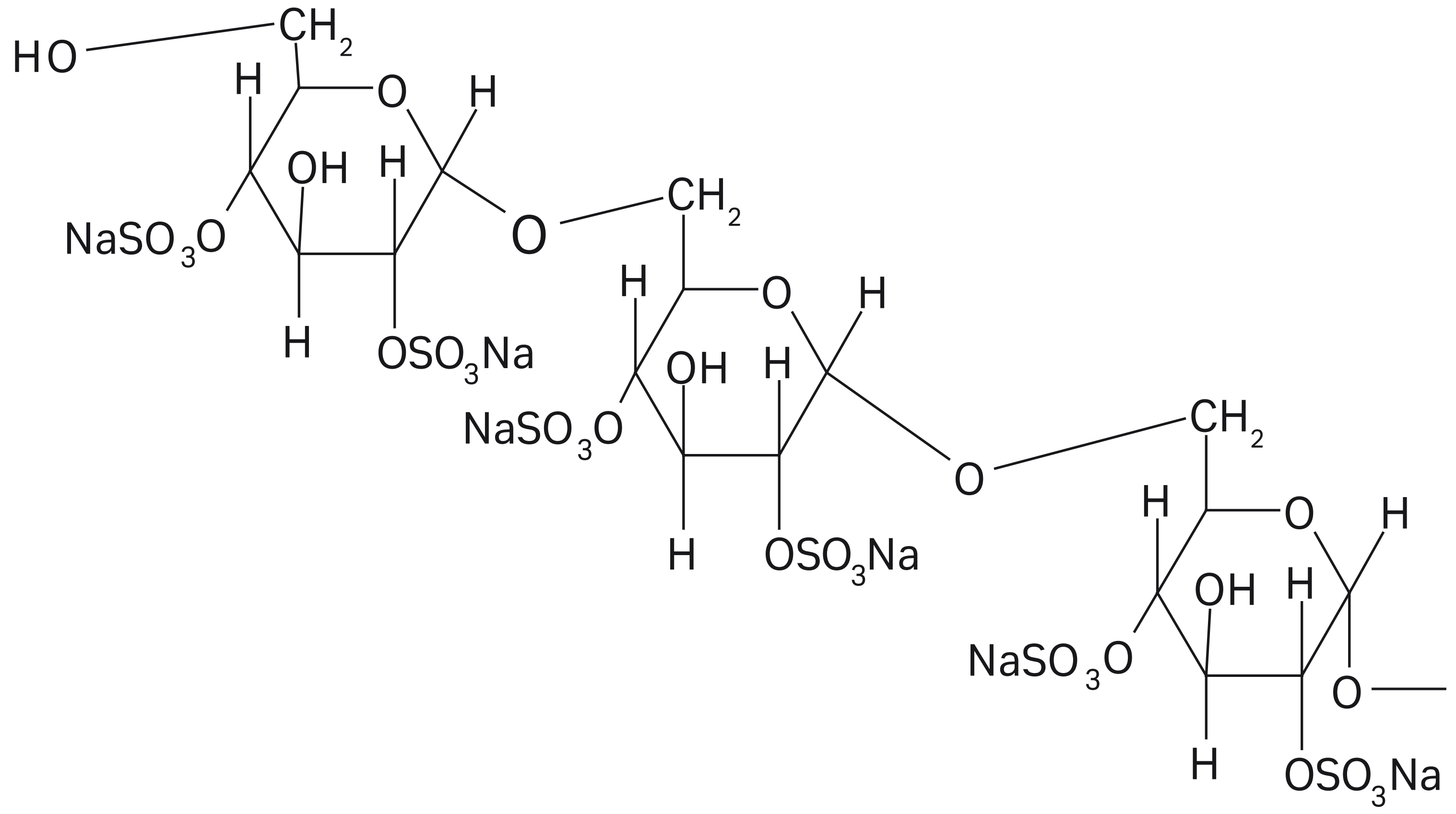


Fig 3.24. Capto DeVirS consists of highly cross-linked agarose base matrix coupled to dextran sulfate ligand.

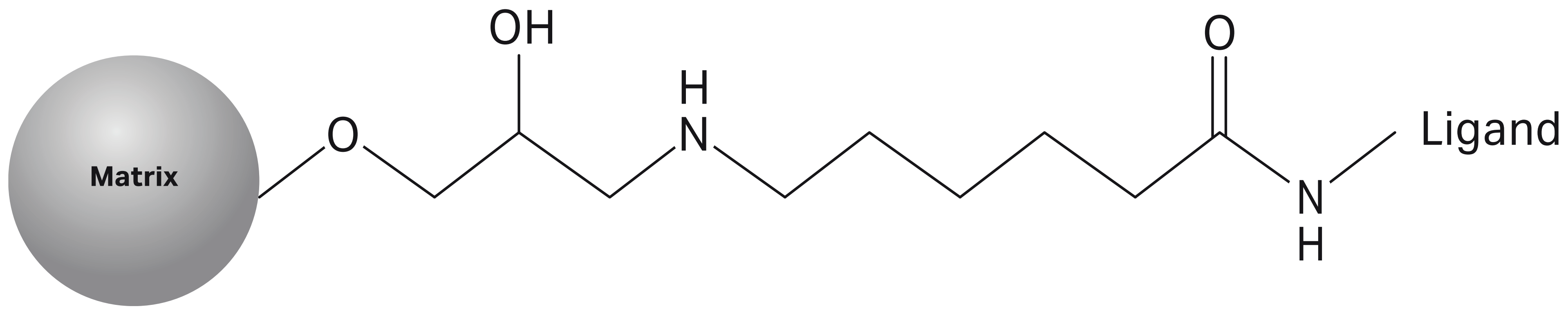


Fig 3.25. Partial structure of AVB Sepharose High Performance.

Chromatography media characteristics

Characteristics of chromatography media for the purification of viruses are described in Table 3.28.

Table 3.28. Characteristics of Capto DeVirS and AVB Sepharose High Performance chromatography media

Product	Ligand	Composition	pH stability[1]	Average particle size (µm)
Capto DeVirS	Dextran sulfate	Ligand coupled to Capto	Short term: 6 to 14 Long term: 7 to 13	75
AVB Sepharose High Performance	M_r 14 000 recombinant protein produced in *S. cerevisiae*. Binds AAV of subclasses 1, 2, 3, and 5	Ligand coupled to Sepharose High Performance	Short term: 2 to 12 Long term: 3 to 10	34

[1] Short term refers to the pH interval for regeneration, cleaning in place, and sanitization procedures. 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.

Purification options

Purification options for Capto DeVirS and AVB Sepharose High Performance and prepacked columns are shown in Table 3.29.

Table 3.29. Purification options for Capto DeVirS, AVB Sepharose High Performance and prepacked columns

	Binding capacity	Maximum operating flow	Comments
Capto DeVirs	Influenza virus: up to 9 $\log_{10}$ FFU[1]/mL	600 cm/h[2]	Media suspension ready for column packing
HiTrap AVB Sepharose HP, 1 mL	> 1012 genome copies/column	1 mL/min	Prepacked 1 mL column
HiTrap AVB Sepharose HP, 5 mL	> 5060 genome copies/column	5 mL/min	Prepacked 5 mL column
AVB Sepharose High Performance	> 1012 genome copies/mL medium	150 cm/h[3]	Media suspension ready for column packing

[1] FFU: Fluorescence Focal Unit.

[2] 1 m diameter column with a 20 cm bed height at 20°C using process buffers with the same viscosity as water.

[3] Bed height 30 cm.

Purification examples

Capto DeVirS was used as a capture step in the purification of influenza virus. In order to establish the optimal purification protocol, Design of Experiments (DoE) was used. The effect of running parameters on virus binding, recovery, and clearance of host cell protein (HCP) and DNA was investigated. The evaluation showed that optimum conductivity for the binding of influenza virus to Capto DeVirS was below 5 mS/cm, while optimum pH for binding and elution was pH 6.8 and pH 7.8, respectively.

Table 3.30 shows the results for the different strains of influenza virus in optimized Capto DeVirs purification.

Table 3.30. Purification of different influenza strains on Capto DeVirS in an XK50 column (5 × 17 cm) with 330 mL of medium and a flow velocity of 150 cm/h

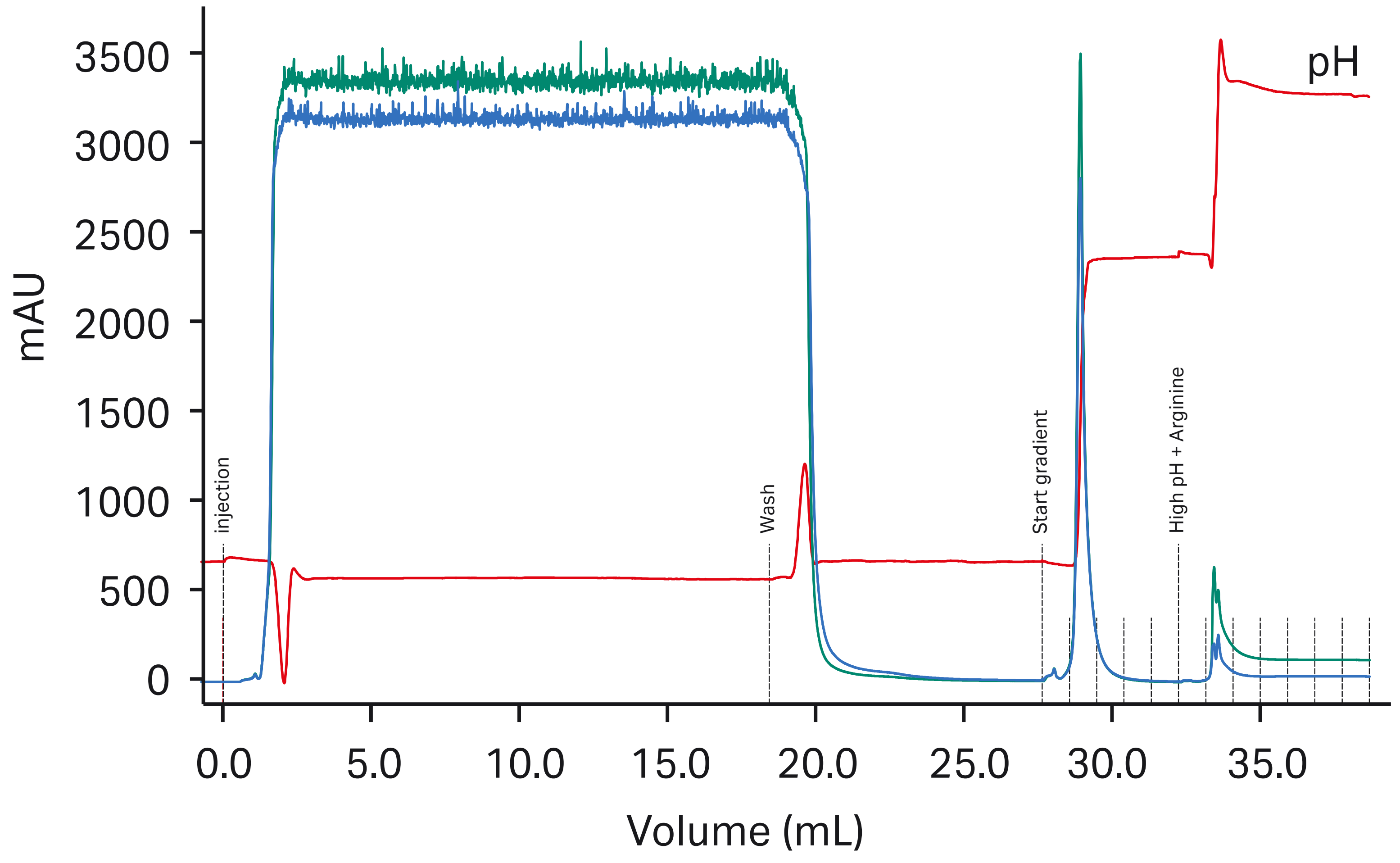
Influenza strain	A/South Dakota	A/Uruguay	B/Florida
Loading titer			
($\log_{10}$ FFU[1]/mL)	9.3	6.6	7.9
Step yield (%)	76	77	84
HCD[2] level (ng/dose)	0.20	N/A	0.93

[1] FFU = Fluorescence Focal Unit.

[2] HCD = Host-cell DNA

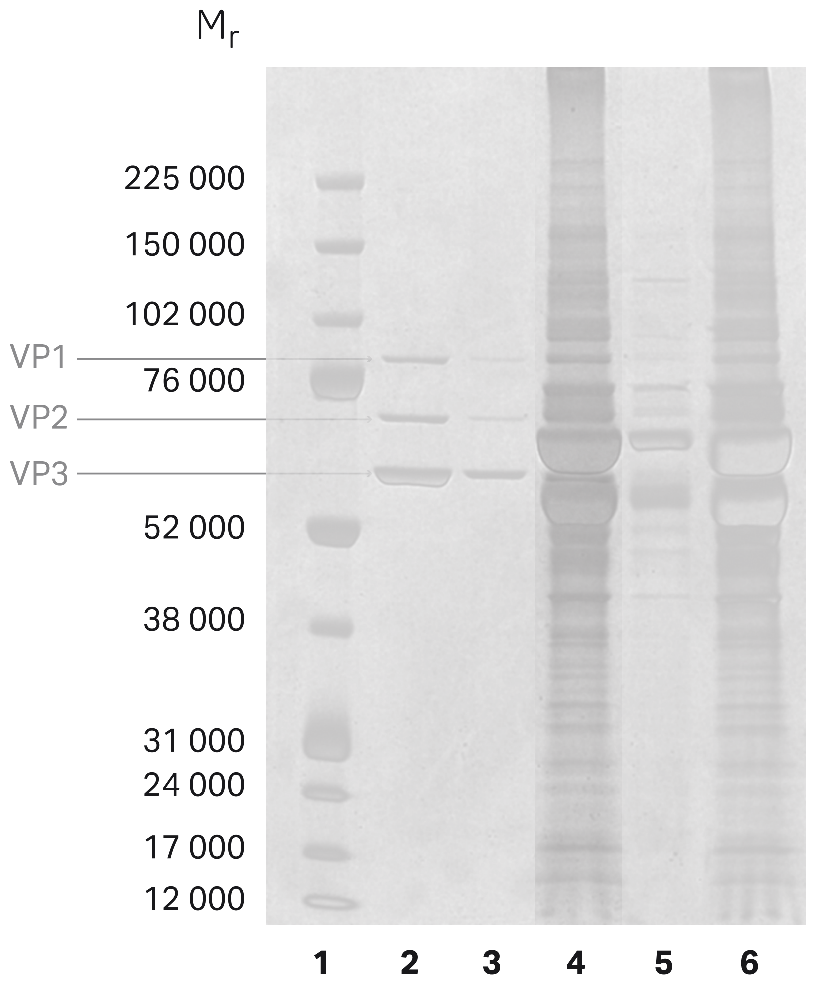
Figure 3.26A shows an example of a purification of adeno-associated virus using AVB Sepharose High Performance. Recombinant AAV1 was eluted using low pH followed by a second elution buffer containing arginine (high pH). Eluted virus was detected by ELISA and SDS-PAGE analysis (Fig 3.26B). SDS-PAGE showed three AAV viral capsid proteins, VP1, VP2, and VP3 (at M_r 87 000, 73 000, and 62 000, respectively) eluted in the initial low pH elution. An additional 6% of the bound virus eluted with the second high-pH elution containing arginine.

(A)



Column:	Tricorn 5/50 (1 mL CV)
Chromatography medium:	AVB Sepharose High Performance, 1 mL
Sample:	rAAV1 (7.0 × 10^{10} viral genomes/mL)
Sample load:	20 mL
Wash buffer:	20 mM Tris-HCl, 500 mM NaCl, pH 8.0
First elution buffer:	100 mM sodium acetate, 500 mM NaCl, pH 2.5
Second elution buffer:	20 mM Tris-HCl, 500 mM NaCl, 500 mM arginine, pH 10.5
Flow velocity:	153 cm/h
System:	ÄKTA system

(B)



Lanes
1. High molecular weight (HMW) markers
2. Fraction from peak eluted at low pH
3. Fraction from peak eluted at high pH (500 mM arginine)
4. Flowthrough
5. Wash
6. Loaded sample

Fig 3.26. (A) Purification of rAAV on AVB Sepharose High Performance using low pH elution followed by high pH elution with 500 mM arginine. The absorbance at 260 and 280 nm is shown in green and blue, respectively. Conductivity is shown in red. (B) SDS-PAGE analysis on fractions collected during purification of rAAV1 on AVB Sepharose High Performance.

Performing a separation

Capto DeVirS

Sample preparation: Concentrate the clarified feed with ultrafiltration and perform a buffer exchange to the start buffer

Binding buffer:	20 mM sodium phosphate, pH 6.8
Elution buffer:	20 mM sodium phosphate, 1.5 M NaCl pH 7.4

1. Equilibrate with 10 CV of binding buffer.
2. Load the clarified sample.
3. Wash with binding buffer until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
4. Elute with 5 to 10 CV of elution buffer.

AVB Sepharose High Performance

1. Equilibrate with 10 CV of binding buffer.
2. Load the clarified sample.
3. Wash with binding buffer until no material appears in the eluent (monitored by UV absorption at $A_{280\ nm}$).
4. Elute with 5 to 10 CV of elution buffer.

Note: Elution at high pH can be performed as an alternative if the virus is sensitive to low pH. The recommended elution buffer in this case is 20 mM Tris-HCl, 500 mM NaCl, 500 mM arginine, pH 10.8.

Cleaning

Cleaning and sanitization protocols should be designed for each process as the efficiency of the protocol is strongly associated with the feedstock and other related operating conditions. Suggested solutions for a contact time of at least 30 min:

Capto DeVirS:	1 M NaOH
AVB Sepharose High Performance:	PAB (120 mM phosphoric acid, 167 mM acetic acid, 2.2% benzyl alcohol)

Storage

Store at 4°C to 8°C in 20% ethanol.

BioProcess chromatography media (resins) for AC

BioProcess™ chromatography media family includes chromatography media widely used by biopharmaceutical manufacturers. Support for these products includes validated manufacturing methods, secure long-term medium supply, safe and easy handling, and regulatory support files (RSF) to assist process validation and submissions to regulatory authorities. In addition, the Fast Trak Training & Education team provides high-level hands-on training for all key aspects of bioprocess development and manufacturing. All BioProcess media have high chemical stability to allow efficient cleaning/sanitization procedures and validated packing methods established for a wide range of large-scale columns.

The range of BioProcess chromatography media for large-scale purification includes Capto media such as Capto Blue for removal or purification of albumin, and a large number of Sepharose Fast Flow media for purification of specific groups of molecules. These AC media can be run at high flow rates and have high dynamic binding capacities.

Most of the chromatography media are available in HiTrap and HiScreen prepacked columns for development of efficient and robust purification parameters before scaling up. By using these small-scale formats in the early stages of process development, valuable time is saved and buffer and sample consumption reduced.

Custom Designed Media

Custom Designed Media (CDM) can be produced for specific industrial process separations when suitable chromatography media are not available from the standard range. The Custom Designed Media group (CDM group) works in close collaboration with the user to design, manufacture, test, and deliver chromatography media for specialized purification requirements. Visit www.cytiva.com/cdm for more information.

04

Designing affinity chromatography media using preactivated matrices

Designing affinity chromatography media using preactivated matrices

Chapter 3 in this handbook covers a wide range of ligands that have been coupled to matrices to provide ready-to-use AC media for specific groups of molecules. However, it is also possible to design new media for special purposes. When a ready-to-use AC 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 preactivated 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 AC 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 nonspecific 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 preactivated Sepharose matrix depends on the functional groups available on the ligand and whether or not a spacer arm is required. Table 4.1 reviews the preactivated 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 4.1).

If possible, test the affinity of the ligand: target molecule interaction. Too low affinity will result in poor yields since the target protein can wash through or leak from the column during sample application. Too high affinity will result in low yields since the target molecule might 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 1, 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 preactivated matrix with a spacer arm. For ligands with $M_r > 5000$, no spacer arm is necessary.

Choosing the coupling method

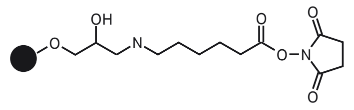
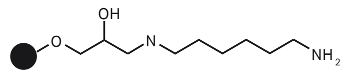
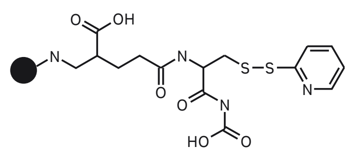
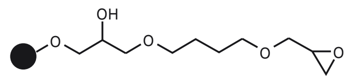
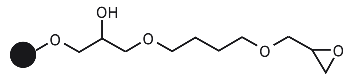
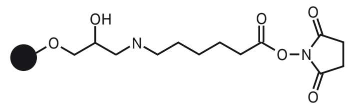
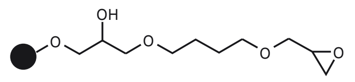
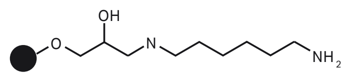
Ligands are coupled via reactive functional groups such as amino, carboxyl, hydroxyl, thiol, and aldehyde moieties. In the absence of information on the location of binding sites in the ligand, a systematic trial-and-error approach should be used.

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, nonessential, 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 4.1. Chemical groups on ligands and spacer arms for preactivated chromatography media

Chemical group on ligand	Length of spacer arm	Structure of spacer arm	Product
Proteins, peptides, amino acids			
amino	10-atom 14-atom		NHS-activated High Performance NHS-activated Sepharose 4 Fast Flow
	None	–	CNBr-activated Sepharose 4B
	None	–	CNBr-activated Sepharose 4 Fast Flow
carboxyl	11-atom		EAH Sepharose 4B
	9-atom		Activated Thiol Sepharose 4B
	12-atom		Epoxy-activated Sepharose 6B
Sugars			
hydroxyl	12-atom		Epoxy-activated Sepharose 6B
amino	10-atom		NHS-activated High Performance
	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
Coenzymes, cofactors, antibiotics, steroids			
amino, carboxyl, thiol, or hydroxyl			Use matrix with spacer arm (see above)

Coupling the ligand

☞ The principle for coupling of ligand is described in this protocol (see also specific protocols for each preactivated medium).

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 preactivated matrix according to the supplied 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 AC medium. After coupling, wash the medium thoroughly using buffers of alternating low and high pH to remove noncovalently bound ligand.

✋ A high concentration of coupled ligand is likely to have adverse effects on AC. 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 can bind more strongly to the ligand making elution difficult. The extent of nonspecific binding increases at very high ligand concentrations thus reducing the selectivity of the medium.

☞ Remember that the useful capacity of an AC medium can 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 13.0.

Figure 4.1 shows the effect of ligand concentration on the final amount of ligand coupled to a matrix.

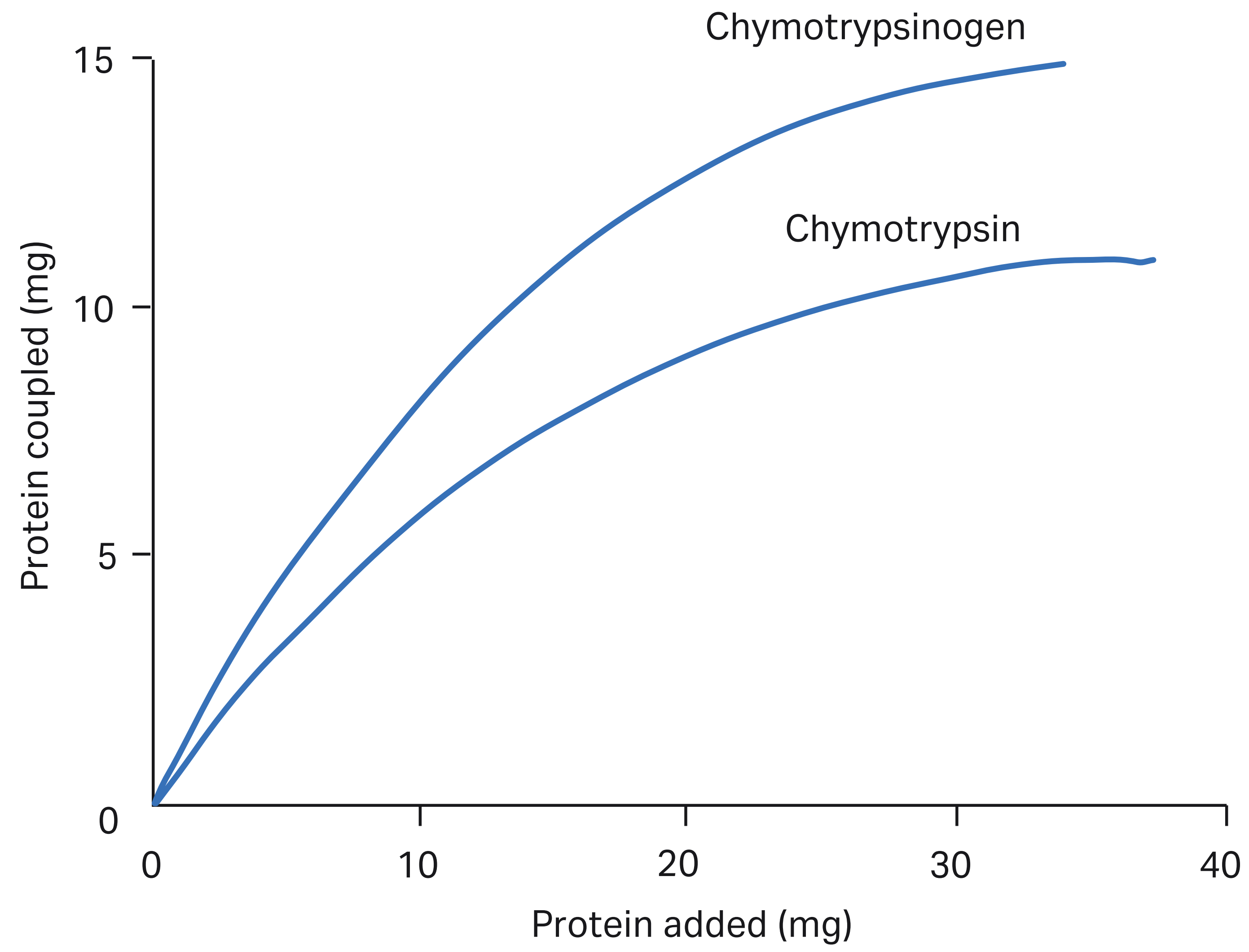


Fig 4.1 Effect of protein concentration on amount of protein coupled. Protein was coupled to 2 mL CNBr-activated Sepharose 4B in $NaHCO_3$, NaCl solution, pH 8.0.

Table 4.2 summarizes recommended ligand concentrations according to various ligand types.

Table 4.2. Recommended ligand concentrations for coupling

Experimental condition	Recommended concentration for coupling
Readily available ligands	10- to 100-fold molar excess of ligand over available groups
Small ligands	1 to 20 µmol/mL medium (typically 2 µmol/mL medium)
Protein ligands	5 to 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 preactivated 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 can 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 (500 mM 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 AC medium.

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

- The fastest and easiest, but least accurate way to quantitate 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 can also be used if the ligand has been suitably prelabeled. The coupled ligand can be quantitated by direct spectroscopy of the AC 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-hydroxysuccinimide in the case of NHS-activated matrices, can be quantitated 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 might 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 11.0)
- Substances that reduce the polarity of the buffer can 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 CV of binding buffer.
   B. Wash with 3 CV of elution buffer.
2. Equilibrate with 10 CV 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 to 1 mL/ min on a HiTrap NHS-activated HP 1 mL column.
4. Wash with 5 to 10 CV of binding buffer, or until no material appears in the eluent, as monitored by absorption at $A_{280\ nm}$.
5. Elute with 1 to 3 CV of elution buffer (larger volumes might be necessary).
6. If required purified fractions can be desalted and transferred into the buffer of choice using prepacked desalting columns (see *Buffer exchange and desalting*, Appendix 1).
7. Re-equilibrate the column immediately by washing with 5 to 10 CV of binding buffer.

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

Coupling through the primary amine of a ligand

NHS-activated Sepharose High Performance, 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). The matrix of NHS-activated Sepharose High Performance is based on highly cross-linked agarose beads with 10-atom spacer arms (6-aminohexanoic acid) attached by epichlorohydrin and activated by N-hydroxysuccinimide (Fig 4.2). The matrix of NHS-activated Sepharose 4 Fast Flow is based on highly cross-linked agarose beads with 14-atom spacer arms. Nonspecific adsorption of proteins to NHS-activated Sepharose (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).

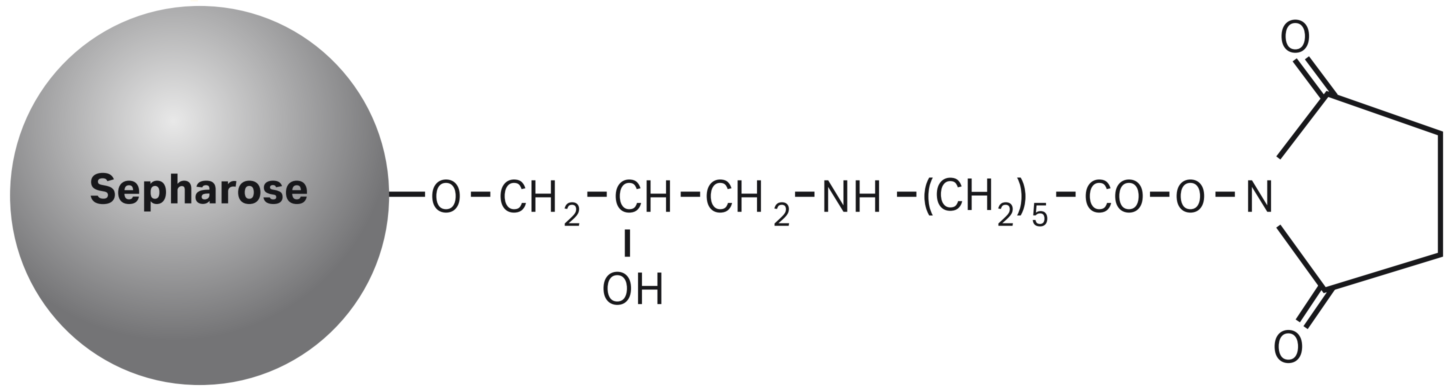


Fig 4.2. Partial structure of NHS-activated Sepharose High Performance bearing activated spacer arms.

Ligands containing amino groups couple rapidly and spontaneously by nucleophilic attack at the ester linkage to give a very stable amide linkage (Fig 4.3). The amide bond is stable up to pH 13.0 making NHS-activated Sepharose suitable for applications that require conditions at high pH.

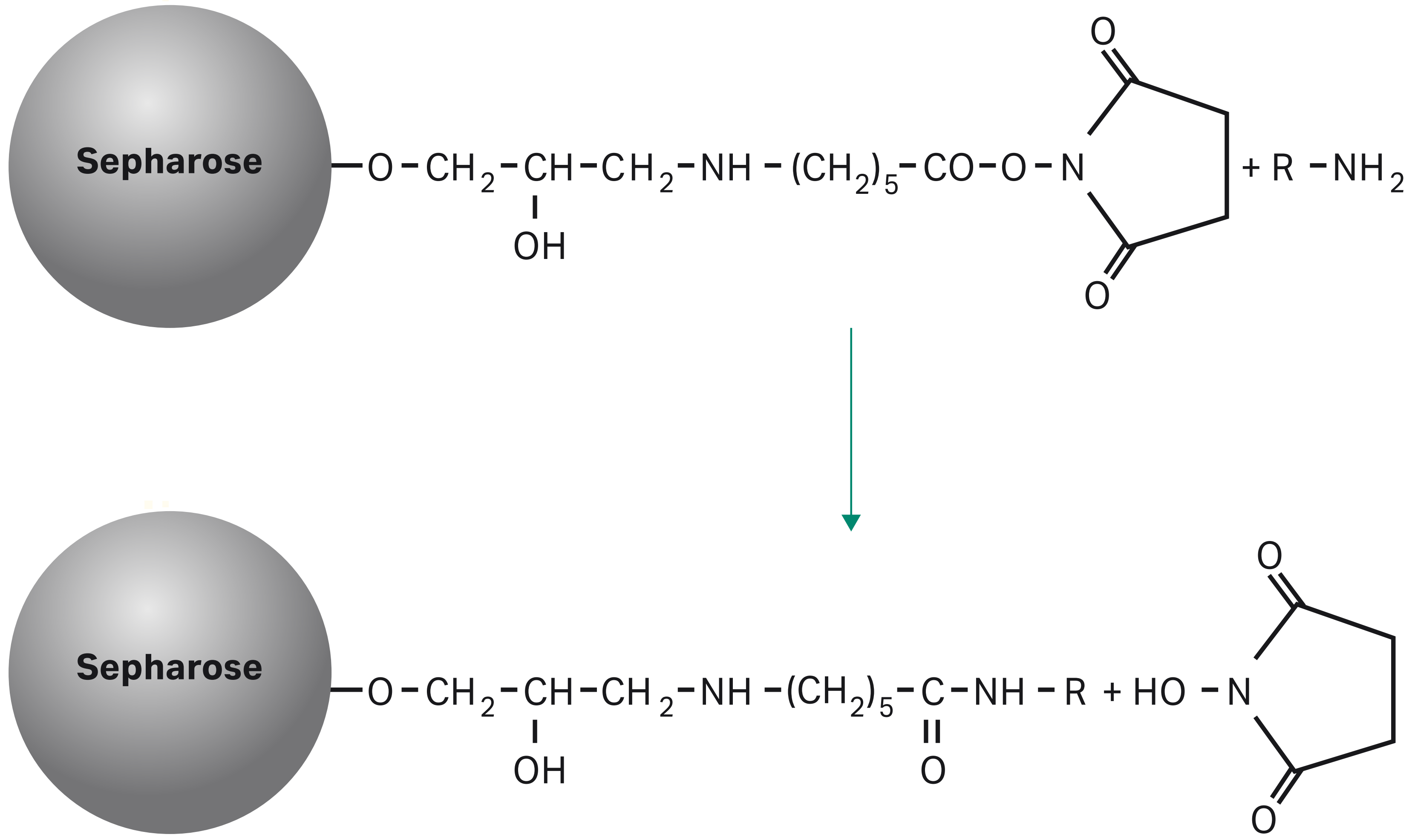


Fig 4.3. Coupling a ligand to NHS-activated Sepharose High Performance.

Chromatography media characteristics

Characteristics of NHS-activated Sepharose chromatography media are shown in Table 4.3.

Table 4.3. Characteristics of NHS-activated Sepharose chromatography media

Product	Ligand density (µmol/mL)	Composition	pH stability[1]	Average particle size (µm)
NHS-activated Sepharose High Performance	10	6-aminohexanoic acid linked by epoxy coupling to Sepharose High Performance, terminal carboxyl group esterified with NHS	Short term: 3 to 12 Long term: 3 to 12	34
NHS-activated Sepharose 4 Fast Flow	16 to 23	As above	Short term: 3 to 13 Long term: 3 to 13	90

[1] 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. 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.

Purification options

NHS-activated Sepharose chromatography media and prepacked columns are described in Table 4.4.

Table 4.4. Purification options for NHS-activated chromatography media and prepacked columns

Product	Spacer arm	Coupling conditions	Maximum operating flow	Comments
HiTrap NHS-activated HP	10-atom	pH 6.5 to 9.0, 15 to 30 min at room temp. or 4 h at 4°C	4 mL/min (1 mL column) 20 mL/min (5 mL column)	Prepacked 1 mL column Prepacked 5 mL column
NHS-activated Sepharose 4 Fast Flow	14-atom	pH 6 to 9, 16 h 4°C to room temp	300 cm/h[1]	Supplied as suspension ready for column packing

[1] See Appendix 4 to convert flow velocity (cm/h) to volumetric flow rate (mL/min). 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 4.4 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 min. The AC medium is then ready to use for antigen purification.

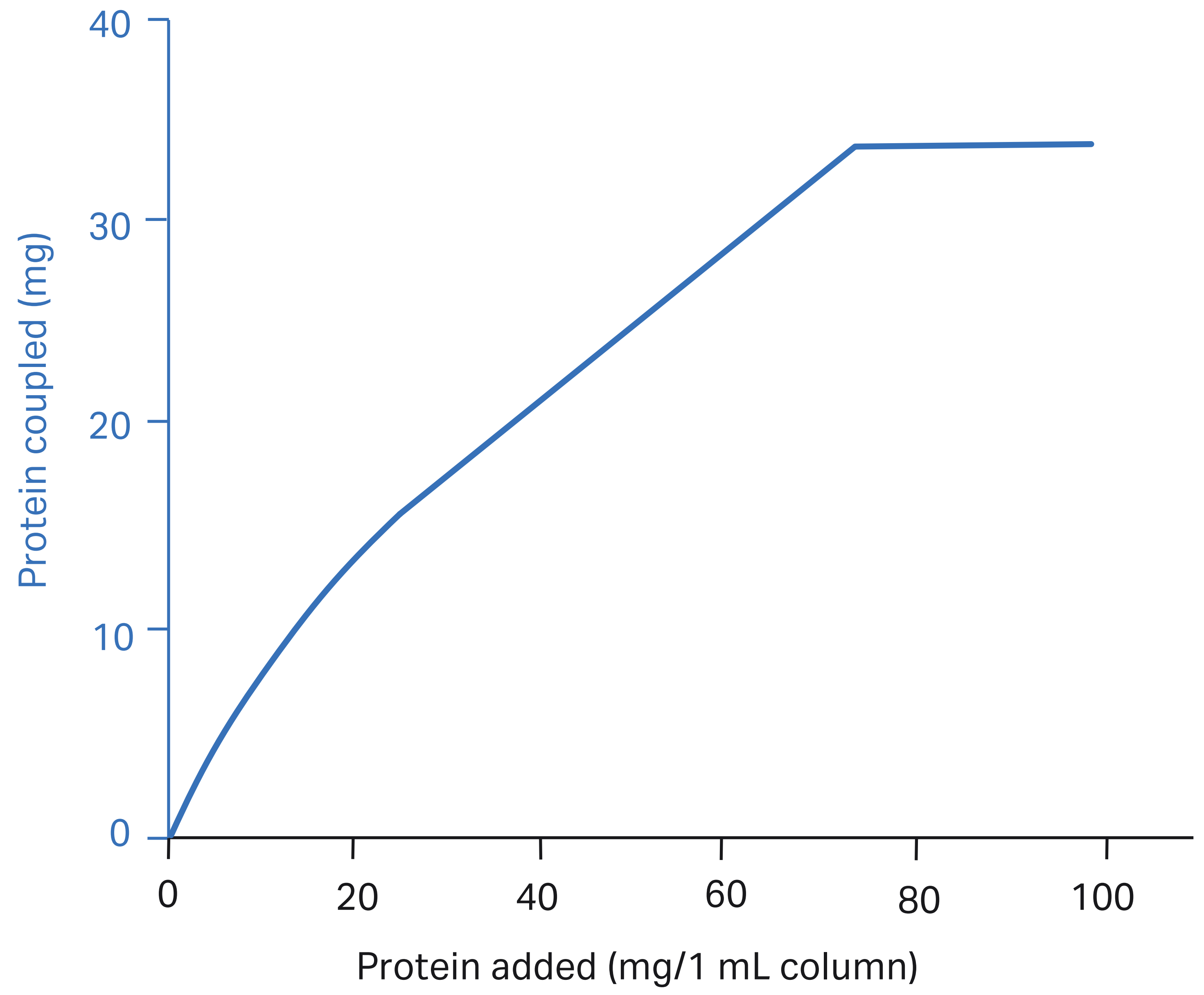


Fig 4.4. Ligand coupling to HiTrap NHS-activated HP.

Purification example

AC can be used to produce monospecific antibodies from polyclonal sera. This approach was taken by the Human Protein Atlas project (www.proteinatlas.org) on a proteome-wide scale. Polyclonal antibodies were raised in rabbits to all proteins encoded for by the human genome. Each antibody serum was purified using HiTrap NHS-activated columns to which antigen epitopes had been immobilized (Fig 4.5). Elution was achieved by lowering the pH to 2.5. The required high throughput was obtained by using 12 modules of an ÄKTA system to purify 48 polyclonal antisera per day.

AC

Columns:	4 × HiTrap NHS-activated HP 1 mL, with immobilized antigens
Sample:	12 mL antiserum applied to each column
Binding buffer:	2 mM NaH_2PO_4, 8 mM Na_2HPO_4, 150 mM NaCl, 0.05% Tween, pH 7.0 to 7.5
Elution buffer:	200 mM glycine, 1 mM EDTA, pH 2.5
Flow rate:	0.7 mL/min (sample loading); 1mL/min (elution)
System:	ÄKTA system

DS

Columns:	2 × HiTrap Desalting, 5 mL
Flow rate:	5 mL/min
Buffer:	2 mM NaH_2PO_4, 8 mM Na_2HPO_4, 150 mM NaCl
System:	ÄKTA system

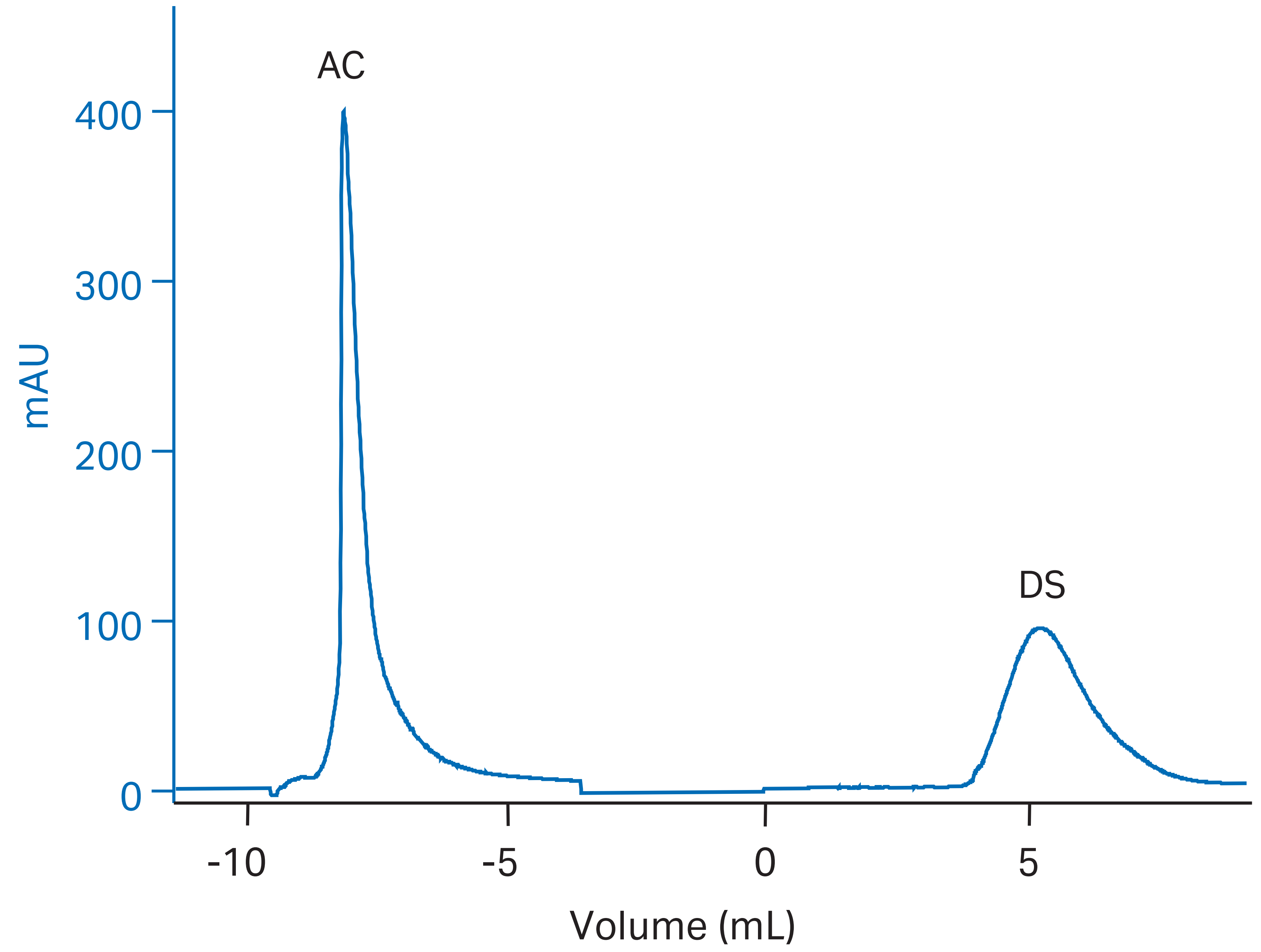


Fig 4.5. Elution of purified antibody from the antigen-specific column and desalting on HiTrap Desalting, 5 mL (courtesy of the Human Protein Atlas project).

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 chromatography 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:	200 mM $NaHCO_3$, 500 mM NaCl, pH 8.3
Blocking buffer:	500 mM ethanolamine, 500 mM NaCl, pH 8.3
Wash buffer:	100 mM acetate, 500 mM NaCl, pH 4.0

☞ Coupling within pH range 6.5 to 9.0, maximum yield is achieved at around pH 8.0.

Ligand and column preparation

1. Dissolve the ligand in the coupling buffer to a final concentration of 0.5 to 10 mg/mL (for protein ligands) or perform a buffer exchange using a desalting column (see *Buffer exchange and desalting* in Appendix 1). 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 snap-off end.

Ligand coupling

1. Wash out the isopropanol with 3 × 2 CV of ice-cold 1 mM HCl.
2. Inject 1 CV of ligand solution onto the column.
3. Seal the column. Leave for 15 to 30 min at 25°C (or 4 h at 4°C).

☞ Recirculate 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 to 30 min 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 columns. For HiTrap 5 mL columns, the recommended flow rate is 5 mL/min (equivalent to approximately 120 drops/min when using a syringe). 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-hydroxysuccinimide, 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 *Buffer exchange and desalting*, Appendix 1) to remove N-hydroxysuccinimide from protein ligands. Alternative methods for the measurement of coupling efficiency are described in *Binding capacity, ligand density, and coupling efficiency* earlier in this chapter and in the HiTrap NHS-activated HP instructions, 71700600.

Washing and deactivation

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

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

The column is now ready for use.

Storage

Store the column in a solution that maintains the stability of the ligand and contains a bacteriostatic agent, see Appendix 8.

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

CNBr-activated Sepharose

CNBr-activated Sepharose offers a well-established option for the attachment of larger ligands and is 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 (Fig 4.6). The coupling reaction is spontaneous and requires no special chemicals or equipment. The resulting multipoint 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.

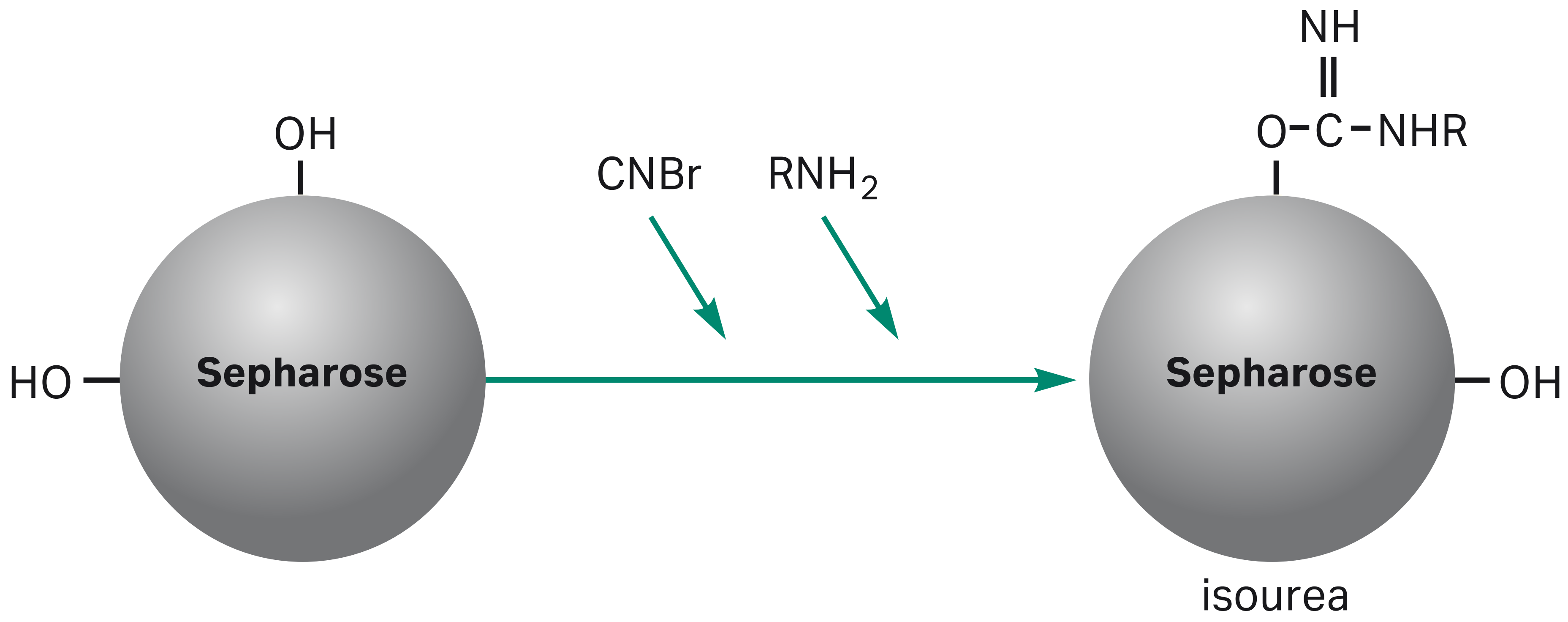


Fig 4.6 Activation by cyanogen bromide and coupling to the activated matrix.

Chromatography media characteristics

Characteristics of CNBr-activated Sepharose chromatography media are shown in Table 4.5.

Table 4.5. Characteristics of CNBr-activated Sepharose chromatography media

Product	Composition	Binding capacity/mL medium	pH stability[1]	Average particle size (µm)
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 to 26 mg	Short term: 3 to 11 Long term: 3 to 11	90
CNBr-activated Sepharose 4B		α-chymotrypsinogen, 25 to 60 mg	Short term: 3 to 11 Long term: 3 to 11	90

[1] 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. 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.

Purification options

Available CNBr-activated Sepharose chromatography media are shown in Table 4.6.

Table 4.6. Purification options for CNBr-activated Sepharose chromatography media

Product	Spacer arm	Coupling conditions	Maximum operating flow velocity (cm/h)[1]	Comments
CNBr-activated Sepharose 4 Fast Flow	None	pH 7 to 9; 2 to 16 h; 4°C to room temp	400	Supplied as a freeze-dried powder
CNBr-activated Sepharose 4B	None	pH 8 to 10; 2 to 16 h; 4°C to room temp	75	Supplied as a freeze-dried powder

[1] See Appendix 4 to convert flow velocity (cm/h) to volumetric flow rate (mL/min). 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

There are many examples in the literature of the use of CNBr-activated Sepharose. Figure 4.7 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 AC medium.

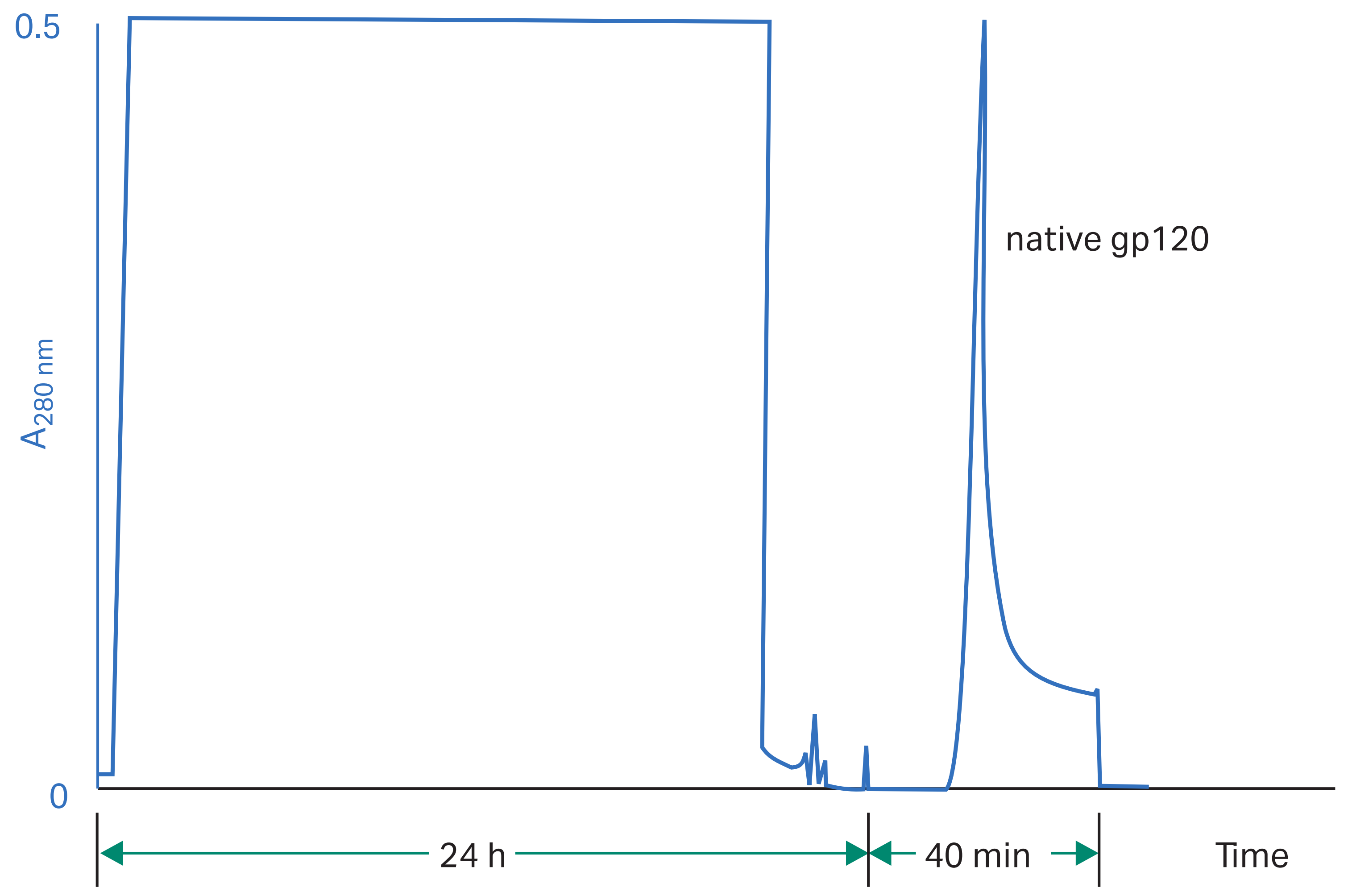


Fig 4.7. Separation of native gp120 protein on GNA coupled to CNBr-activated Sepharose 4 Fast Flow.
From Gilljam, G. *et al.*, Purification of native gp120 from HIV-1 infected T-cells; poster presented at Recovery of Biological Products VII, San Diego, CA, USA (1994).

Performing a separation

Buffer preparation

Acidification solution:	1 mM HCl (kept on ice)
Coupling buffer:	200 mM $NaHCO_3$, 500 mM NaCl, pH 8.3
Blocking buffer:	1 M ethanolamine or 200 mM glycine, pH 8.0
Wash buffer:	100 mM acetate, 500 mM NaCl, pH 4.0

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 of 1 mM HCl per gram dry powder, added and removed by suction in several aliquots. The final aliquot of 1 mM HCl is removed by suction 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 to 10 mg/mL (for protein ligands) or perform a buffer exchange using a desalting column (see page 146). The optimal concentration depends on the ligand. Use a matrix: buffer ratio of 1:0.5 to 1:1.

Ligand coupling

1. Mix the ligand solution with suspension in an end-over-end or similar mixer for 2 h at room temperature or overnight at 4°C. A matrix: buffer ratio of 1:0.5 to 1:1 gives a suitable suspension for coupling.
2. Transfer the medium to blocking buffer for 16 h at 4°C or 2 h at room temperature to block any remaining active groups. Alternatively, leave the medium for 2 h in Tris-HCl buffer, pH 8.0.
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 can 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 might 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. Prehydrolysis 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 can be obtained. At pH 3.0, 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 h of prehydrolysis at pH 8.3 (Fig 4.8).

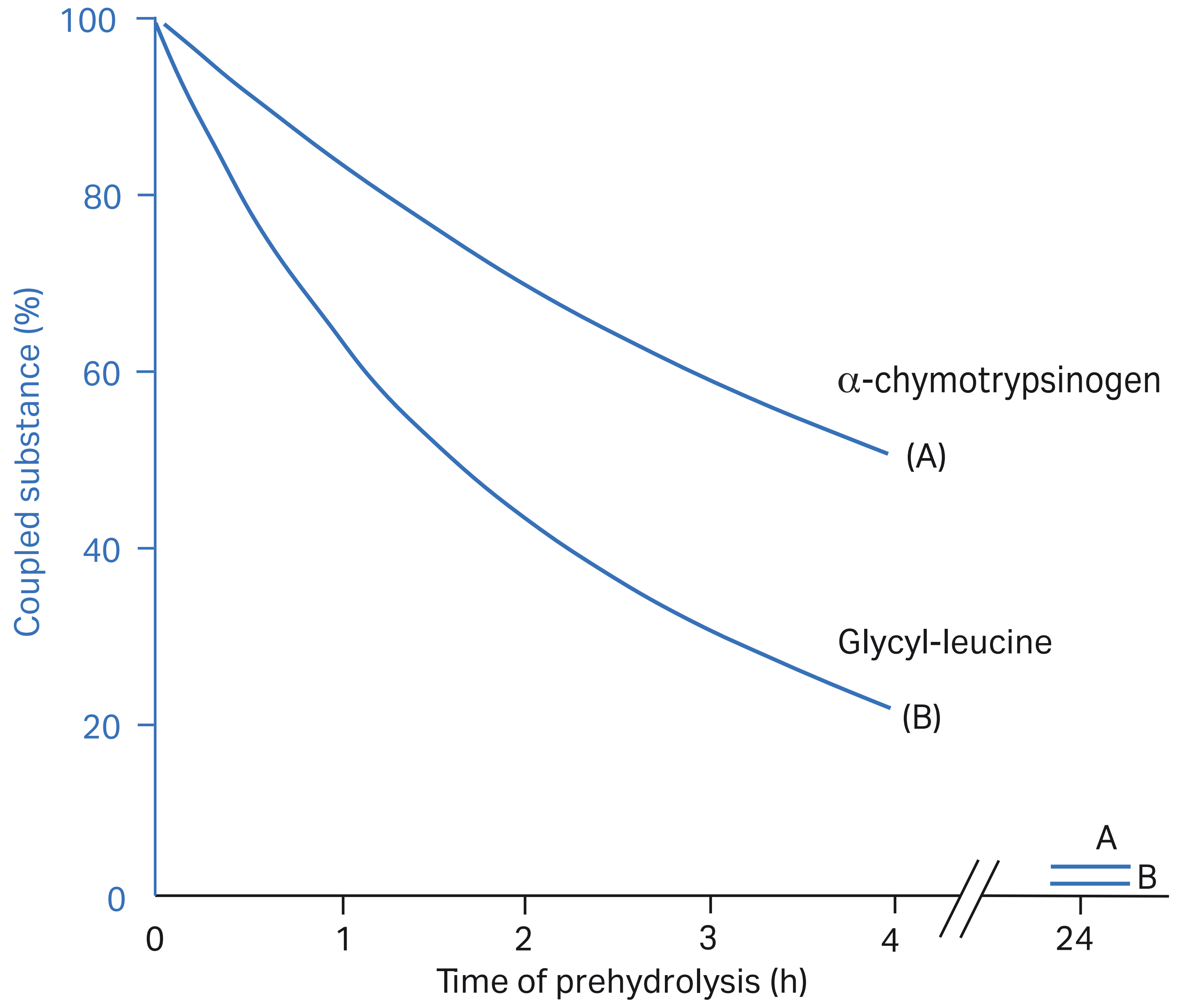


Fig 4.8. Variation of coupling activity with time of pre-hydrolysis at pH 8.3. CNBr-activated Sepharose 4B was washed at pH 3.0 and transferred to 100 mM $NaHCO_3$, pH 8.3 for prehydrolysis. Samples were removed after different times and tested for coupling activity towards α-chymotrypsinogen (A) and glycyl-leucine (B).

☞ Coupling at low pH is less efficient, but can be advantageous if the ligand loses biological activity when it is fixed too firmly, for example, by multipoint 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 6.0.

☞ IgG is often coupled at a slightly higher pH, for example in 200 to 250 mM $NaHCO_3$, 500 mM NaCl, pH 8.5 to 9.0.

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, see Appendix 8, or 20% ethanol in a suitable buffer.

✋ The pH stability of the chromatography 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 chromatography 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 handbook *Affinity Chromatography Vol. 1: Antibodies*, 18103746 from Cytiva 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 antiantibody) 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 4.9 illustrates a typical immunoaffinity purification.

If there is no primary amine available (this group might be required for the specific interaction), then preactivated medium for ligand attachment via carboxyl, thiol, or hydroxyl groups can be considered.

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.

Column:	HiTrap NHS-activated HP 1 mL. Mouse IgG, (10 mg, 3.2 mL) was coupled in 200 mM $NaHCO_3$, 500 mM NaCl, pH 8.3, room temp., recycled with a peristaltic pump for 1 h. The coupling yield was 95% (9.5 mg).
Sample:	50 mL sheep antimouse Fc serum, filtered 0.45 µm
Binding buffer:	75 mM Tris-HCl, pH 8.0
Elution buffer:	100 mM glycine-HCl, 500 mM NaCl, pH 2.7
Flow rate:	1 mL/min
Electrophoresis:	SDS-PAGE, ExcelGel SDS Gradient 8–25, 1 µL sample, Coomassie stained

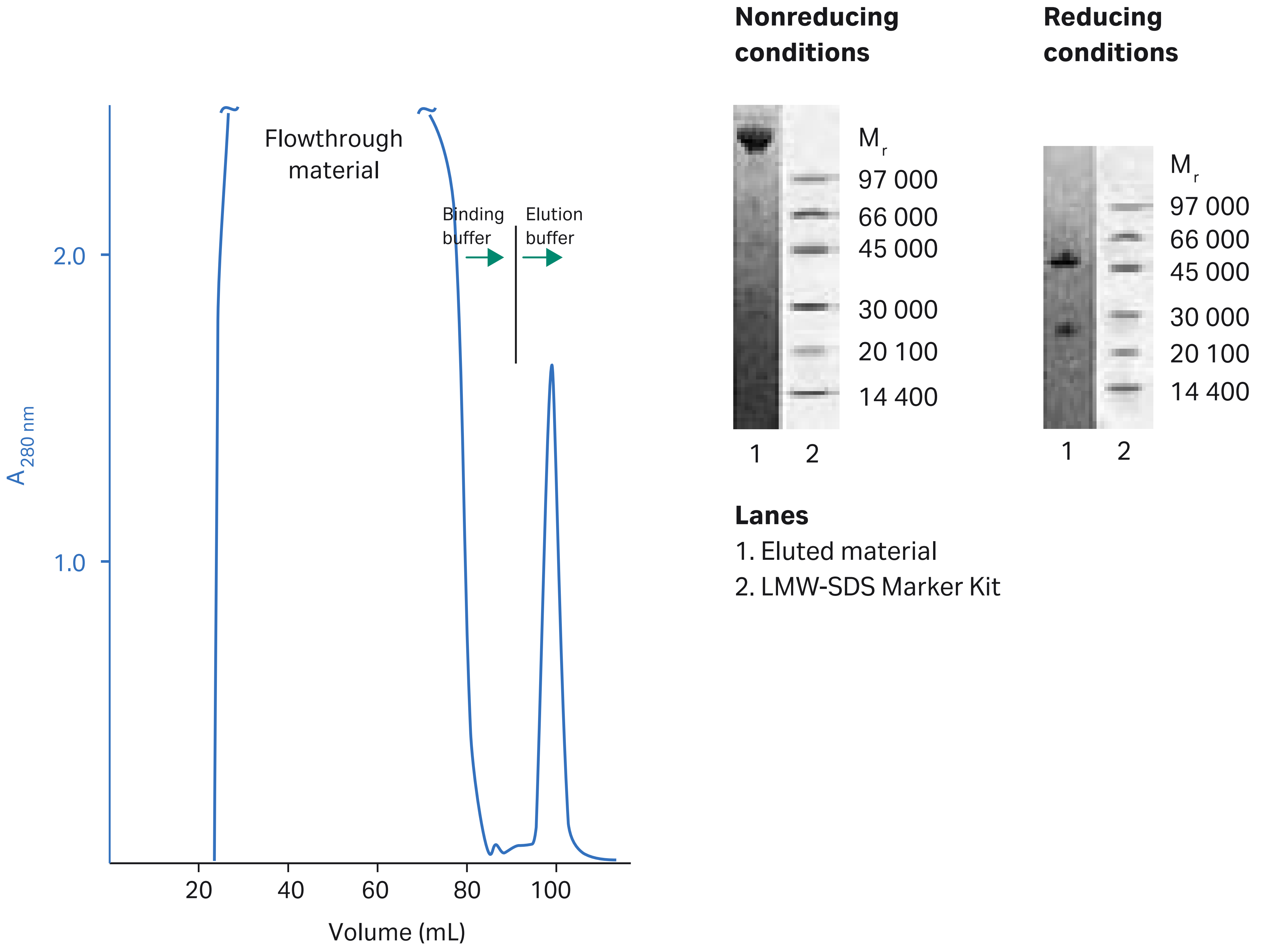


Fig 4.9. Purification of antimouse Fc-IgG from sheep antiserum.

Coupling small ligands through carboxyl groups via a spacer arm

EAH Sepharose 4B

The partial structure of EAH Sepharose 4B is shown in Figure 4.10.

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. The carbodiimide yields an isourea upon hydration. The coupling reaction is shown in Figure 4.11.

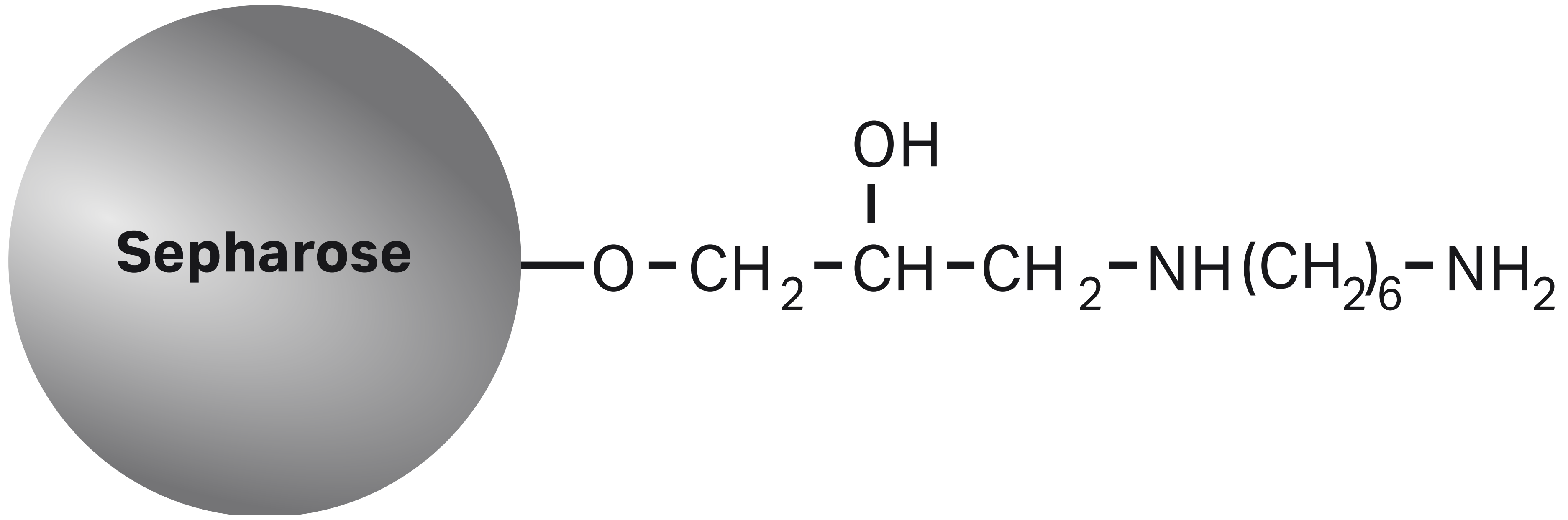
Chromatography medium characteristics

Characteristics of EAH Sepharose 4B chromatography medium are shown in Table 4.7.

Table 4.7. Characteristics of EAH 4B chromatography medium

Product	Composition	pH stability[1]	Average particle size (µm)
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 to 14 Long term: 3 to 14	90

[1] Stability data refers to the coupled medium provided that the ligand can withstand the pH. Short term refers to the pH interval for regeneration, cleaning in place, and sanitization procedures. 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.



$$\text{Sepharose}-O-CH_2-\overset{OH}{\overset{|}{CH}}-CH_2-NH(CH_2)_6-NH_2$$

Fig 4.10. Partial structure of EAH Sepharose 4B.

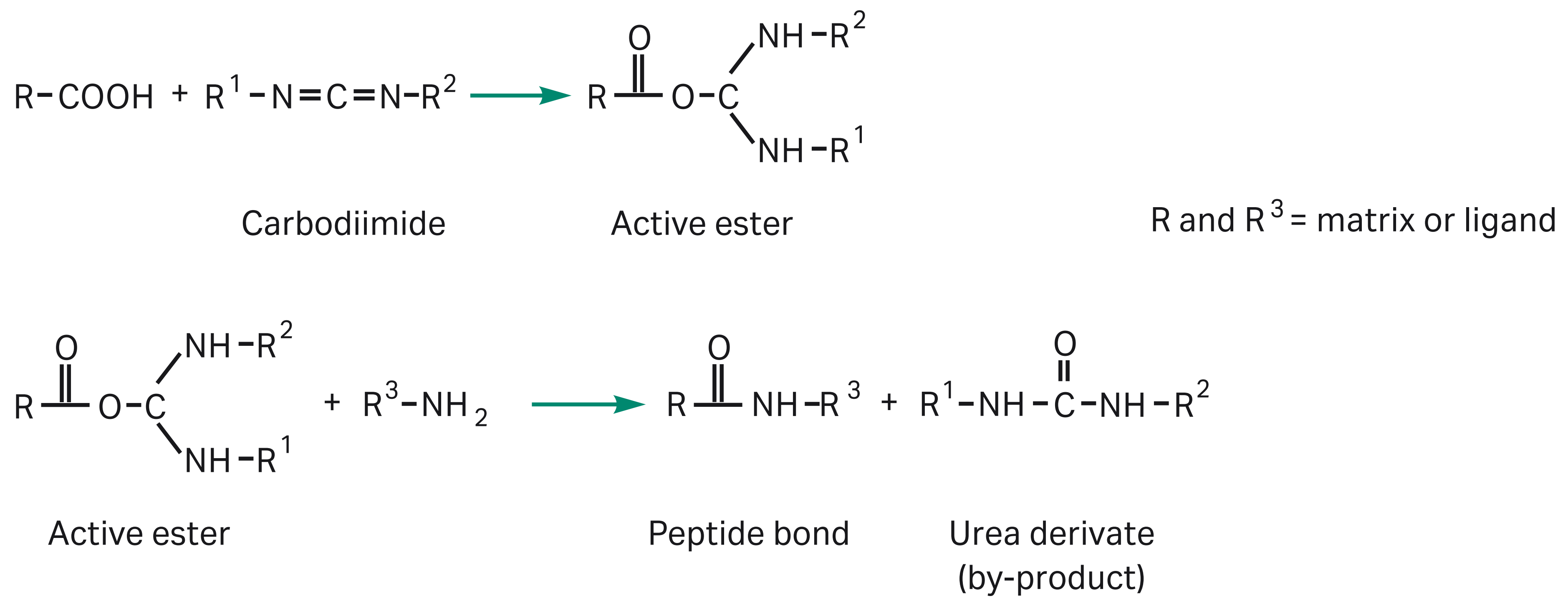


Fig 4.11. Carbodiimide coupling reaction.

Purification options

The purification options for EAH Sepharose 4B are shown in Table 4.8

Table 4.8. Purification options for EAH Sepharose 4B

Product	Spacer arm	Substitution (µmol/mL of medium)	Coupling conditions	Maximum operating flow velocity (cm/h)[1]	Comments
EAH Sepharose 4B	11-atom	7 to 11 amino groups	pH 4.5, 1.5 to 24 h, 4°C to room temp	75	Couple ligands containing free carboxyl groups. Supplied as suspension ready for use.

[1] See Appendix 4 to convert flow velocity (cm/h) to volumetric flow rate (mL/min). 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 sulfonate (CMC). These two carbodiimides have been used in a variety of experimental conditions and at a wide range of concentrations (Table 4.9). EDC often gives better coupling yields than CMC.

Table 4.9. Examples of conditions used during coupling via carbodiimides

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

Use a concentration of carbodiimide greater than the stoichiometric concentration, usually 10- to 100-fold greater than the concentration of spacer groups.

The coupling reaction is normally performed in distilled water adjusted to pH 4.5 to 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:	100 mM acetate, 500 mM NaCl, pH 4.0

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

Preparation of EAH 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 500 mM 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 can 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 100 mM sodium hydroxide.
3. Wash at pH 8.0 and pH 4.0 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 can disrupt the Sepharose matrix.

Storage

Store preactivated matrices 4°C to 8°C in 20% ethanol.

Store the column in a solution that maintains the stability of the ligand and contains a bacteriostatic agent, see Appendix 8, or 20% ethanol in a suitable buffer.

✋ The pH stability of the chromatography medium when coupled to a ligand will depend upon the stability of the ligand.

Performing a separation

See *Binding and elution conditions* earlier in this chapter 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 preactivated 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 4.12.

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.

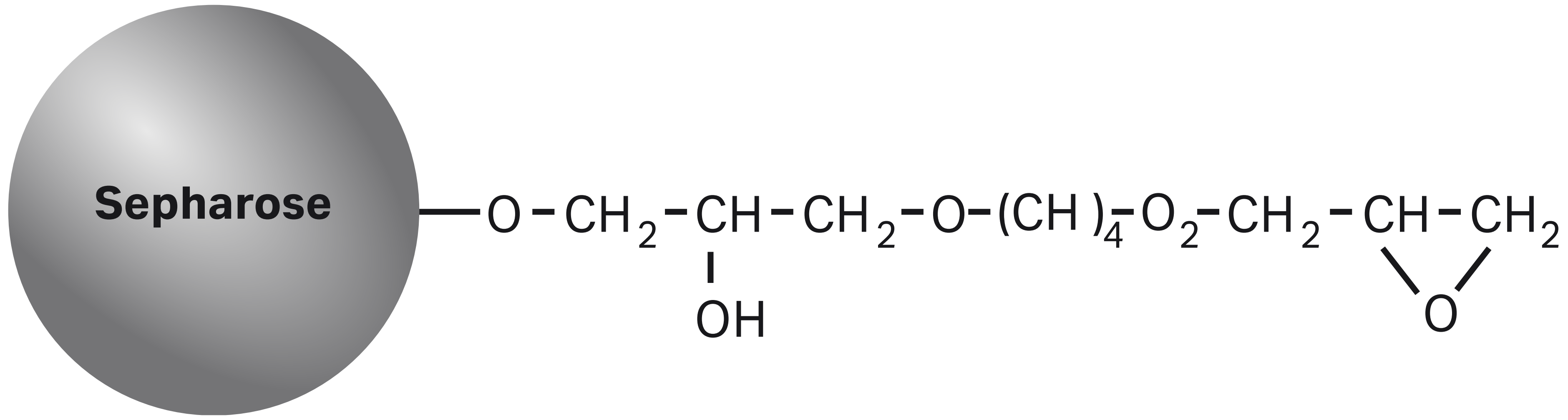


Fig 4.12. Partial structure of Epoxy-activated Sepharose 6B.

Chromatography medium characteristics

Characteristics of Epoxy-activated Sepharose 6B are shown in Table 4.11.

Table 4.11. Characteristics of Epoxy-activated Sepharose 6B medium

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

[1] 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. 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.

Purification options

Purification options for Epoxy-activated Sepharose 6B are shown in Table 4.12.

Table 4.12. Purification options for Epoxy-activated Sepharose 6B medium

Product	Spacer arm	Substitution (µmol/mL medium)	Coupling conditions	Maximum operating flow velocity (cm/h)[1]	Comments
Epoxy-activated Sepharose 6B	12-atom	19 to 40 epoxy groups	pH 9 to 13, 1 h to several days, 20°C to 40°C	75	Supplied as a freeze-dried powder

[1] See Appendix 4 to convert flow velocity (cm/h) to volumetric flow rate (mL/min). 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

Capture and purification of fucose-specific lectin from a crude plant extract using Epoxy-activated Sepharose 6B is shown in Figure 4.13.

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 of final medium volume).
2. Wash immediately for 1 h 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 to 10 mg/mL (for protein ligands) or transfer solubilized ligands into the coupling buffer using a desalting column (see *Buffer exchange and desalting* in Appendix 1). Adjust the pH of the aqueous phase.
4. Use a medium: buffer ratio of 1:0.5 to 1:1, mix the medium suspension with the ligand solution for 16 h at 20°C 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°C to 50°C.
6. Wash away excess ligand with coupling solution followed by distilled water, 100 mM $NaHCO_3$, 500 mM NaCl, pH 8.0, and 100 mM NaCl, 100 mM acetate, pH 4.0.

☞ If organic solvents have been used, use pH paper to measure pH since solvents can 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 can disrupt the Sepharose medium.

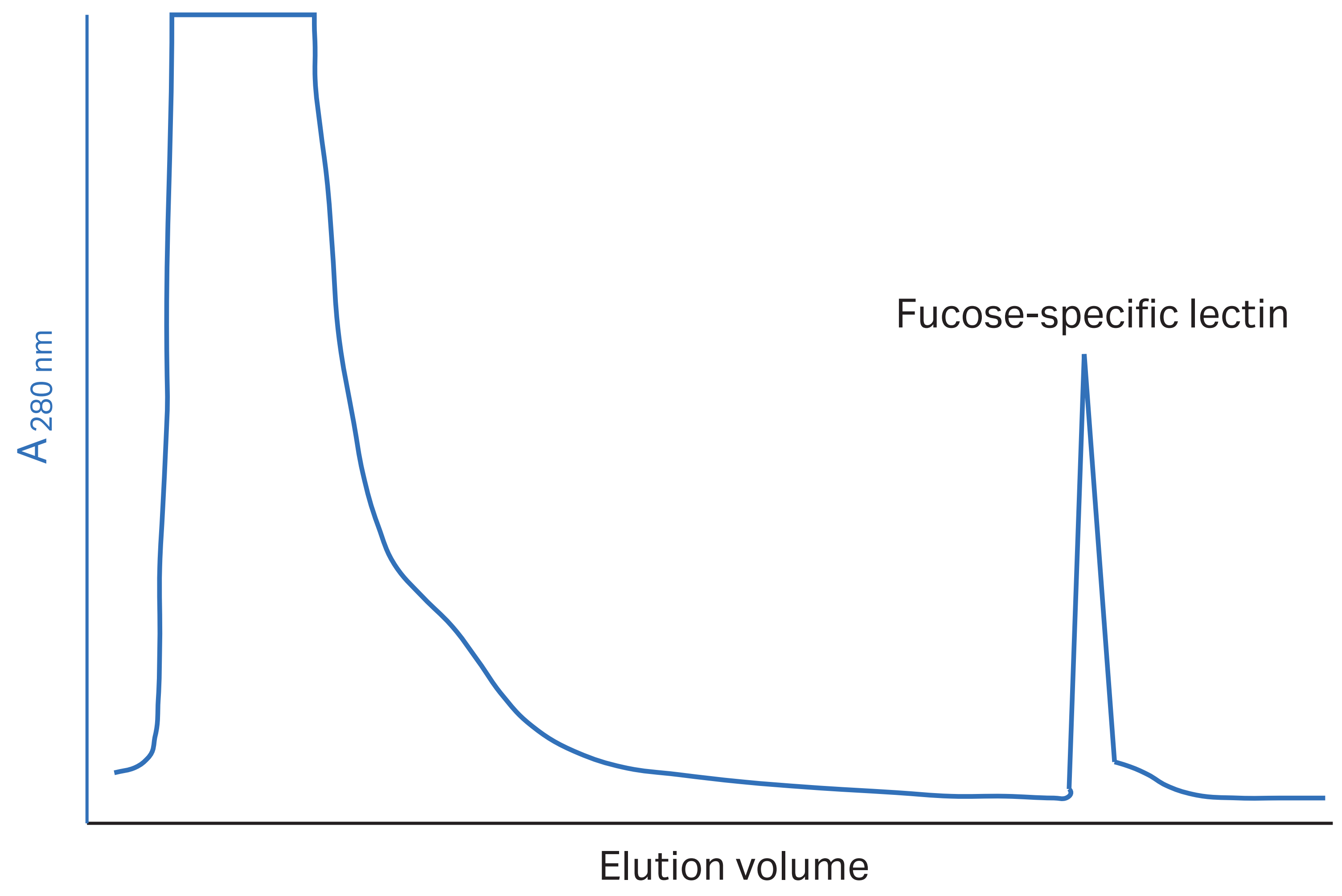


Fig 4.13. Chromatography of a crude extract of Ulex europaeus on fucose coupled to Epoxy-activated Sepharose 6B, column volume 11 mL. Extract was applied in 0.9% NaCl. Fucose-specific lectin was eluted with 5 mL fucose (50 mg/mL).

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 pH range of 9.0 to 13.0 as shown in Figure 4.14 and the efficiency of coupling is pH- and temperature-dependent (Fig 4.15).

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, see Appendix 8, or 20% ethanol in a suitable buffer.

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

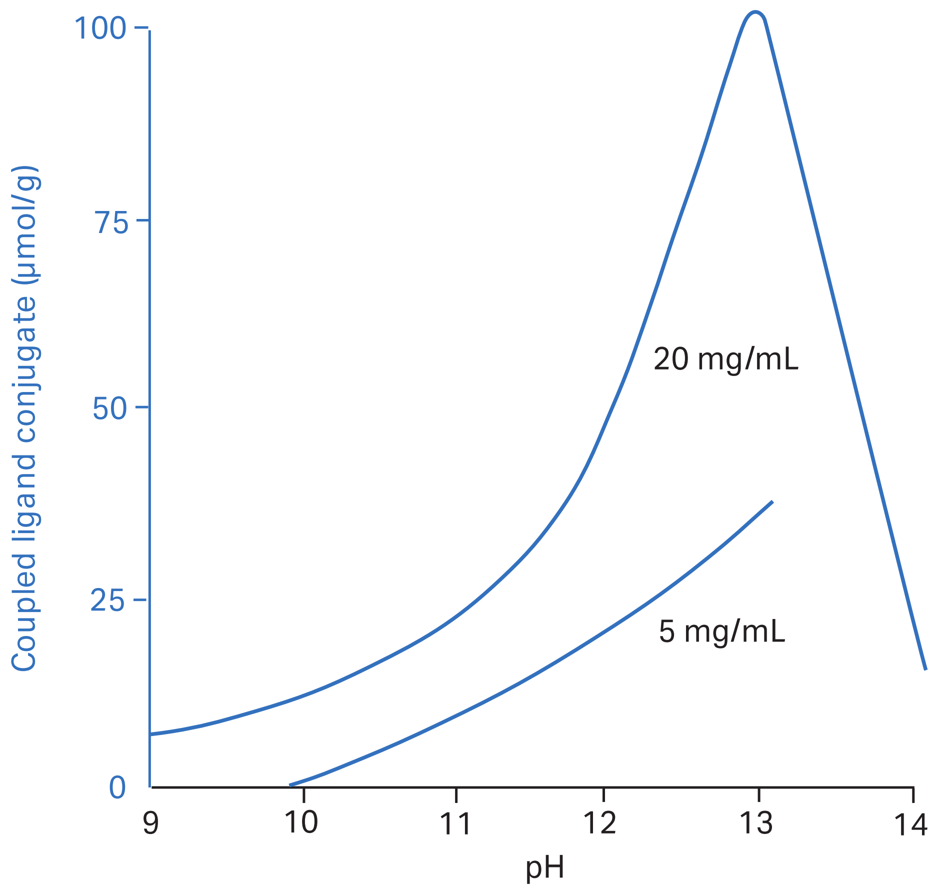


Fig 4.14. pH dependence of coupling N-acetyl-D-galactosamine to Epoxy-activated Sepharose 6B. Carbonate/bicarbonate buffers were used in the pH range of 9.0 to 11.0, sodium hydroxide solution in the pH range of 12.0 to 14.0. Ligand concentrations: 5 mg/mL and 20 mg/mL.

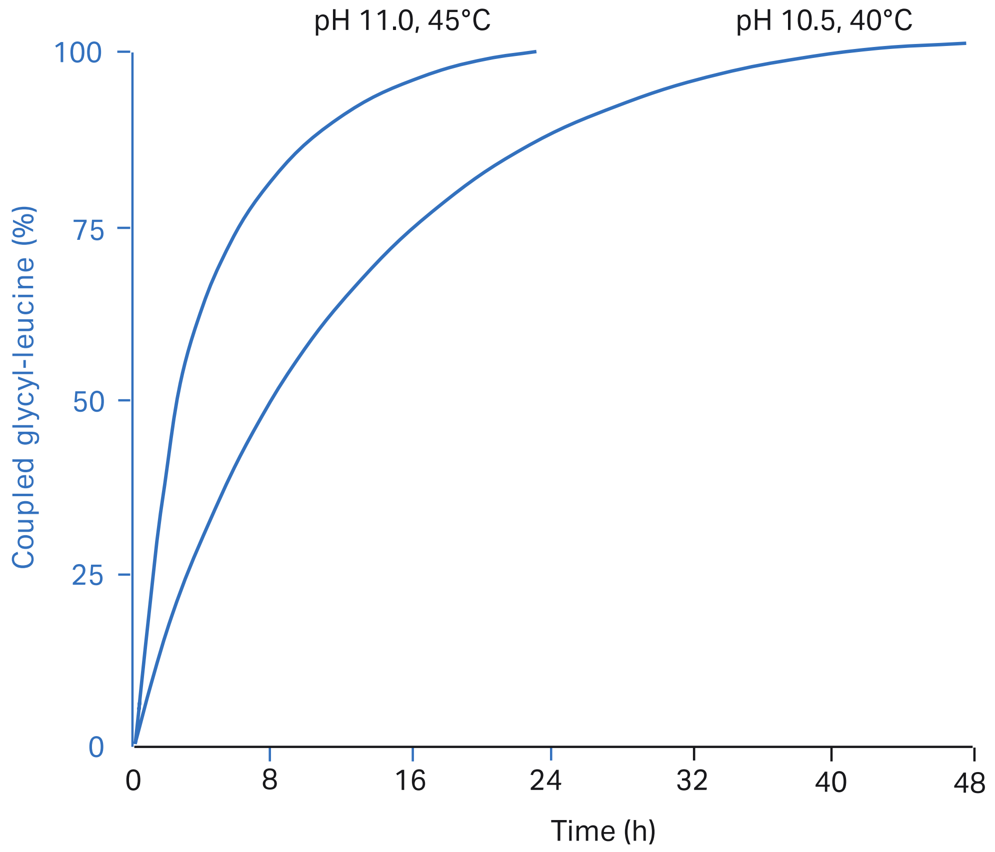


Fig 4.15. Efficiency of coupling glycyl-leucine to Epoxy-activated Sepharose 6B.

Coupling other functional groups

EAH Sepharose 4B may be used as a starting material for coupling via alternative functional groups (Fig 4.16). 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 6.0 (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.

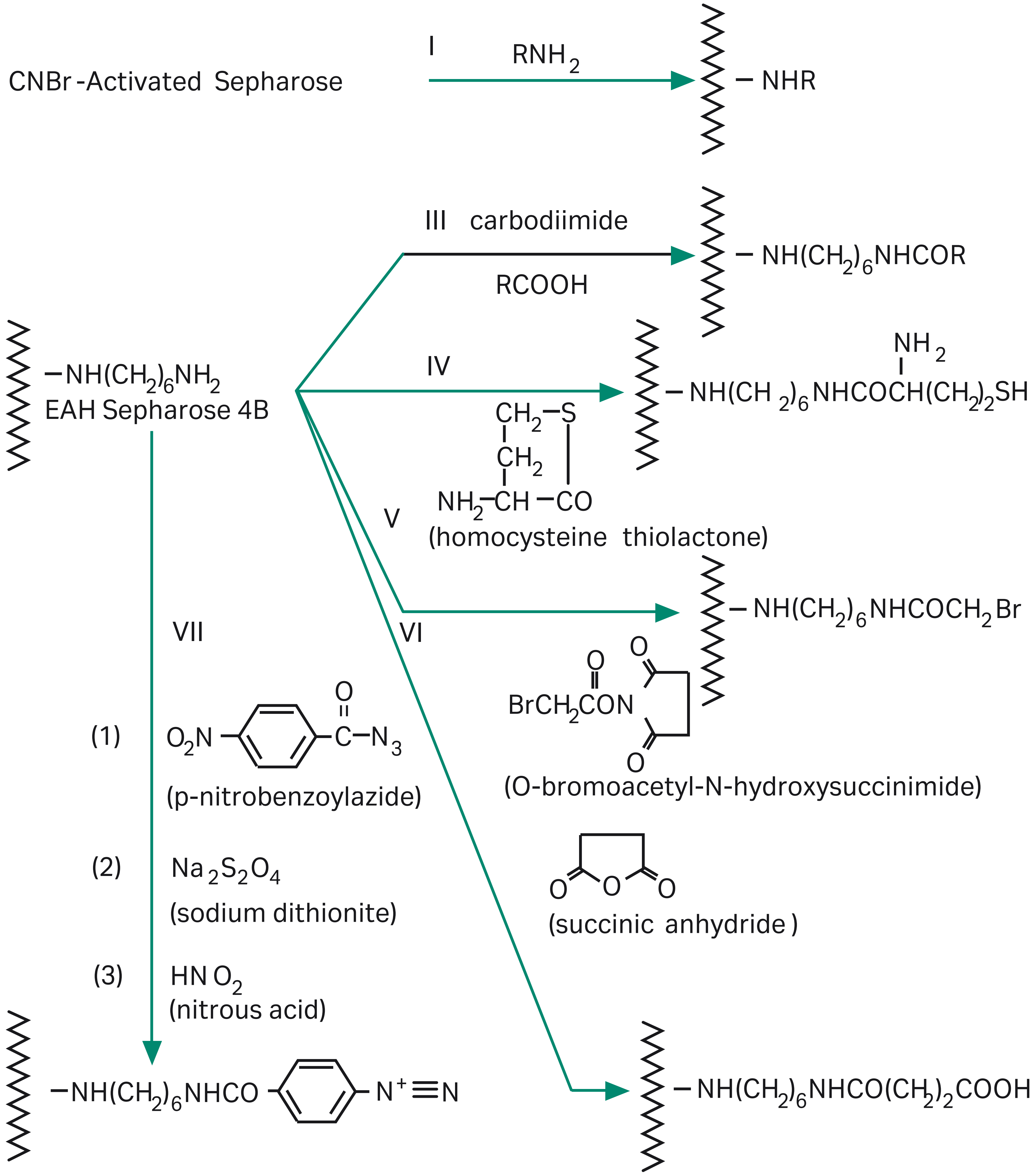


Fig 4.16. Reactions used to couple ligands to Sepharose.

05

Magnetic beads for affinity chromatography

Magnetic beads for affinity chromatography

The magnetic bead format facilitates fast and efficient small-scale experiments without the need for a chromatography system. The purification can simply be performed at the lab bench in combination with a magnetic device. Magnetic beads also provide flexibility, allowing a wide range of sample volumes and easy scaling up by varying the bead quantity.

Magnetic beads for AC from Cytiva include Streptavidin beads for biotinylated biomolecules, TiO_2 beads for phosphorylated biomolecules, and NHS- and carboxy-preactivated beads for covalent coupling to obtain highly specific chromatography media. The beads are superparamagnetic, which means that they do not retain magnetism once removed from a magnetic field.

Magnetic beads for purification of antibodies and histidine-tagged proteins are also available from Cytiva. These products are described in *Affinity Chromatography Handbook Vol. 1: Antibodies*, 18103746 and *Affinity Chromatography Handbook Vol. 2: Tagged Proteins*, 18114275.

The magnetic beads are based on two different matrices: Mag Sepharose and Sera-Mag. The major application area for Mag Sepharose beads is purification of proteins. The beads have a particle size of 37 to 100 µm and consist of highly cross-linked spherical agarose (Sepharose) containing magnetite. Since the beads share properties such as high porosity and high capacity with Sepharose chromatography media, Mag Sepharose beads can also be used for fast screening before scaling up to nonmagnetic Sepharose media with the same ligand.

Sera-Mag beads are small polymer based beads with a particle size of 1 µm. The beads possess colloidal stability, resisting flocculation and aggregation, and have high monodispersity with a narrow particle size range. Sera-Mag beads contains a single layer of magnetite, while Sera-Mag SpeedBeads have two layers of magnetite which increases the speed in response to the magnetic field (Fig 5.1). Sera-Mag and Sera-Mag SpeedBeads are designed for diagnostic kits and for purification of proteins, nucleic acids, and peptides.

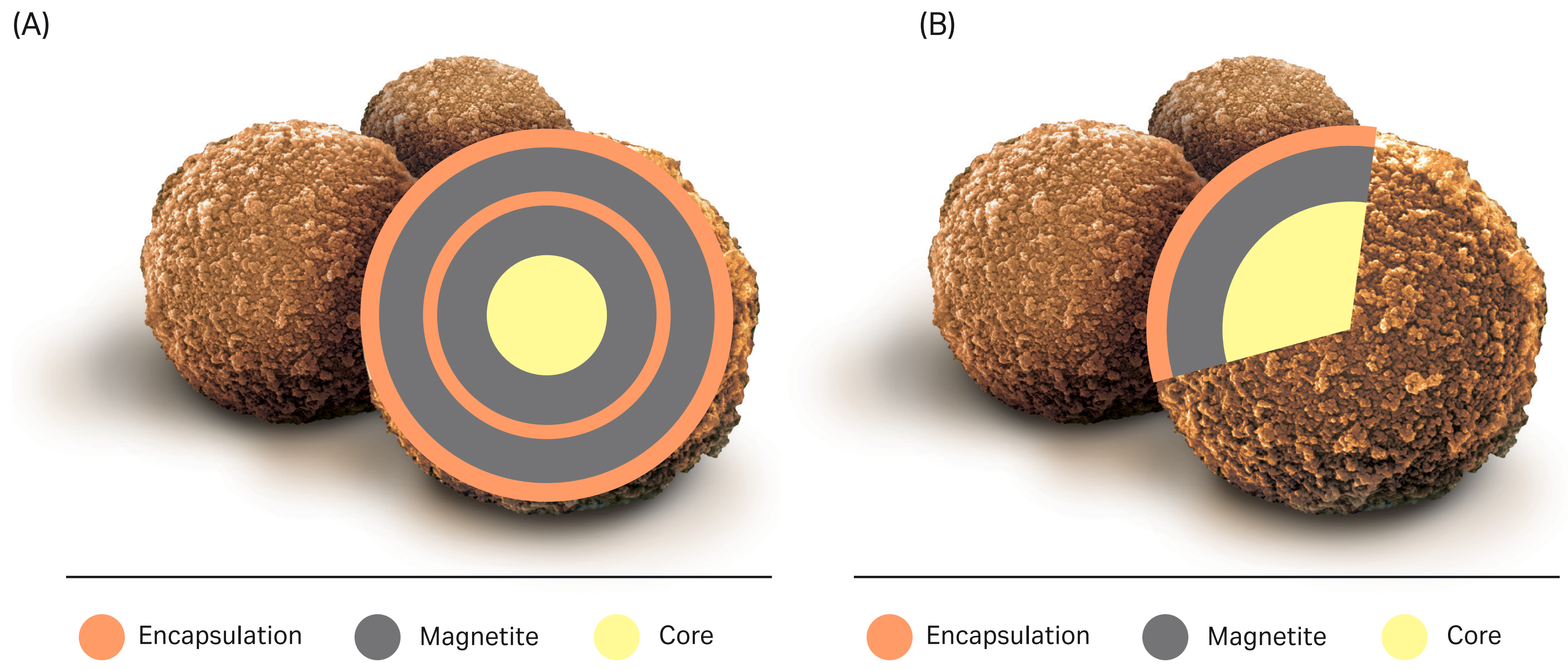


Fig 5.1. Sera-Mag SpeedBeads (A) have a second layer of magnetite within the bead, resulting in twice the speed in a magnetic field compared with Sera-Mag beads (B).

The handling of magnetic beads is simple, and efficient purifications are quickly performed in combination with a magnetic device such as MagRack 6 and MagRack Maxi (Fig 5.2). MagRack 6 enables preparation of up to six samples in 1.5 mL microcentrifuge tubes. The larger MagRack Maxi is designed for sample volumes up to 50 mL. When the tubes are placed in the rack, the magnetic beads are attracted to the magnet within a few seconds, allowing easy removal of the supernatant from the magnetic beads, which remain in the tube (see description on the following page). Except for MagRack 6 and MagRack Maxi, a robotic device can be used for a large number of samples for high throughput purification.

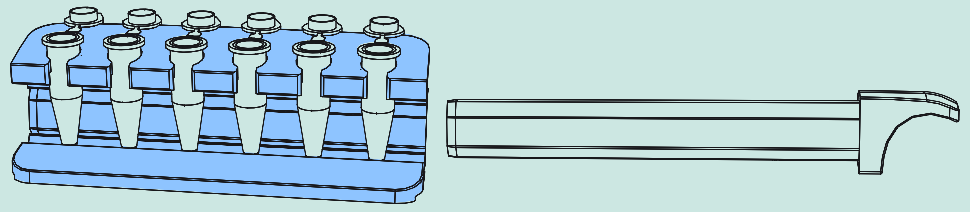
Although using a magnet is usually the most convenient and straightforward way to achieve the separation, magnetic beads have a high density and can alternatively be separated from liquid using centrifugation.

Fig 5.2. MagRack 6 (lower) and MagRack Maxi (upper) are designed for efficient small-scale purification using magnetic beads.

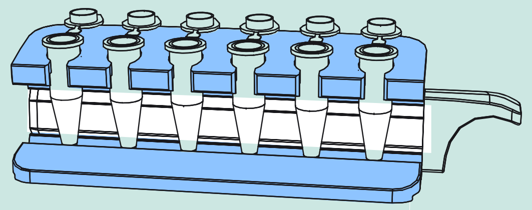
General magnetic bead separation steps

When performing magnetic bead separation, it is recommended to use MagRack 6 for test tubes up to 1.5 mL and MagRack Maxi for test tubes up to 50 mL.

1. Remove the magnet before adding liquid.



2. Insert the magnet before removing liquid.



When using volumes above 50 mL, the beads can be spun down using a swing-out centrifuge.

Dispensing the medium slurry

1. Prior to dispensing the medium slurry, make sure it is homogeneous by vortexing the vial thoroughly.
2. When the medium slurry is resuspended, immediately pipette the required amount of medium slurry into the desired tube.
3. Due to the fast sedimentation of the beads, it is important to repeat the resuspension between each pipetting.

Handling liquids

1. Before application of liquid, remove the magnet from the magnetic rack.
2. After addition of liquid, resuspend the beads by vortexing or manual inversion of the tube. When processing multiple samples, manual inversion of the magnetic rack is recommended.
3. Use the magnetic rack with the magnet in place for each liquid removal step. Pipette or pour off the liquid. If needed, a pipette can be used to remove liquid from the lid of the test tube.

Incubation

During incubation, make sure the magnetic beads are well resuspended and kept in solution by end-over-end mixing or by using a benchtop shaker.

☞ Incubation generally takes place at room temperature. However, incubation can take place at 4°C if this is the recommended condition for the specific sample.

☞ When purifying samples of large volumes, an increase of the incubation time may be necessary.

Purification or removal of biotin and biotinylated biomolecules with magnetic beads

Streptavidin Mag Sepharose, Sera-Mag Streptavidin coated, Sera-Mag SpeedBeads Streptavidin-Coated, Sera-Mag SpeedBeads Streptavidin-Blocked, Sera-Mag SpeedBeads Neutravidin-Coated

The magnetic beads for separation of biotinylated biomolecules have a Streptavidin ligand. Streptavidin is an M_r 60 000 protein from *Streptomyces avidinii*, and is a tetramer containing four biotin binding sites. Since the affinity between streptavidin and biotin is exceptionally high, harsh conditions are required for disruption, such as the use of SDS in the sample buffer. Therefore, it is also possible to use the beads for immunoprecipitation and elute binding partners in an interaction complex without coeluting the biotinylated component. The principle of immunoprecipitation is shown in Figure 5.3.

Streptavidin Mag Sepharose is used for efficient enrichment of biotinylated proteins, such as antibodies and immunoprecipitation. Streptavidin-coated Sera-Mag and Sera-Mag SpeedBeads are designed for isolation of biotinylated targets such as PCR products, oligos, and antibodies. The beads are generally used for increased throughput and precision in immunoassays. Neutravidin-coated and Streptavidin-blocked beads have reduced nonspecific binding, which can be beneficial in some applications.

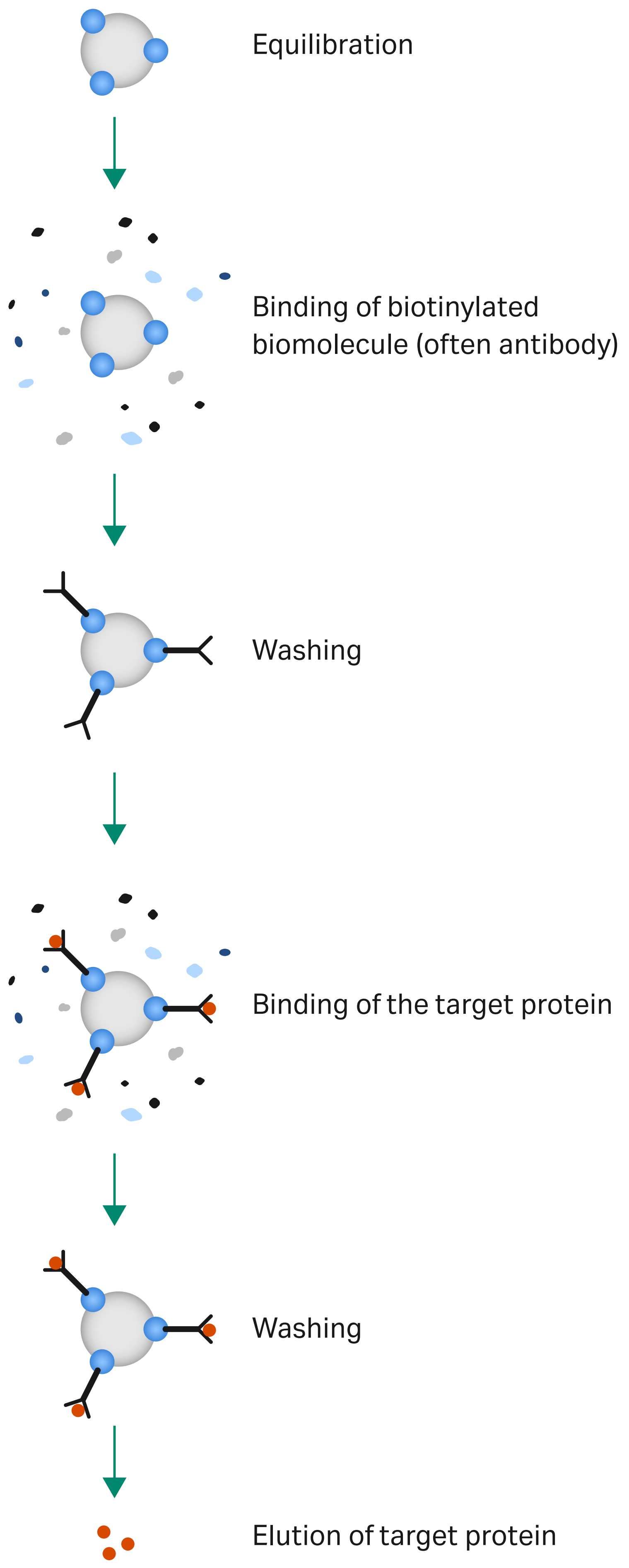


Fig 5.3. Principle of immunoprecipitation.

Bead characteristics

Characteristics of Mag Sepharose, Sera-Mag, and Sera-Mag SpeedBeads for the capture of biotin and biotinylated substances are shown in Table 5.1.

Table 5.1. Characteristics of Mag Sepharose, Sera-Mag, and Sera-Mag SpeedBeads for capture of biotin and biotinylated molecules

Product	Ligand	Matrix	Binding capacity	Average particle size (µm)
Streptavidin Mag Sepharose	Streptavidin	Highly cross-linked agarose with magnetite	300 µg biotinylated BSA/mL medium slurry	37 to 100
Sera-Mag Streptavidin-Coated	Streptavidin	Polymer beads with single layer of magnetite	Biotin (pmol/mg): Low 2500 to 3500 Medium 3500 to 4500 High 4500 to 5500	1
Sera-Mag SpeedBeads Streptavidin-Coated	Streptavidin	Polymer beads with double layer of magnetite	Biotin (pmol/mg): Low 2500 to 3500 Medium 3500 to 4500 High 4500 to 5500	1
Sera-Mag SpeedBeads Streptavidin-Blocked	Streptavidin	Polymer beads with double layer of magnetite	Fluorescein (pmol/mg): Medium ~ 3500	1
Sera-Mag SpeedBeads Neutravidin-Coated	Neutravidin	Polymer beads with double layer of magnetite	Biotin (pmol/mg): Medium 3500 to 4500	1

Purification examples

Immunoprecipitation of low concentration transferrin from large sample volumes

The ability to use different volumes of sample and medium slurry is one of the key advantages of the magnetic beads separation method. Streptavidin Mag Sepharose was used to purify human transferrin spiked in 5 mg/mL of *E. coli* lysate. Purification was scaled up 10-fold from 50 to 500 µL of Streptavidin Mag Sepharose and 3 to 30 mL of sample, respectively. The experiment was performed in duplicate using MagRack Maxi. The transferrin concentration was 0.75 µg/mL, which corresponds to ~ 0.015% of the total *E. coli* protein content. Transferrin was captured by immunoprecipitation using a biotinylated polyclonal rabbit antihuman transferrin immobilized on the medium and the yield of transferrin was estimated by SDS-PAGE of the eluted fractions.

The results (Fig 5.4) show the inherent flexibility of Streptavidin Mag Sepharose. A corresponding increase in the purity and recovery of transferrin was observed when the immunoprecipitation was scaled up 10-fold. The yields of transferrin were 1.2 and 13 µg using 50 and 500 µL of Streptavidin Mag Sepharose slurry, respectively. The average purity was 78% (5200-fold enrichment) and 75% (5000-fold enrichment).

Performing a separation: Streptavidin Mag Sepharose

The following recommended protocols are suitable as starting points for most purifications involving biotinylated biomolecules. However, the optimal parameters depend on the specific biomolecules used and optimization may be required for best results.

Magnetic beads: Strepatvidin Mag Sepharose
Medium slurry volume: 50 or 500 µL
Sample: 0.75 µg/mL human transferrin in 5 mg/mL *E. coli* lysate
Sample volume: 3 or 30 mL
Antibody: Polyclonal rabbit antihuman transferrin (biotinylated)
Binding buffer: Tris-buffered saline (TBS: 50 mM Tris, 150 mM NaCl), pH 7.5
Wash buffer: TBS, 2 M urea, pH 7.5
Elution buffer: 100 mM glycine-HCl, pH 2.9

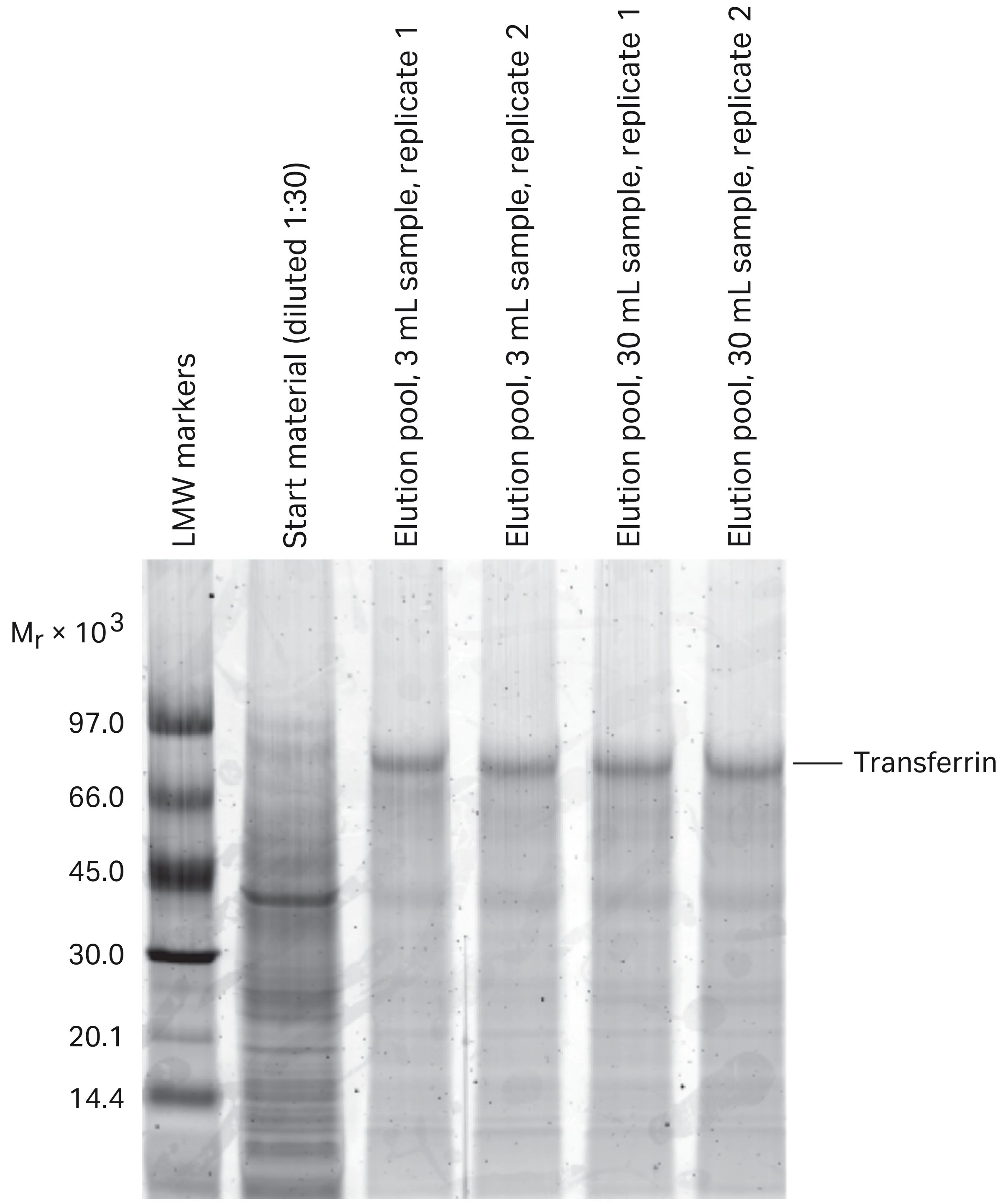


Fig 5.4. SDS-PAGE (reducing conditions) stained with Deep Purple Total Protein Stain. The purity and recovery obtained were equally high when the scale of purification was increased 10-fold. Quantitation of the eluted transferrin was performed using standard curves with known amounts of transferrin (data not shown).

Examples of parameters that might require optimization are:

- Amount of beads
- Amount of biotinylated biomolecules
- Amount of biomolecules to be enriched in immunoprecipitation
- Incubation time
- Number of washes
- Buffer compositions and pH

Recommended buffers for capture and elution of biotinylated proteins:
Binding buffer: Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl), pH 7.5
Washing buffer: TBS, 2 M urea, pH 7.5
Elution buffer: 2% SDS

Recommended buffers for immunoprecipitation:
Binding buffer: TBS, pH 7.5
Washing buffer: TBS, 2 M urea, pH 7.5
Elution buffer: 100 mM glycine-HCl, 2 M urea, pH 2.9

Alternative buffers for optimization:
Wash buffer: 100 mM triethanolamine, 500 mM NaCl, pH 9.0
Elution buffer: - 100 mM glycine, pH 2.5 to pH 3.1
- 100 mM citric acid, pH 2.5 to pH 3.1
- 100 mM ammonium hydroxide, pH 10.0 to pH 11.0

Sample preparation

Adjust the sample to the composition and pH of the binding buffer. pH can be adjusted by either diluting the sample with binding buffer or by buffer exchange using PD-10 MiniTrap™ G-25 or HiTrap Desalting columns (see Appendix 1). Clarify the sample before applying it to the beads, if needed. Inhibiting protease activity in the sample prevents degradation of the target protein.

Purification of biotinylated proteins

☞ Use the magnetic rack with the magnet in place to attract the beads before each liquid removal step.

1. Prepare the Mag Sepharose beads
    - A. Mix the medium slurry thoroughly by vortexing. Dispense 100 μL of the homogenous medium slurry into an Eppendorf™ tube.
    - B. Place the Eppendorf tube in the magnetic rack to attract the beads.
    - C. Remove the storage solution.
2. Equilibration
    - A. Add 500 μL binding buffer and resuspend the medium.
    - B. Remove the liquid.
3. Apply the sample
    - A. Add 300 μL of sample. If the sample volume is less than 300 μL, dilute to 300 μL with binding buffer.
    - B. Resuspend the medium and incubate for 30 min with slow end-over-end mixing or by using a benchtop shaker.
    - C. Remove the sample.
4. Wash (perform this step three times)
    - A. Add 500 μL washing buffer and resuspend the medium.
    - B. Remove the liquid.
5. Elute biotinylated proteins
    - A. Add 100 μL elution buffer.
    - B. Resuspend the medium and incubate at 95°C to 100°C for 5 min.
    - C. Remove and collect the eluted fraction. The collected fraction contains the main part of the protein. If needed, repeat the elution.

☞ The streptavidin-biotin bond can be broken efficiently only by harsh denaturing conditions. Hence, dissociation of biotin from streptavidin will denature both the biotinylated protein and streptavidin, causing a leakage of the streptavidin monomer.

Immunoprecipitation

☞ Use the magnetic rack with the magnet in place to attract the beads before each liquid removal step.

1. Prepare the Mag Sepharose beads
   - A. Mix the medium slurry thoroughly by vortexing. Dispense 50 µL of the homogenous medium slurry into an Eppendorf tube.
   - B. Place the Eppendorf tube in the magnetic rack to attract the beads.
   - C. Remove the storage solution.
2. Equilibration
   - A. Add 500 µL binding buffer and resuspend the medium.
   - B. Remove the liquid.
3. Binding of biotinylated antibody
   - A. Add 300 µL of biotinylated antibody solution (~ 0.2 to 0.4 mg/mL). If the sample volume is less than 300 µL, dilute to 300 µL with the binding buffer.
   - B. Resuspend the medium and incubate for 30 min with slow end-over-end mixing or by using a benchtop shaker.
   - C. Remove the liquid.
4. Wash (perform this step twice)
   - A. Add 500 µL washing buffer and resuspend the medium.
   - B. Remove the liquid.
5. Binding of the target protein
   - A. Add 300 µL of sample. If the sample volume is less than 300 µL, dilute to 300 µL with binding buffer.
   - B. Resuspend the medium and incubate for 60 min with slow end-over-end mixing or by using a benchtop shaker.
   - C. Remove the liquid.
6. Wash (perform this step three times)
   - A. Add 500 µL washing buffer and resuspend the medium.
   - B. Remove the liquid.
7. Elution
   - A. Add 50 µL of elution buffer.
   - B. Resuspend the medium and incubate for 2 min.
   - C. Remove and collect the elution fraction. The collected elution fraction contains the main part of the protein. If needed, repeat the elution.

Performing a separation: Sera-Mag SpeedBeads Streptavidin-Blocked

Sample preparation

Combine antigen sample with 10 µg of biotinylated antibody (or biomolecule). Incubate 1 to 2 h at room temperature or overnight at 4°C with mixing.

☞ Dilute each sample to a minimum volume of 300 µL with cell lysis buffer or binding/wash buffer.

Immunoprecipitation

☞ Use the magnetic rack with the magnet in place to attract the beads before each liquid removal step.

☞ See recommended buffers for Streptavidin Mag Sepharose.

☞ If a low pH elution buffer is selected for elution, streptavidin may leach from the particles. Low pH elution buffers are effective for most antibody-antigen interactions. However, to ensure efficient release of target antigen from the antibody, prerinse the beads with 300 µL of 0.1% Tween 20 in water (no buffering capacity) before adding low pH elution buffer.

☞ Alternative elution for recovery of antigen: Add 100 µL of SDS reducing sample buffer to the tube and heat the samples at 96°C to 100°C in a heating block for 5 min. Magnetically separate the particles and save the supernatant containing the target antigen.

1. Prepare the Sera-Mag beads
    - A. Mix the medium slurry thoroughly by vortexing. Dispense 50 µL (0.5 mg) of the homogenous medium slurry into an Eppendorf tube.
    - B. Place the Eppendorf tube in the magnetic rack to attract the beads.
    - C. Remove the storage solution.
2. Equilibration
    - A. Add 1 mL of binding buffer and resuspend the medium.
    - B. Remove the liquid.
3. Binding of biotinylated antibody/antigen sample
    - A. Add 300 µL of biotinylated antibody/antigen mixture.
    - B. Resuspend the medium and incubate at room temperature for 1 h with slow end-over-end mixing or by using a bench top shaker.
    - C. Remove the liquid.
4. Washing (perform this step twice)
    - A. Add 300 µL of wash buffer and resuspend the medium.
    - B. Remove the liquid.
5. Elution
    - A. Add 100 µL of elution buffer.
    - B. Resuspend the medium and incubate at room temperature with mixing for 5 min.
    - C. Remove and collect the elution fraction. The collected fraction contains the main part of the protein. If needed, repeat the elution.

Purification or removal of phosphorylated biomolecules

TiO_2 Mag Sepharose

Phosphorylation is a common reversible post-translational modification involved in the regulation of many essential biological processes. Phosphoproteins and phosphopeptides are usually present at very low concentrations and ionize poorly, making their detection by MS difficult.

TiO_2 Mag Sepharose magnetic beads simplify capture and enrichment of phosphopeptides by titanium dioxide (TiO_2)-based chromatography (Fig 5.5). TiO_2 has high affinity for phosphopeptides and provides efficient enrichment of phosphopeptides from complex samples.

Bead characteristics

Characteristics of TiO_2 Mag Sepharose beads are shown in Table 5.2.

Table 5.2. Characteristics of Mag Sepharose beads

Product	Ligand	Matrix	Binding capacity	Average particle size (µm)
TiO_2 Mag Sepharose	TiO_2	Highly cross-linked agarose with magnetite	~ 35 µg phosphopeptide/mL medium slurry	37 to 100

Purification examples

Two phosphorylated proteins (α-casein and β-casein) and one nonphosphorylated protein (bovine serum albumin) were reduced and alkylated with Tris(2-carboxyethyl) phosphine (TCEP) and iodoacetamide, respectively, followed by trypsin-digestion. A total of 50 pmol of each protein digest was mixed and applied to the magnetic beads. After enrichment, the eluates were lyophilized and dissolved in 20% acetonitrile with 0.1% trifluoroacetic acid (TFA, 20 µL) and analyzed by MALDI-ToF MS.

TiO_2 Mag Sepharose detected five peptides, with a ratio of 2.5 between phosphopeptides and nonphosphorylated peptides. Also, after a 100-fold dilution of the eluate, two phosphopeptides could still be detected. The experimental conditions and mass spectrograms are shown in Figure 5.6.

Fig 5.5. TiO_2 Mag Sepharose is designed for efficient small-scale enrichment of phosphopeptides.

Performing a separation

Binding buffer:	1 M glycolic acid in 80% acetonitrile, 5% trifluoroacetic acid
Wash buffer:	80% acetonitrile, 1% trifluoroacetic acid
Elution buffer:	5% ammonium hydroxide, pH ~ 12.0

Use high-purity water and chemicals for buffer preparation.

Sample preparation

For complex samples, such as cell lysate digests, it is recommended to perform a desalting step by use of, for example, an RPC/C18 cartridge or similar for efficient phosphopeptide enrichment.

Dilute the sample with a minimum of four volumes of binding buffer or dissolve lyophilized sample in binding buffer. Keep sample volumes small, preferably max 100 µL, however, up to 250 µL may be used.

Magnetic beads:	TiO_2 Mag Sepharose
Sample:	100 µL mix of tryptic fragments from BSA, α-casein, and β-casein (50 pmol each)
Conditioning/equilibration:	1 × 500 µL binding buffer (1 M glycolic acid, 5% TFA, 80% acetonitrile)
Binding:	1 × 30 min
Washing:	1 × 500 µL binding buffer 2 × 500 µL washing buffer (1% TFA, 80% acetonitrile)
Elution:	2 × 50 µL elution buffer (5% ammonia)
Analysis:	MALDI-ToF MS

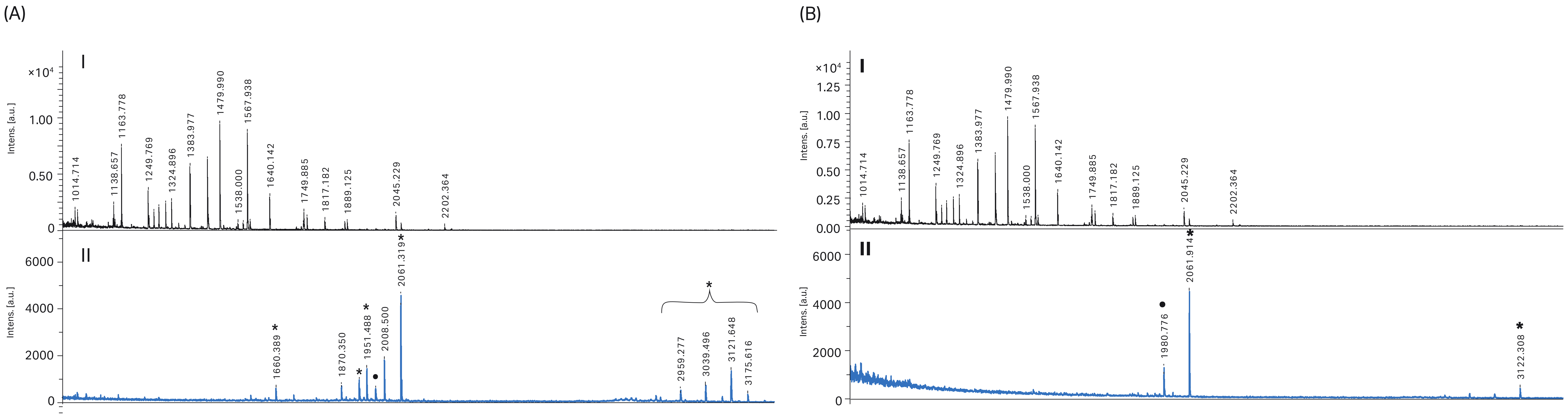


Fig 5.6. MALDI-ToF MS analysis of trypsin-digested protein mix (50 pmol each of BSA, α-casein, and β-casein) enriched using three different chromatographic media. (A) Spotting from lyophilized eluates dissolved in 20 µL and (B) eluates diluted 100-fold before spotting. The spectra show start material (Panel I) and eluates from TiO_2 Mag Sepharose (Panel II). Identified phosphorylated peptides are marked with asterisks*.

Enrichment of phosphorylated proteins

☞ Use the magnetic rack with the magnet in place to attract the beads before each liquid removal step.

MS analysis

Eluates must be evaporated or neutralized with formic acid or trifluoroacetic acid before analysis with MALDI-ToF. Suitable solvent for evaporated samples is 20% acetonitrile acidified with 0.1% trifluoroacetic acid. For LC-MS analysis using reversed phase chromatography (RPC) the eluates must first be evaporated and resuspended in formic acid to a final concentration of 0.1%.

1. Prepare the Mag Sepharose beads
   A. Mix the medium slurry thoroughly by vortexing. Dispense 50 µL of the homogenous medium slurry into an Eppendorf tube.
   B. Place the Eppendorf tube in the magnetic rack to attract the beads.
   C. Remove the storage solution.
2. Equilibration
   A. Add 500 µL binding buffer and resuspend the medium.
   B. Remove the liquid.
3. Apply the sample
   A. Add 50 µL to 250 µL sample.
   B. Resuspend the medium and incubate for 30 min with slow end-over-end mixing or by using a benchtop shaker.
   C. Remove the liquid.
4. Wash 1
   A. Add 500 µL binding buffer and resuspend the medium.
   B. Remove the liquid.
5. Wash 2 and 3 (perform this step twice)
   A. Add 500 µL wash buffer and resuspend the medium.
   B. Remove the liquid.
6. Elution
   A. Add 50 µL elution buffer.
   B. Resuspend the medium and incubate for 5 min.
   C. Remove and collect the eluted fraction. The collected fraction contains the main part of the protein. If needed repeat the elution.

Preactivated magnetic beads

NHS Mag Sepharose, Sera-Mag Carboxylate-Modified, Sera-Mag SpeedBeads Carboxylate-Modified

Preactivated magnetic beads are designed for covalent coupling of antibodies, aptamers, and proteins. After coupling is performed, proteins of interest can be affinity captured and enriched using immunoprecipitation (see example in Fig 5.7). The preactivated magnetic beads include NHS Mag Sepharose, Sera-Mag Carboxylate-Modified, and Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles.

NHS Mag Sepharose has an N-hydroxysuccinimide ligand to which molecules with primary amino groups bind covalently. This enables enrichment of target protein for further downstream analyses such as LC-MS and electrophoresis techniques.

Sera-Mag and Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles have carboxylic groups on the surface that permit easy covalent coupling using simple carbodiimide chemistry.

Proteins bind to carboxylate-modified particles by adsorption. Adsorption is mediated by hydrophobic and ionic interactions between the protein and the surface of the particles.

In addition to being adsorbed, proteins can be covalently attached to the surface of carboxylate-modified particles. Carboxyl groups on the particles, activated by the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), react with free amino groups of the adsorbed protein to form amide bonds.

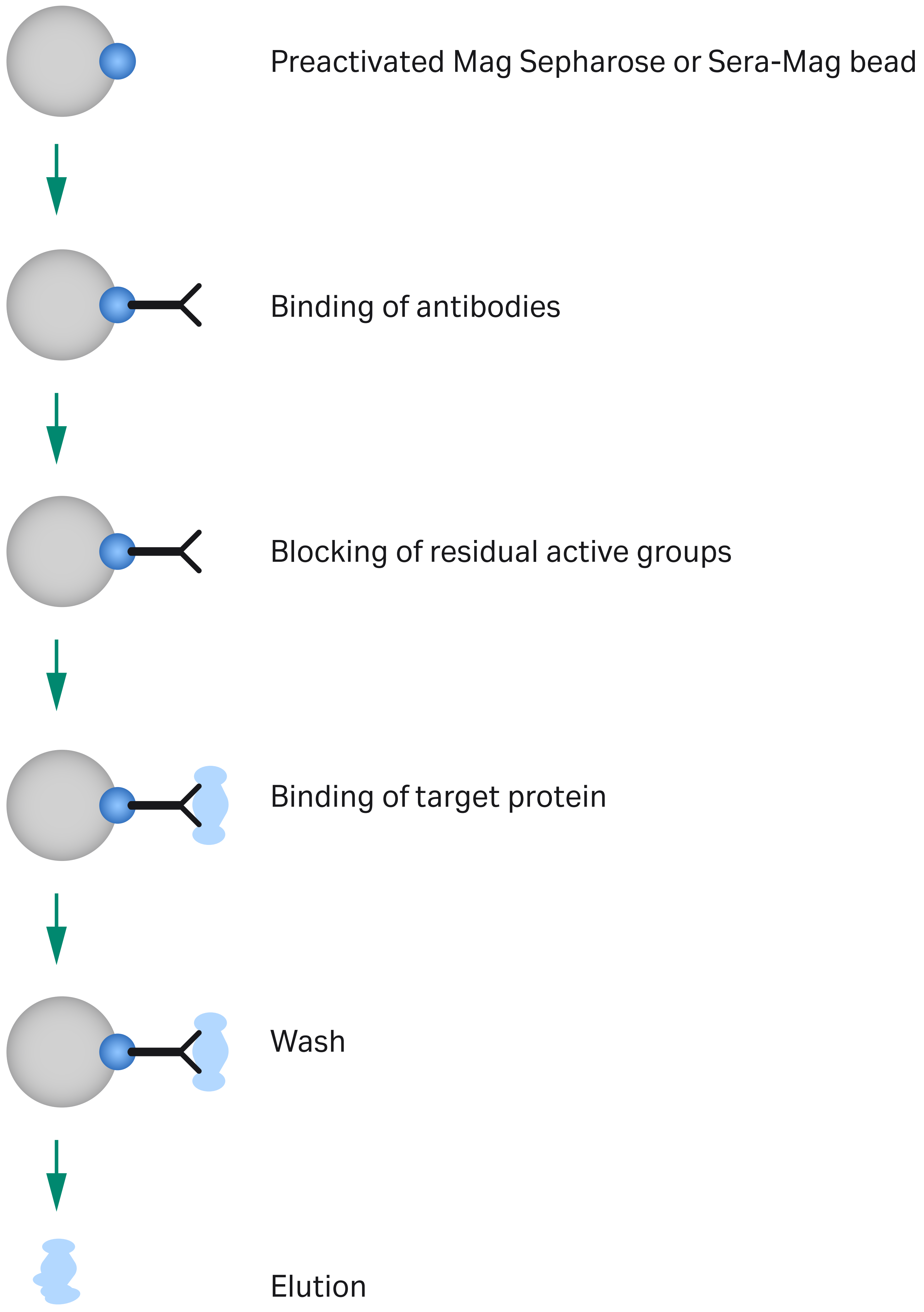


Fig 5.7. Principle for coupling of biomolecules.

Bead characteristics

Characteristics of Mag Sepharose, Sera-Mag, and Sera-Mag SpeedBeads preactivated magnetic beads are shown in Table 5.3.

Table 5.3. Characteristics of Mag Sepharose, Sera-Mag, and Sera-Mag SpeedBeads preactivated magnetic beads

Product	Ligand	Matrix	Binding capacity	Average particle size (µm)
NHS Mag Sepharose	N-hydroxysuccinimide	Highly cross-linked agarose with magnetite	8 to 14 µmol/mL medium	37 to 100
Sera-Mag Carboxylate-Modified	Carboxylate	Polymer beads with single layer of magnetite	Sample dependent, no data available	1
Sera-Mag SpeedBeads Carboxylate-Modified	Carboxylate	Polymer beads with double layers of magnetite	Sample dependent, no data available	1

Purification example

Enrichment of plasminogen from human plasma

Human plasma contains a vast number of proteins and can be difficult to work with due to the great range of protein concentrations. In this experiment, plasminogen was enriched from human plasma by immunoprecipitation. Monoclonal antiplasminogen mouse IgG1 was covalently coupled to NHS Mag Sepharose for capture of plasminogen. The eluted fractions were analyzed by SDS-PAGE (Fig 5.8) and showed efficient enrichment of the target protein. Enrichment and identification of plasminogen were confirmed by LC-MS/MS analysis.

Magnetic beads:	25 µL of NHS Mag Sepharose
Sample:	Human plasma, 6 mL
Antibodies:	Antiplasminogen mouse monoclonal IgG_1
Coupling buffer:	150 mM triethanolamine, 500 mM NaCl, pH 8.3
Binding buffer:	Tris buffered saline (TBS: 50 mM Tris, 150 mM NaCl, pH 7.5)
Wash buffer:	TBS, 2 M urea, pH 7.5
Elution buffer:	2 × 50 µL of 100 mM glycine/HCl, 2 M urea, pH 2.9

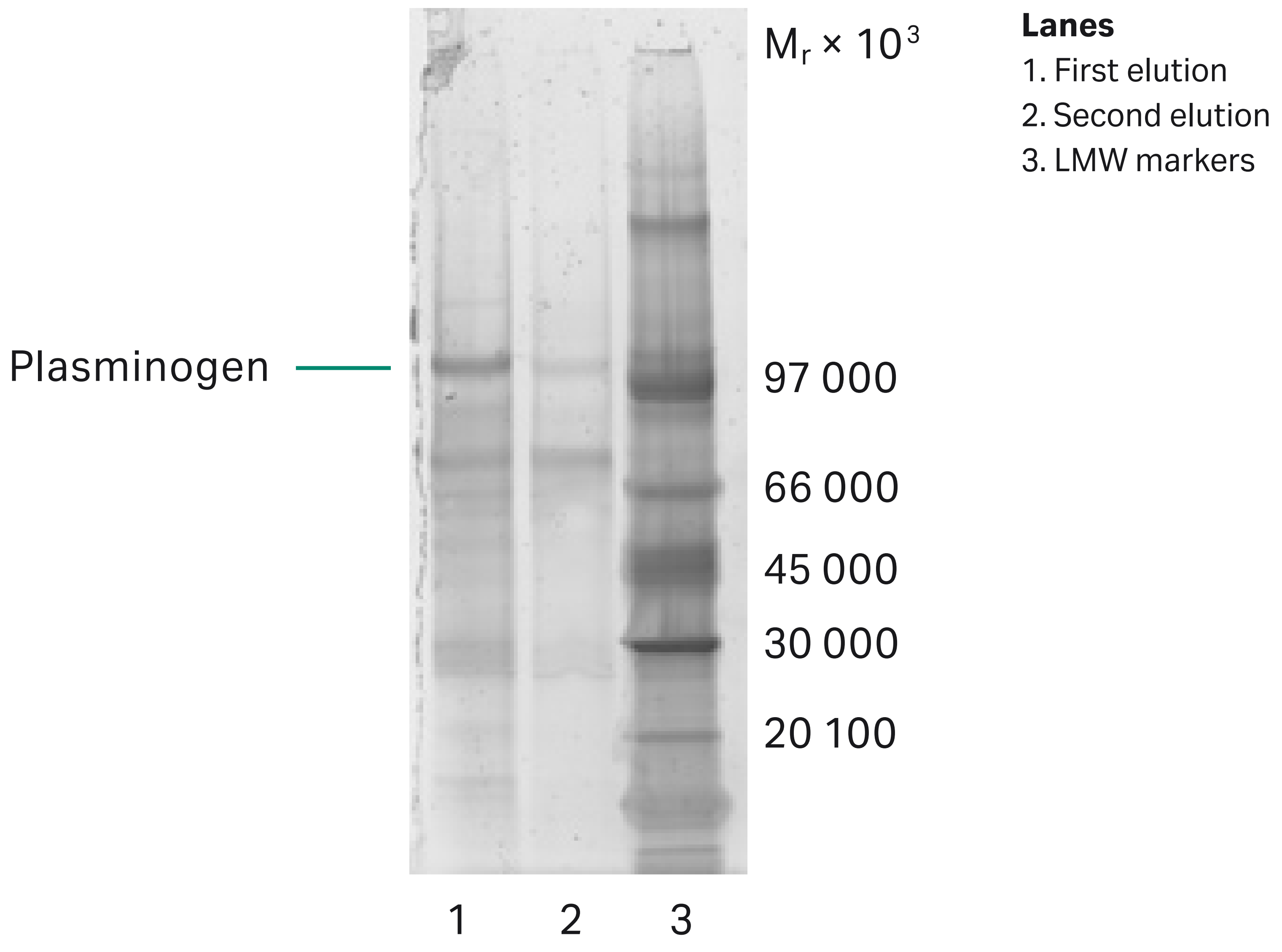


Fig 5.8. SDS-PAGE results of the enrichment of plasminogen from human plasma using NHS Mag Sepharose magnetic beads. The gel was post-stained with Deep Purple Total Protein Stain and scanned.

Performing a separation

NHS Mag Sepharose

The optimal parameters for protein enrichment are dependent on the specific combination of biomolecules used. Optimization may be required for each specific combination to obtain the best result. Examples of parameters which may require optimization are:

- Amount of beads
- Amount of antibodies
- Amount of protein (antigen) to be enriched
- Incubation times
- Choice of buffers
- Number of washes

Equilibration buffer:	1 mM HCl (ice cold)
Coupling buffers:	150 mM triethanolamine, 500 mM NaCl, pH 8.3
	200 mM $NaHCO_3$, 500 mM NaCl, pH 8.3
Blocking buffer A:	500 mM ethanolamine, 500 mM NaCl, pH 8.3
Blocking buffer B:	100 mM Na-acetate, 500 mM NaCl, pH 4.0
Binding buffer:	TBS (50 mM Tris, 150 mM NaCl, pH 7.5)
Wash buffer:	TBS with 2 M urea, pH 7.5
Elution buffer:	100 mM glycine-HCl, 2 M urea, pH 2.9
Alternative buffers:	
Blocking buffers:	50 mM Tris-HCl, 1 M NaCl, pH 8.0
	50 mM glycine-HCl, 1 M NaCl, pH 3.0
Elution buffers:	100 mM glycine-HCl, pH 2.5 to 3.1
	100 mM citric acid, pH 2.5 to 3.1
	2.5% acetic acid
	2% SDS
	100 mM ammonium hydroxide, pH 10.0 to 11.0

Preparation of antibody solution

Prepare the antibody solution by dilution in coupling buffer and keep it on ice.

Coupling and purification of target protein

☞ Use the magnetic rack with the magnet in place to attract the beads before each liquid removal step.

✋ Do not use an amine-containing buffer (e.g., Tris or glycine) for the antibody/protein that is to be coupled since the amines in the buffer will compete for the coupling sites.

☞ Remove potential amines before coupling with antibody solution, for example by dialysis or buffer exchange with a desalting column.

1. Prepare the Mag Sepharose beads
   - A. Mix the medium slurry thoroughly by vortexing. Dispense 25 μL of medium slurry into an Eppendorf tube.
   - B. Place the Eppendorf tube in the magnetic rack to attract the beads.
   - C. Remove the storage solution.
2. Equilibration
   - A. Add 500 μL ice cold equilibration buffer and resuspend the medium.
   - B. Remove the liquid.
3. Binding of antibody
   - A. Immediately after equilibration, add the antibody solution (at least 50 μL).
   - B. Resuspend the medium and incubate at least 15 min with slow end-over-end mixing or by using a benchtop shaker.
   - C. Remove the liquid.
4. Blocking of residual active groups
   - A. Add 500 μL blocking buffer A and remove the liquid.
   - B. Add 500 μL blocking buffer B and remove the liquid.
   - C. Add 500 μL blocking buffer A.
   - D. Incubate for 15 min with slow end-over-end mixing.
   - E. Remove the liquid.
   - F. Add 500 μL blocking buffer B and remove the liquid.
   - G. Add 500 μL blocking buffer A and remove the liquid.
   - H. Add 500 μL blocking buffer B and remove the liquid.
5. Equilibration for binding
   - A. Add 500 μL binding buffer and resuspend the medium.
   - B. Remove the liquid.
6. Binding of target protein
   - A. Add sample, diluted in, for example, binding buffer.
   - B. Resuspend the medium and incubate for 10 to 60 min with slow end-over-end mixing.
   - C. Remove the liquid.
7. Wash (perform this step three times)
   - A. Add 500 μL wash buffer.
   - B. Remove the liquid.
8. Elution (perform this step twice)
   - A. Add 50 μL elution buffer.
   - B. Resuspend the medium and incubate for at least 2 min.
   - C. Remove and collect the elution fraction. The collected elution fraction contains the main part of the protein. If needed, repeat the elution.

Sera-Mag and Sera-Mag SpeedBeads Carboxylate-Modified

Sera-Mag and Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles feature carboxylic groups on the surface that permit easy covalent coupling using simple carbodiimide chemistry (Fig 5.9).

Two procedures exist for coupling of proteins and there is also a method for coupling of oligonucleotides. The one-step covalent coupling procedure with EDAC is recommended for covalent coupling of most proteins. However, if EDAC is found to damage the protein of interest, the two-step procedure, which includes a preactivation (active ester) step prior to introducing the protein, can be used for covalent coupling.

Coupling buffer:	2-(N-morpholino)-ethanesulfonic acid (MES) buffer. Prepare a 10× stock buffer of 500 mM MES, pH 6.1. Store the 10× stock at 4°C and discard if yellowed or contaminated.
Activation solution:	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) Just before use, weigh approximately 10 mg of EDAC on an analytical balance. Add 1 mL of deionized water for each 10.0 mg to obtain a final concentration of approximately 52 µmol/mL.
For two-step coupling procedure only:	N-hydroxysuccinimide (NHS), 50 mg/mL in water.
Additional buffers for coupling of oligonucleotides:	100 mM imidazole at pH 6.0 and 100 mM sodium bicarbonate buffer (pH-adjustment not needed).

☞ EDAC is very sensitive to moisture and undergoes rapid hydrolysis in aqueous solutions. Therefore, EDAC should be stored in a desiccator at -20°C and brought to room temperature just before weighing.

Sample preparation

The protein used to coat the particles should be completely dissolved and not too concentrated. A concentration of 1 to 10 mg/mL in water is recommended for most proteins.

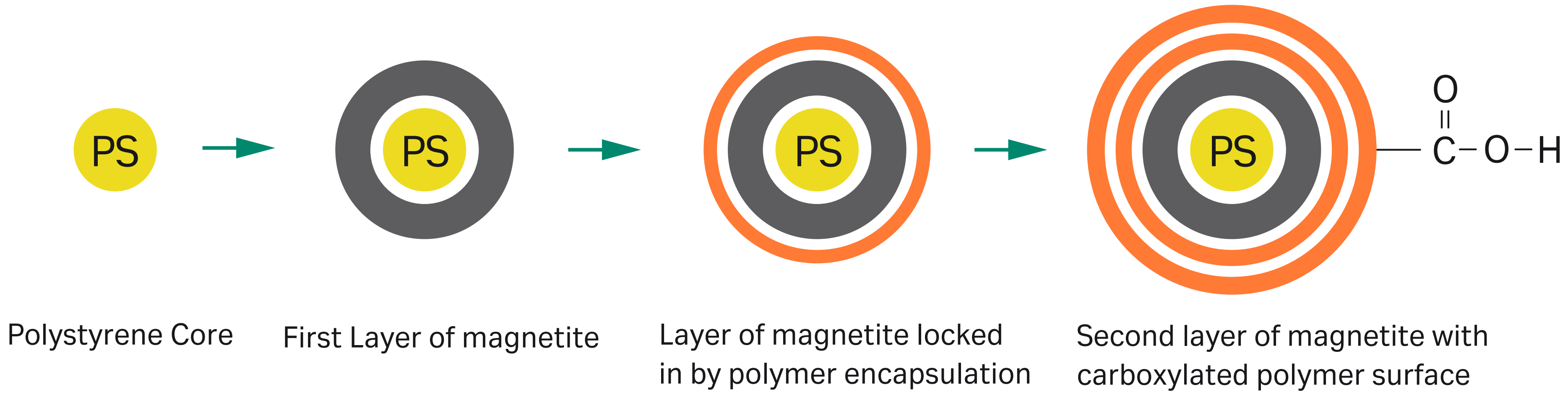


Fig 5.9. Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles feature carboxylic groups on the surface that permit easy covalent coupling using simple carbodiimide chemistry.

Optimizing the amount of EDAC and protein

1. Determine the optimal molar ratio of EDAC:COOH.

   For one-step coupling: Perform an EDAC titration while holding the amount of protein constant. We recommend EDAC:COOH ratios of 0, 0.5, 1, 2.5, 5 and 10:1 for optimization.

   For two-step coupling: Optimize the EDAC:COOH ratio, starting with a recommended 2.5:1 ratio. A molar ratio of 20:1 NHS:COOH is recommended for all reactions.

2. Determine the amount of protein to add.

   Perform a protein titration, holding the determined EDAC concentration fixed. The optimal amount of protein to use depends on several factors:

   - Surface area available: surface area/mg of particles increases linearly with decreasing particle diameter
   - Colloidal stability: proteins can have stabilizing or destabilizing effects on the particles
   - Immunoreactivity: the optimal amount of bound sensitizing protein must ultimately be determined by functional assay. Performing a protein titration or binding isotherm is a good first experiment. It is recommended to start with protein concentrations of 0, 25, 50, 75, 100, and 150 or 200 µg/mg of particle.

Calculation of required amount of EDAC

Use the following information to calculate the amount of EDAC required. See *Optimizing the amount of EDAC and protein* above for details. Reactions of 1 mL are recommended for optimization.

The magnetic bead acid content, provided in mEq/g, is equivalent to µmol/mg.

Note: 1 mL of 1% medium slurry contains 10 mg of magnetic beads.

The µmol EDAC required = (acid content, in µmol/mg) × 10 mg of magnetic beads × desired ratio.

Use this value in the following equation to determine how much of the EDAC stock to add:

$$\frac{\mu\text{mol EDAC required}}{52\ \mu\text{mol/mL}} = \text{mL of EDAC stock for a 1 mL reaction}$$

One-step coupling procedure for proteins

☞ Use the magnetic rack with the magnet in place to attract the beads before each liquid removal step.

1. Prepare Sera-Mag beads
   A. Mix the 10% medium slurry thoroughly by vortexing. Dispense 100 µL of the homogenous medium slurry into an Eppendorf tube.
   B. Add 50 µL of 10 × MES buffer (to 25 mM final concentration) and water to bring reaction up to 1.0 mL final volume.
   C. Add protein stock solution (see *Optimizing the amount of EDAC and protein* for details).
   D. Resuspend the medium and incubate for approximately 15 min by end-over-end mixing or a benchtop device.
2. Activation and coupling
   A. Prepare the EDAC solution immediately before use.
   B. Mix the calculated volume of EDAC solution rapidly into the reaction by pipetting up and down repeatedly.
   C. Incubate for 1 h by end-over-end mixing. The beads may flocculate during this time, but this is not unusual or harmful.
   D. Perform centrifugation (10 to 30 min in a standard microcentrifuge).
   E. Decant the supernatant.
3. Wash (perform this step twice)
   A. Add 50 µL of 10 × MES buffer (to 25 mM final concentration) and water to bring reaction up to 1.0 mL, or use a higher pH buffer of choice. Use ultrasonication for resuspension.
   B. Perform centrifugation as in previous step.
   C. Decant the supernatant.
4. Resuspension
   A. Add buffer that does not contain blocking proteins (use MES buffer or a higher pH buffer of choice).
   B. Resuspend the coupled magnetic beads to the desired medium slurry concentration. For example, if the target of solid concentration is 1.0%, add 0.97 mL of the buffer, which accounts for a small amount of liquid that will remain after pellet formation.

The amount of protein bound on the magnetic beads is determined by a BCA protein assay. For long-term colloidal stability, a stabilizing storage buffer will be needed. After performing the protein analysis, coated beads can be centrifuged and resuspended in a variety of storage buffers, and the colloidal stability and reactivity can be optimized.

☞ Covalently bound protein will not elute when subjected to detergent washes or buffer changes. Thus, covalently coupled reagents are compatible with a wide variety of buffer additives.

Active ester two-step coupling procedure for proteins

Use the magnetic rack with the magnet in place to attract the beads before each liquid removal step.

1. Prepare Sera-Mag beads
   - A. Mix the 10% medium slurry thoroughly by vortexing. Dispense 100 µL of the homogenous medium slurry into an Eppendorf tube.
   - B. Add 100 µL of 10 × MES buffer (to 50 mM final concentration) and water to bring reaction up to 1.0 mL final volume.
   - C. Add 230 µL NHS solution: 100 mM final concentration and resuspend the medium.
2. Activation
   - A. Prepare the EDAC solution immediately before use.
   - B. Mix the calculated volume of EDAC solution rapidly into the reaction by pipetting up and down repeatedly.
   - C. Incubate for 30 min by end-over-end mixing. The beads may flocculate during this time, but this is not unusual or harmful.
   - D. Perform centrifugation (10 to 30 min in a standard microcentrifuge).
   - E. Decant the supernatant.
3. Wash (perform this step twice)
   - A. Add 1 mL of 50 mM MES buffer, pH 6.1. Use ultrasonication for resuspension.
   - B. Perform centrifugation as in previous step.
   - C. Decant the supernatant.
4. Coupling
   - A. Add the protein stock solution (see *Optimizing the amount of EDAC and protein* for details).
   - B. Resuspend the medium and incubate for 1 h by end-over-end mixing.
   - C. Perform centrifugation as in previous step.
   - D. Decant the supernatant.
5. Wash (perform this step twice)
   - A. Add 100 µL of 500 mM MES buffer and fill up to 1 mL with water. Use ultrasonication to resuspend pellet.
   - B. Perform centrifugation as in previous step.
   - C. Decant the supernatant.
6. Resuspension
   - A. Add buffer that does not contain blocking proteins (use MES buffer or a higher pH buffer of choice)
   - B. Resuspend the coupled magnetic beads to the desired medium slurry concentration. For example, if the target concentration for solids is 1.0%, add 0.97 mL of the buffer, which accounts for a small amount of liquid that will remain after pellet formation.

The amount of protein bound on the magnetic beads is determined by a BCA protein assay. For long-term colloidal stability, a stabilizing storage buffer will be needed. After performing the protein analysis, coated beads can be centrifuged and resuspended in a variety of storage buffers, and the colloidal stability and reactivity can be optimized.

Note that covalently bound protein will not elute when subjected to detergent washes or buffer changes. Thus, covalently coupled reagents are compatible with a wide variety of buffer additives.

Covalent coupling of oligonucleotides

☞ Use the magnetic rack with the magnet in place to attract the beads before each liquid removal step.

1. Prepare the Sera-Mag beads
   - A. Mix the 10% medium slurry thoroughly by vortexing. Dispense 200 μL of the homogenous medium slurry into an Eppendorf tube.
   - B. Add 100 μL of 10 × MES buffer (to 50 mM final concentration).
   - C. Add amine-modified oligonucleotide modified in water.
   - D. Add DNase/RNase-free water to 1 mL final volume.
2. Activation and coupling
   - A. Prepare the EDAC solution immediately before use.
   - B. Add 100 μL EDAC solution rapidly into the reaction by pipetting up and down repeatedly.
   - C. Incubate for overnight by end-over-end mixing at 37°C. The beads might flocculate during this time, but this is not unusual or harmful.
   - D. Remove the liquid.
3. Wash 1 (perform this step twice)
   - A. Add 1 mL of DNase/RNase-free water and resuspend the medium.
   - B. Remove the liquid.
4. Wash 2 (perform this step twice)
   - A. Add 1 mL of 100 mM imidazole (pH 6.0).
   - B. Resuspend the medium and incubate for 5 min at 37°C by end-over-end mixing.
   - C. Remove the liquid.
5. Wash 3 (perform this step twice)
   - A. Add 1 mL of 100 mM sodium bicarbonate.
   - B. Resuspend the medium and incubate for 5 min at 37°C by end-over-end mixing.
   - C. Remove the liquid.
6. Wash 4 (perform this step twice)
   - A. Add 1 mL of 100 mM sodium bicarbonate.
   - B. Resuspend the medium and incubate for 30 min at 65°C by end-over-end mixing.
   - C. Remove the liquid.
7. Resuspension
   - A. Add DNase/RNase-free water or buffer of choice for storage.

06

Affinity chromatography in a purification strategy (CIPP)

Affinity chromatography in a purification strategy (CIPP)

AC separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix. 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. AC can therefore offer immense time-saving over less selective multistep procedures.

In many cases, the high level of purity achieved in affinity purification requires, at most, only a second step on a SEC 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 multistep process can be developed using the purification strategy of capture, intermediate purification, and polishing (CIPP), shown in Figure 6.1.

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. AC 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 multistep protein purification. *The Strategies for Protein Purification Handbook*, 28983331 from Cytiva is highly recommended as a guide to planning efficient and effective protein purification strategies and for the selection of the correct chromatography medium for each step and scale of purification.

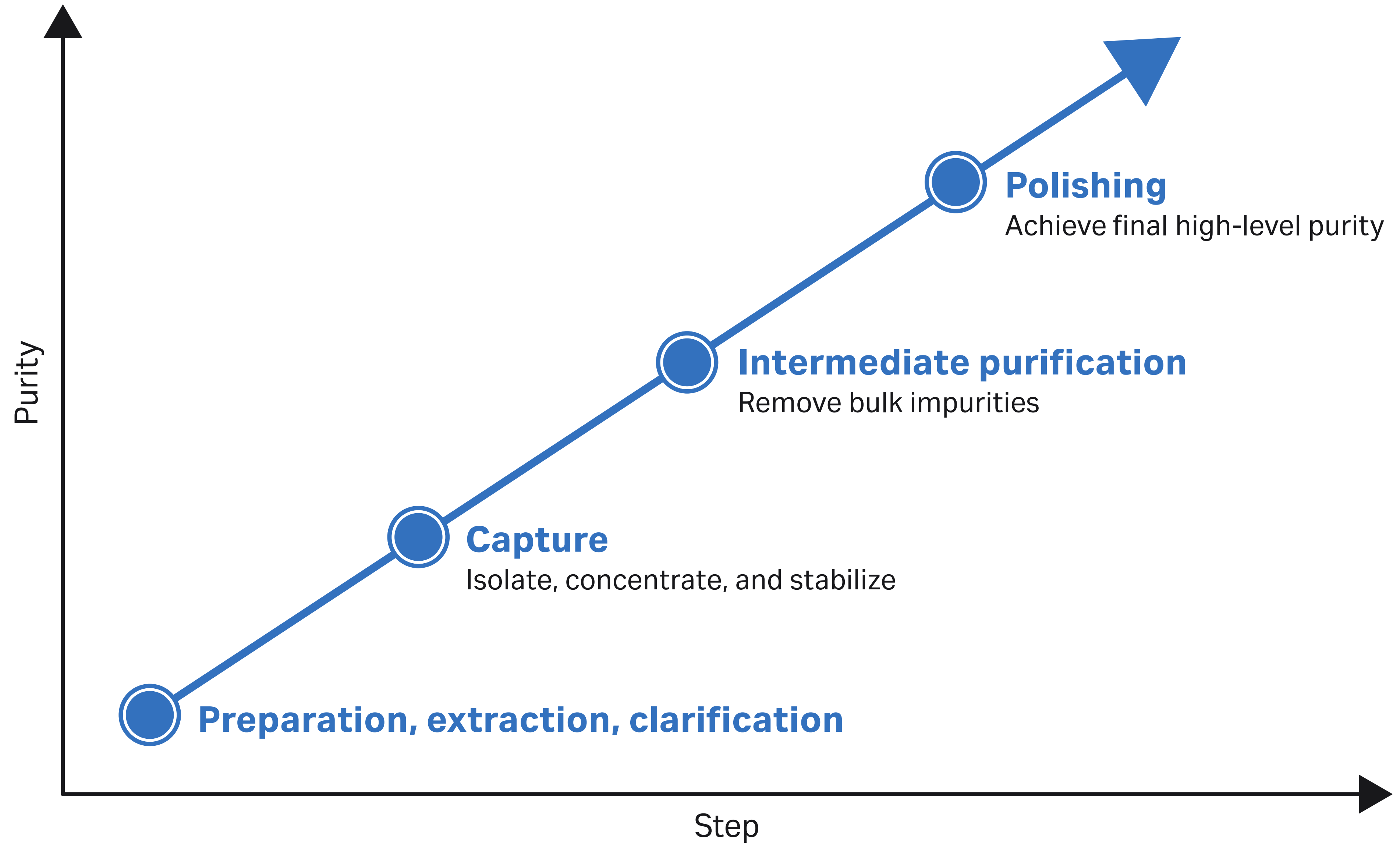


Fig 6.1. Preparation and CIPP.

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

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 objectives are 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. 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.

☞ CIPP does not mean that there must always be three purification steps. For example, capture and intermediate purification might be achievable in a single step, as might intermediate purification and polishing. Similarly, purity demands can 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 might be required to fulfill the highest purity and safety demands. The number of steps used will always depend upon the purity requirements and intended use of the protein.

Selection and combination of purification techniques

Proteins are purified using purification techniques that separate according to differences in specific properties, as shown in Table 6.1.

Table 6.1. Protein properties used during purification

Protein property	Chromatography technique
Size	Size exclusion chromatography (SEC)
Charge	Ion exchange chromatography (IEX)
Hydrophobicity	Hydrophobic interaction chromatography (HIC) Reversed phase chromatography (RPC)
Biorecognition (ligand specificity)	Affinity chromatography (AC)

There are four important performance parameters to consider when planning each purification step: resolution, capacity, speed, and recovery. Optimization of any one of these four parameters can be achieved only at the expense of the others, and each purification step will be a compromise (Fig 6.2). The importance of each parameter will vary depending on whether a purification step is used for capture, intermediate purification, or polishing. Purification methods should be selected and optimized to meet the objectives for each purification step.

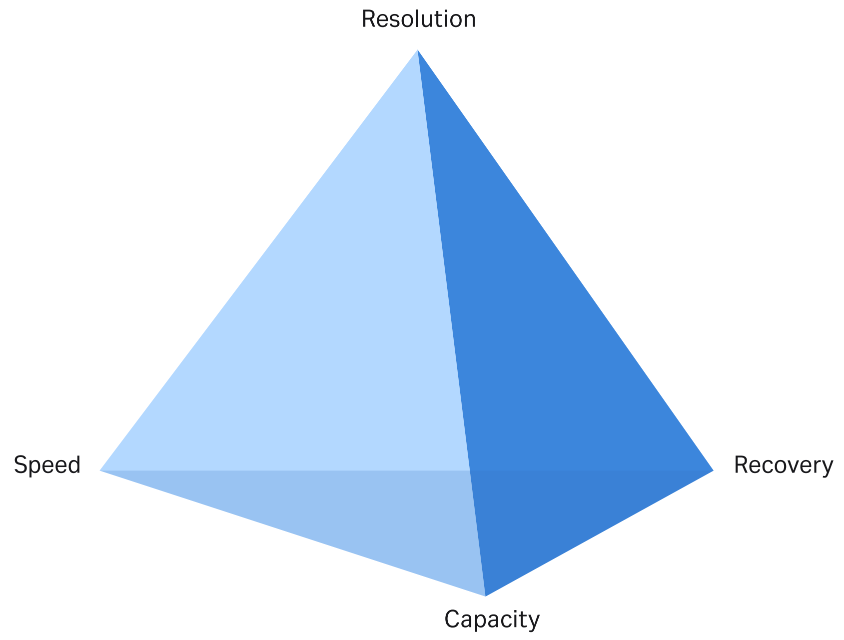


Fig 6.2. Key performance parameters for protein purification. Each purification step should be optimized for one or two of the parameters.

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

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

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

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

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

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

A guide to the suitability of each purification technique for the stages in CIPP is shown in Table 6.2.

☞ Minimize sample handling between purification steps by combining techniques to avoid the need for sample conditioning. The product should be eluted from the first column in conditions suitable for the start conditions of the next column (see Table 6.2).

☞ Ammonium sulfate, often used for sample clarification and concentration (see Appendix 1), leaves the sample in a high salt environment. Consequently HIC, which requires high salt to enhance binding to the chromatography media, becomes the excellent choice 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.

☞ SEC is a nonbinding technique unaffected by buffer conditions, but with limited volume capacity. SEC 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 buffer will not affect the size exclusion process.

Table 6.2. Suitability of purification techniques for CIPP

	Typical characteristics		Purification phase				
Method	**Resolution**	**Capacity**	**Capture**	**Intermediate**	**Polishing**	**Sample start conditions**	**Sample end conditions**
AC	+++ or ++	+++ or ++	+++	++	+	Various binding conditions	Specific elution conditions
SEC	++	+	+		+++	Most conditions acceptable, limited sample volume	Buffer exchange possible, diluted sample
IEX	+++	+++	+++	+++	+++	Low ionic strength. pH depends on protein and IEX type.	High ionic strength or pH changed
HIC	+++	++	++	+++	+++	High ionic strength, addition of salt required	Low ionic strength
RPC	+++	++		+	++	Ion-pair reagents and organic modifiers might be required	Organic solvents (risk for loss of biological activity)

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 6.3.

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, that is, 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-SEC 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 (SEC).

If nothing is known about the target protein, use IEX-HIC-SEC. This combination of techniques can be regarded as a standard protocol.

Consider the use of both AIEX and CIEX to give different selectivities within the same purification strategy.

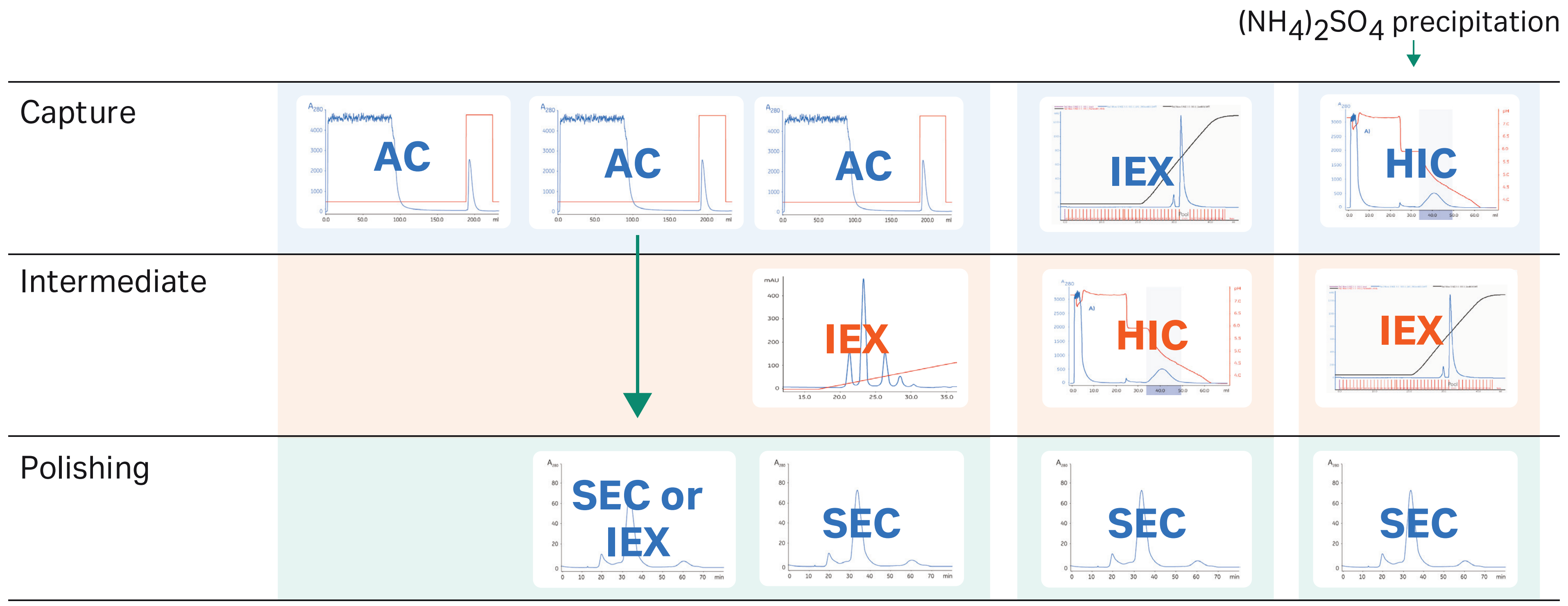


Fig 6.3. Examples of logical combinations of chromatography steps.

07

Appendix

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, reduce the need for stringent washing procedures, and 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 *Strategies for Protein Purification Handbook* and more specifically according to target molecule in the handbooks *Affinity Chromatography, Vol. 1: Antibodies*, 18103746 and *Vol. 2: Tagged Proteins*, 18114275 available from Cytiva.

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 nonspecific 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 can 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 some stability tests before beginning to develop a purification protocol. The list below shows examples of such testing:

- Test pH stability in steps of one pH unit between pH 2.0 and pH 9.0
- Test salt stability with 0 to 2 M NaCl and 0 to 2 M $(NH_4)_2SO_4$ in steps of 500 mM
- Test the stability towards acetonitrile and methanol in 10% steps between 0% and 50%
- Test the temperature stability in 10°C steps from 4°C to 40°C
- Test the stability and occurrence of proteolytic activity by leaving an aliquot of the sample at room temperature overnight. Centrifuge each sample and measure activity and UV absorbance at 280 nm in the supernatant

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 samples 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 min
- For cell lysates, centrifuge at 40 000 to 50 000 × g for 30 min
- Serum samples can be filtered through glass wool after centrifugation to remove remaining lipids

Filtration

Filtration removes particulate matter. Whatman™ syringe filters, which give the least amount of nonspecific binding of proteins, are composed of cellulose acetate (CA), regenerated cellulose (RA), or polyvinylidene fluoride (PVDF) (Table A1.1).

Table A1.1. Whatman syringe filters for filtration of samples

Filter pore size (μm)	Up to sample volume (mL)	Whatman syringe filter[1]	Membrane
0.8	100	Puradisc FP 30	CA
0.45	1	Puradisc 4	PVDF
0.45	10	Puradisc 13	PVDF
0.45	100	Puradisc 25	PVDF
0.45	10	SPARTAN™ 13	RC
0.45	100	SPARTAN 30	RC
0.45	100	Puradisc FP 30	CA
0.2	1	Puradisc 4	PVDF
0.2	10	Puradisc 13	PVDF
0.2	100	Puradisc 25	PVDF
0.2	10	SPARTAN 13	RC
0.2	100	SPARTAN 30	RC
0.2	100	Puradisc FP 30	CA

[1] The number indicates the diameter (mm) of the syringe filter.

For sample preparation before chromatography, select a filter pore size in relation to the bead size of the chromatographic medium (Table A1.2).

Table A1.2. Selecting a sample filter based on the bead size of the chromatographic medium used

Nominal pore size of filter (μm)	Particle size of chromatographic medium (μm)
1.0	90 and upwards
0.45	30 or 34
0.22	3, 10, 15 or when extra clean samples or sterile filtration is required

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

Desalting

Desalting columns are suitable for any sample volume and will rapidly remove LMW 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 in the section *Buffer exchange and desalting* later in this appendix.

At laboratory scale, when samples are reasonably clean after filtration or centrifugation, the buffer exchange and desalting step can be avoided. For AC or HIC, it might be sufficient to adjust the pH of the sample. For IEX, it might be sufficient to dilute the sample to reduce the ionic strength.

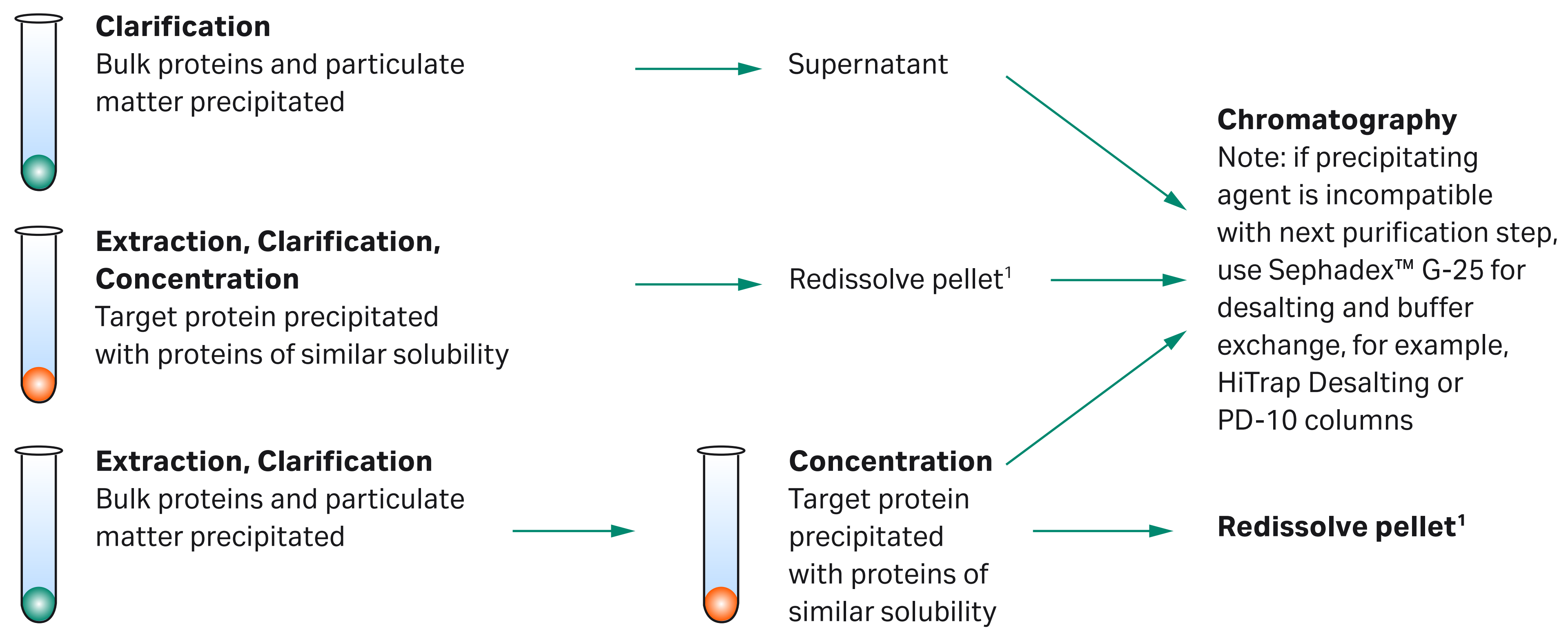
Specific sample preparation steps

Specific sample preparation steps might be required if the crude sample is known to contain contaminants such as lipids, lipoproteins, or phenol red that might build up on a column. Gross impurities, such as bulk protein, should be removed before any chromatographic step.

Fractional precipitation

Fractional precipitation is occasionally used at laboratory scale to remove gross impurities from the sample. Precipitation techniques separate fractions by the principle of differential solubility. Because proteins 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 A1.1.

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



[1] Remember: not all proteins are easy to redissolve, yield can be reduced

Fig A1.1. Three ways to use precipitation.

Examples of precipitation agents are reviewed in Table A1.3. The most common precipitation method using ammonium sulfate is described in more detail.

Table A1.3. Examples of precipitation techniques

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulfate	As described below	> 1 mg/mL proteins especially immuno-globulins	Stabilizes proteins, no denaturation, supernatant can go directly to HIC. Helps to reduce lipid content.
Dextran sulfate	Add 0.04 mL 10% dextran sulfate and 1 mL of 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 h, centrifuge 17 000 × g, discard pellet	Samples with high levels of lipoprotein, e.g., ascites	Alternative to dextran sulfate
Polyethylene glycol (PEG, M_r > 4000)	Up to 20% w/v	Plasma proteins	No denaturation, supernatant goes directly to IEX or AC, complete removal might be difficult. Stabilizes proteins.
Acetone (cold)	Up to 80% v/v at ± 0°C. Collect pellet after centrifugation at full speed in a microcentrifuge		Can denature protein irreversibly. Useful for peptide precipitation or concentration of sample for electrophoresis.
Polyethyleneimine	0.1% w/v		Precipitates aggregated nucleoproteins
Protamine sulfate	1% w/v		Precipitates aggregated nucleoproteins
Streptomycin sulfate	1% w/v		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, second ed. Wiley Inc, (1998). Personal communications.*

Ammonium sulfate precipitation

✋ Some proteins can be damaged by ammonium sulfate. Take care when adding crystalline ammonium sulfate; high local concentrations can cause contamination of the precipitate with unwanted proteins.

☞ For routine, reproducible purification, precipitation with ammonium sulfate 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 sulfate solution (add 100 g ammonium sulfate 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 sulfate solution, drop by drop. Add up to 50% saturation[1]. Stir for 1 h.
4. Centrifuge 20 min at 10 000 × g.
5. Remove supernatant. Wash the pellet twice by resuspension in an equal volume of ammonium sulfate solution of the same concentration (i.e., a solution that will not redissolve the precipitated protein or cause further precipitation). Centrifuge again.
6. Dissolve pellet in a small volume of the buffer to be used for the next step.
7. Ammonium sulfate is removed during clarification/buffer exchange steps with Sephadex G-25, using desalting columns (see *Buffer exchange and desalting* later in this appendix).

[1] The percent saturation can be adjusted either to precipitate a target molecule or to precipitate contaminants.

The quantity of ammonium sulfate required to reach a given degree of saturation varies according to temperature. Table A1.4 shows the quantities required at 20°C.

Table A1.4. Quantities of ammonium sulfate 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 sulfate to add (gram) 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 A1.5 gives examples of common denaturing agents.

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. There is also a risk of losing material during handling or as a result of proteolytic breakdown or nonspecific binding to the dialysis membranes. A simpler and much faster technique is to use a desalting column, packed with Sephadex G-25, to perform a group separation between HMW and LMW 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 LMW materials are removed.

Desalting columns are used not only to remove LMW 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 is possible to dilute the sample with the start buffer that is to be used for chromatographic purification, but cell debris and particulate matter must still be removed.

☞ 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 A1.2 shows a typical buffer exchange and desalting separation. The process can be monitored by following changes in UV absorption and conductivity.

Table A1.5. Denaturing agents used for resolubilization of relatively insoluble proteins

Denaturing agent	Typical conditions for use (molar, M)	Removal/comment
Urea	2 to 8	Remove using Sephadex G-25
Guanidine hydrochloride	3 to 6	Remove using Sephadex G-25

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, second ed. Wiley Inc, (1998)* and other sources.

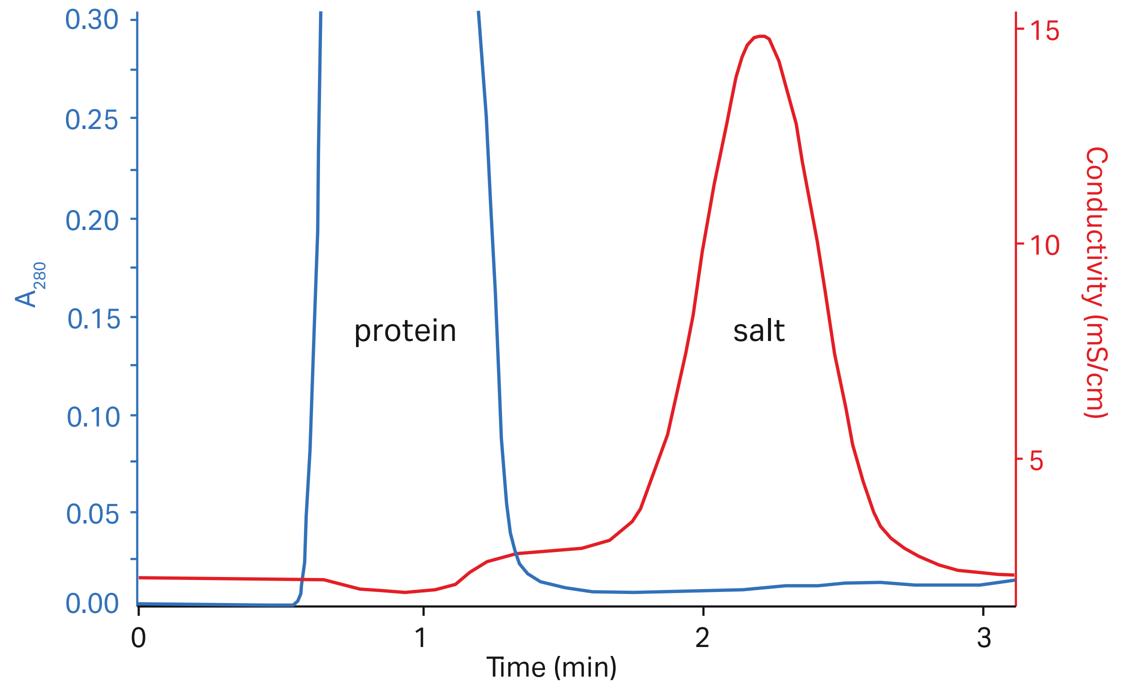


Fig A1.2. Buffer exchange of mouse plasma (10 mL) on HiPrep 26/10 Desalting.

For laboratory-scale operations, Table A1.6 shows examples of prepacked, ready-to-use desalting and buffer exchange columns (see *Size Exclusion Chromatography Handbook*, 18102218, for additional formats).

To desalt larger sample volumes:

- Connect up to five HiTrap Desalting 5 mL columns in series to increase the sample volume capacity, for example, two columns: sample volume 3 mL, five columns: sample volume 7.5 mL
- Connect up to four HiPrep 26/10 Desalting columns in series to increase the sample volume capacity, for example, two columns: sample volume 30 mL, four columns: sample volume 60 mL. Even with four columns in series, the sample can be processed in 20 to 30 min, at room temperature, in aqueous buffers

Instructions are supplied with each column. Desalting and buffer exchange can take less than 5 min per sample with greater than 95% recovery for most proteins.

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 snap-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 (0.25 to 1.5 mL) using a 2 to 5 mL syringe at a flow rate between 1 to 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 A1.7 and collect the desalted protein.

Note: 5 mL/min corresponds to approximately 120 drops/min when using a HiTrap 5 mL column. A simple peristaltic pump or a chromatography system can also be used for the desalting procedure.

☞ The maximum recommended sample volume is 1.5 mL. See Table A1.7 for the effect of reducing the sample volume applied to the column.

Table A1.6. Examples of desalting and buffer exchange columns

Column	Sample volume (mL)	Sample elution volume (mL)
PD MiniTrap G-25	0.2 to 0.5	0.1 to 0.5
PD-10 (gravity feed column)	1.0 to 2.5	3.5
HiTrap Desalting, 5 mL	0.25 to 1.5	1.0 to 2.0
HiPrep 26/10 Desalting	2.5 to 15	7.5 to 20

Table A1.7. Recommended sample and elution volumes using a syringe

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

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 sulfate and polyvinylpyrrolidine, described under Fractional precipitation above, are recommended to remove high levels of lipoproteins from samples such as ascitic fluid.

☞ Centrifuge samples when performing precipitation to avoid the risk of nonspecific binding of the target molecule to a filter.

☞ Samples such as serum can be filtered through glass wool to remove remaining lipids.

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 binds to certain purification media and should be removed as early as possible to avoid the risk of contamination. It is known to bind to AIEX media at pH > 7.0.

☞ 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* earlier in this appendix.

Removal of LMW contaminants

☞ If samples contain a high level of LMW contaminants, use a desalting column before the first chromatographic purification step, as described under *Buffer exchange and desalting* earlier in this appendix.

Appendix 2
Selection of purification equipment

Simple AC purification with step elution can be performed using a syringe or peristaltic pump with prepacked HiTrap columns. A chromatography system is required when reproducible results are important and when manual purification becomes too time-consuming and inefficient. This can be the case when large sample volumes are handled, or when there are many different samples to be purified. The progress of the purification can be monitored automatically and high-resolution separations with accurately controlled linear-gradient elution can be performed.

Table A2.1 lists the standard ÄKTA system configurations, see also *ÄKTA Laboratory-scale Systems: Instrument Management Handbook*, 29010831.

Table A2.1. Ways of working with standard ÄKTA chromatography systems

Way of working		 ÄKTA start	 ÄKTAprime plus	 ÄKTAxpress	 ÄKTA pure		 ÄKTA avant
Simple, one-step desalting, buffer exchange	Research →	•	•	•	•	Process development →	•
Automated and reproducible protein purification using all common techniques including support for gradient elution		•	•	•	•		•
Software compatible with regulatory requirements, e.g., good laboratory practice (GLP)				•	•		•
Method development and optimization using design of experiments (DoE)			o	•			•
Automatic buffer preparation including pH scouting				•	o		•
Automatic chromatography medium or column scouting			o	•	o		•
Automatic, multistep purification			o	•	o		o
Scale-up, process development			o	o	o		•
Flow rate (mL/min)		0.5 to 5.0	0.1 to 50.0	0.1 to 65.0	0.001 to 25.0 (ÄKTA pure 25)/ 0.01 to 150 (ÄKTA pure 150)		0.001 to 25/0.01 to 150
Max. operating pressure (MPa)		0.5	1	3	20/5		20/5
Software[1] for system control and data handling		UNICORN™ start	PrimeView™[2]	UNICORN 5	UNICORN 6 or later		UNICORN 6 or later

[1] A specific software version might be needed for the chosen system. See the web page for each respective system at www.cytiva.com/AKTA.

[2] With PrimeView, you can monitor results and evaluate data but not create methods nor control the system.

• = included

o = optional

Appendix 3
Column packing and preparation

Prepacked columns from Cytiva will ensure reproducible results and the highest performance.

☞ Use small prepacked columns for chromatography media scouting and method optimization and to increase efficiency in method development.

Efficient column packing is essential for AC separation, especially when using gradient elution. A poorly packed column gives rise to poor and uneven flow, band broadening, and loss of resolution. If column packing is required, the following guidelines will apply at all scales of operation:

- With a high binding capacity medium, use short, wide columns (typically 5 to 15 cm bed height) for rapid purification, even at low flow velocity
- The amount of AC medium required will depend on the binding capacity of the medium and the amount of sample. Binding capacities for each medium are given in this handbook and supplied with the product instructions. Estimate the amount of medium required to bind the sample of interest and use five times this amount to pack the column. The amount of medium required can be reduced if resolution is satisfactory
- Once separation parameters have been determined, scale up a purification by increasing the diameter of the column to increase column volume. Avoid increasing the length of the column, if possible, as this will alter separation conditions

AC media can be packed in either Tricorn, XK, or HiScale columns available from Cytiva (Fig A3.1).

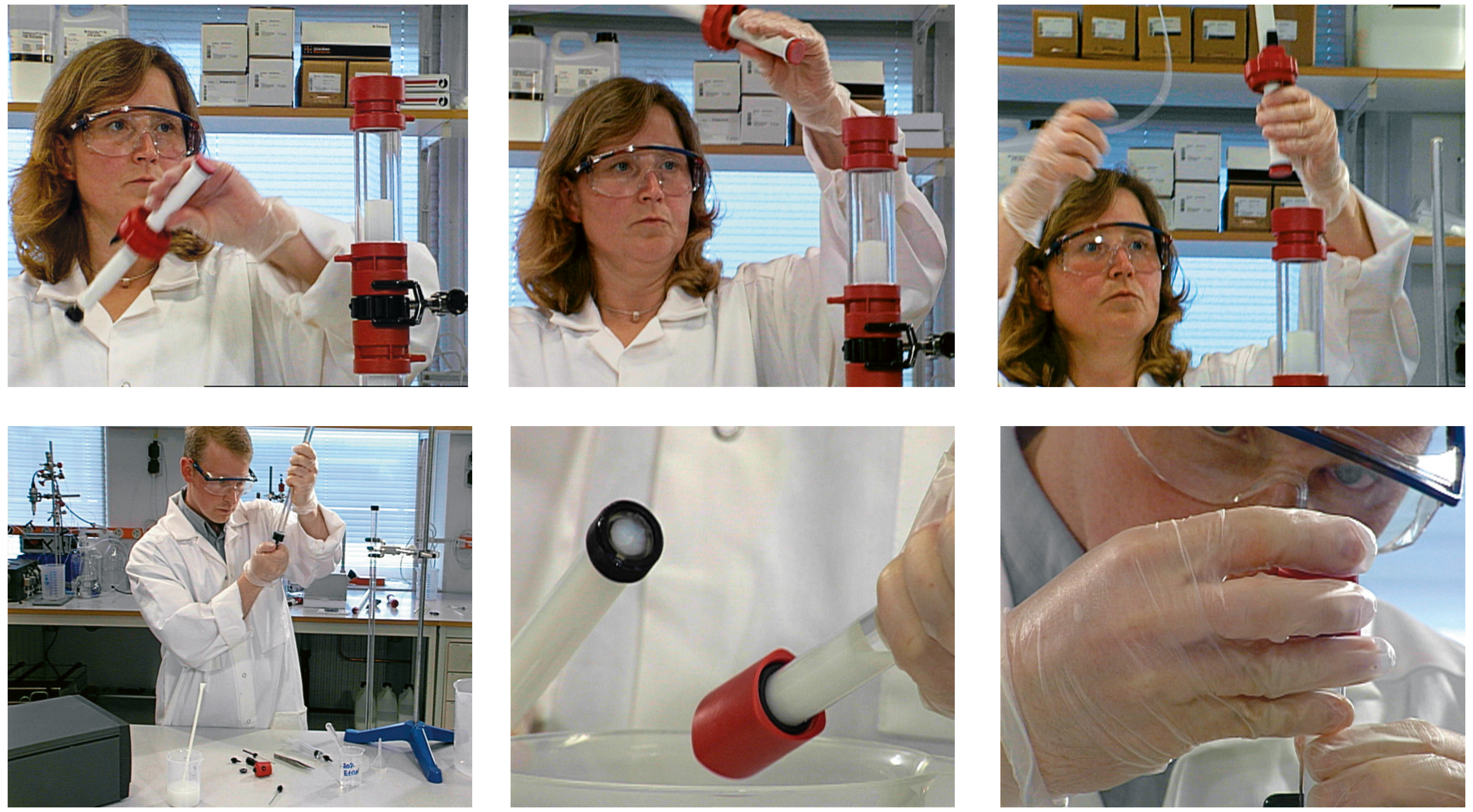


Fig A3.1. Column packing in progress.

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

Note that AC media from Cytiva are supplied ready to use. Decanting of fines that could clog the column is unnecessary.

✋ Avoid using magnetic stirrers since they can damage the chromatography matrix.

4. Estimate the amount of slurry (resuspended medium) required on the basis of the recommendations supplied in the instruction manual.
5. Pour the required volume of slurry into the column. Pouring down a glass rod held against the wall of the column will minimize the introduction of air bubbles.
6. Immediately fill the column with buffer.
7. Mount the column top piece/adapter and connect to a pump.
8. Open the column outlet and set the pump to the desired flow rate (for example, 15 mL/min in an XK 16/20 column).

☞ When slurry volume is greater than the total volume of the column, connect a second glass column to act as a reservoir (see *Ordering information* for details). This ensures that the slurry has a constant diameter during packing, minimizing turbulence and improving column packing conditions.

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

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

✋ Do not exceed 70% of the packing flow rate during any purification.

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

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

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

Column packing and efficiency

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

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

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

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

H is calculated from the expression:

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

L = height of packed bed.

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

Column performance should be checked at regular intervals by injecting acetone to determine column efficiency (N) and peak symmetry (asymmetry factor, A_s). Since the observed value for N depends on experimental factors such as flow rate and sample loading, comparisons must be made under identical conditions. In AC, efficiency is measured under isocratic conditions by injecting acetone (which does not interact with the medium) and measuring the eluted peak as shown in Figure A3.2.

As a general rule, a good H value is about two to three times the average particle diameter of the medium being packed. For a 90 μm particle, this means an H value of 0.018 to 0.027 cm.

The asymmetry factor (A_s) is expressed as:

$$A_s = \frac{b}{a}$$

where

a = First half peak width at 10% of peak height

b = Second half peak width at 10% of peak height

A_s should be as close as possible to 1.0. A reasonable A_s value for a short column as used in AC is 0.80 to 1.80.

☞ An extensive leading edge is usually a sign that the medium is packed too tightly and extensive tailing is usually a sign that the medium is packed too loosely.

☞ Run at least 2 CV of buffer through a newly packed column to ensure that the medium is equilibrated with start buffer. Use pH monitoring to check the pH of the eluent.

Custom column packing

A service for packing of laboratory columns or filling of 96-well plates is supplied when columns or plates with suitable chromatography media are not available from the standard portfolio. The Custom Products group works in close collaboration with you to deliver packed columns for specialized purification requirements. Visit www.cytiva.com/custom-column-packing for more information.

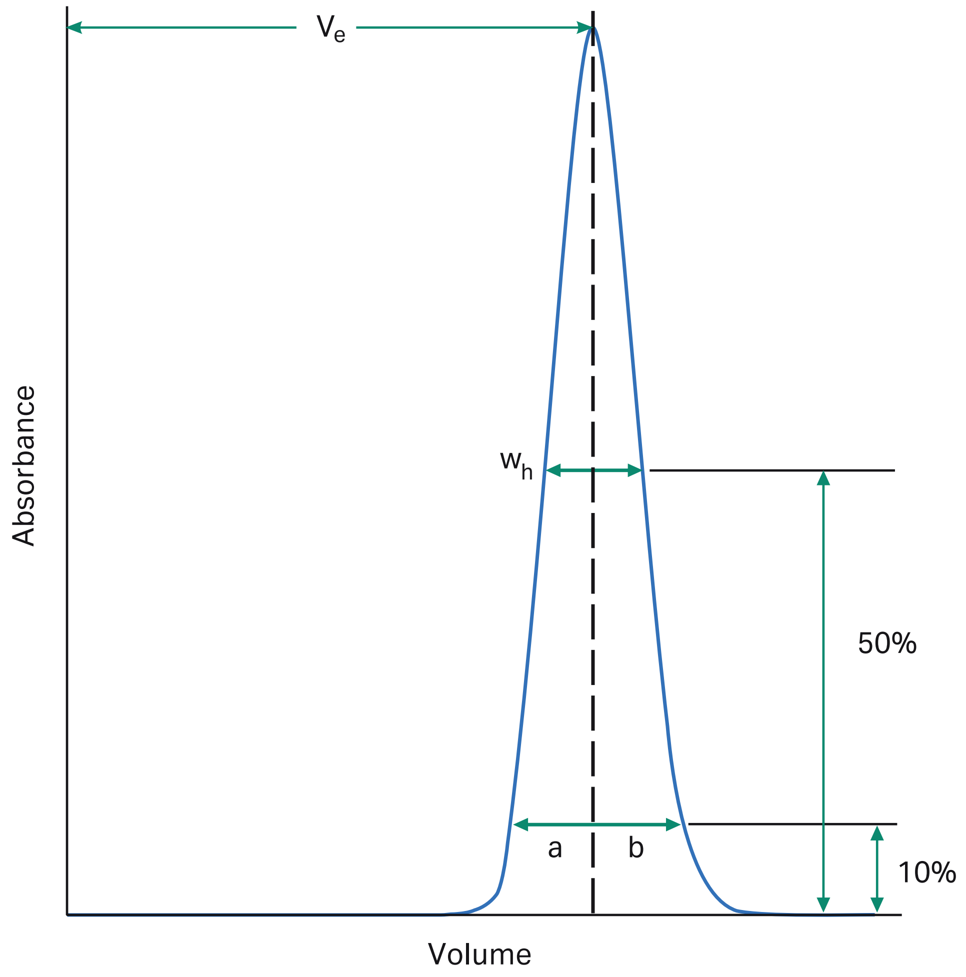


Fig A3.2. Measurements taken to calculate column efficiency.

Appendix 4
Converting from flow velocity to volumetric flow rates

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

From flow velocity (cm/h) to volumetric flow rate (mL/min)

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

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

where

Y = flow velocity 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 flow velocity is 150 cm/h?

Y = flow velocity = 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 flow velocity (cm/h)

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

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

where

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

Example:

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

Z = volumetric flow rate = 1 mL/min
d = column inner diameter = 0.5 cm

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

From volumetric flow rate (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

Proteins

Mass (g/mol)	**1 µg**	**1 nmol**	**Protein**	**A_{280} for 1 mg/mL**
10 000	100 pmol; 6×10^{13} molecules	10 µg	IgG	1.35
50 000	20 pmol; 1.2×10^{13} molecules	50 µg	IgM	1.20
100 000	10 pmol; 6.0×10^{12} molecules	100 µg	IgA	1.30
150 000	6.7 pmol; 4.0×10^{12} molecules	150 µg	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.

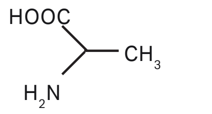
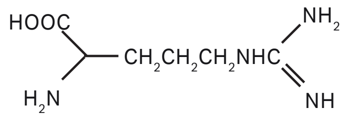
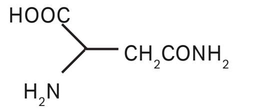
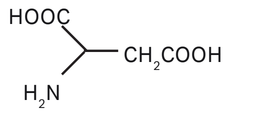
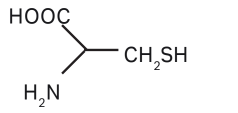
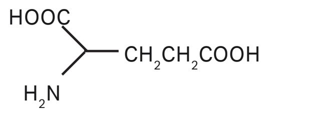
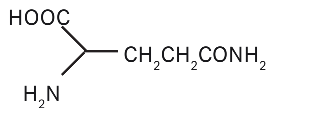
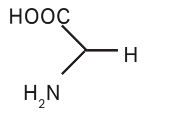
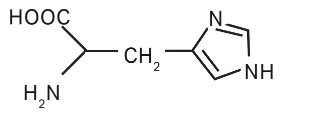
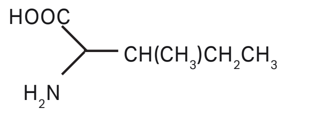
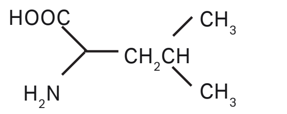
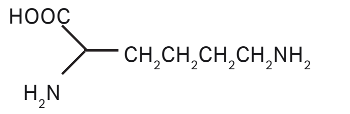
Column pressures

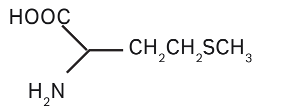
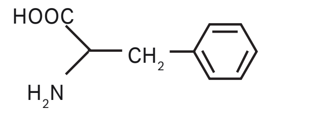
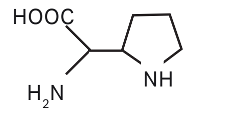
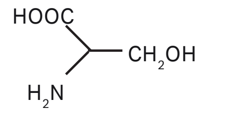
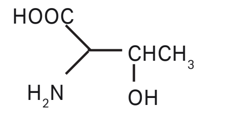
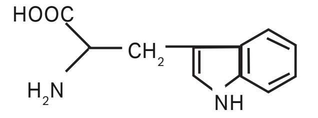
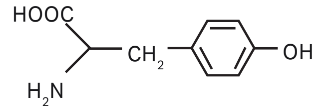
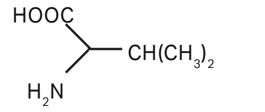
The maximum pressure drop over the packed bed refers to the pressure above which the column contents might begin to compress.

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

Appendix 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	

Amino acid	Three-letter code	Single-letter code	Structure
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	
Serine	Ser	S	
Threonine	Thr	T	
Tryptophan	Trp	W	
Tyrosine	Tyr	Y	
Valine	Val	V	

		Middle unit residue (-$H_2$0)					
Formula	M_r	Formula	M_r	Charge at pH 6.0 to 7.0	Hydrophobic (nonpolar)	Uncharged (polar)	Hydrophilic (polar)
$C_3H_7NO_2$	89.1	C_3H_5NO	71.1	Neutral	•		
$C_6H_{14}N_4O_2$	174.2	$C_6H_{12}N_4O$	156.2	Basic (+ve)			•
$C_4H_8N_2O_3$	132.1	$C_4H_6N_2O_2$	114.1	Neutral		•	
$C_4H_7NO_4$	133.1	$C_4H_5NO_3$	115.1	Acidic(-ve)			•
$C_3H_7NO_2S$	121.2	C_3H_5NOS	103.2	Neutral	•		
$C_5H_9NO_4$	147.1	$C_5H_7NO_3$	129.1	Acidic (-ve)			•
$C_5H_{10}N_2O_3$	146.1	$C_5H_8N_2O_2$	128.1	Neutral		•	
$C_2H_5NO_2$	75.1	C_2H_3NO	57.1	Neutral		•	
$C_6H_9N_3O_2$	155.2	$C_6H_7N_3O$	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
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, and recovery as well as to help during optimization of experimental conditions. The importance of a reliable assay for the target molecule cannot be overemphasized.

☞ 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 can interfere with the Lowry assay.

Purity determination

Purity is most often estimated by SDS-PAGE. Alternatively, IEF, capillary electrophoresis, RPC, or MS may be used.

SDS-PAGE analysis

The general steps involved in SDS-PAGE analysis are summarized below.

1. Prepare samples by mixing with equal volume of 2× SDS loading buffer
2. Vortex briefly and heat for 5 min at 90°C to 100°C.
3. Load the samples and, optionally, a MW marker onto a SDS-polyacrylamide gel.
4. Run the gel.
5. Stain the gel with Coomassie Blue (Coomassie Blue Tablets, PhastGel Blue R-350) 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 A8.1).

Table A8.1. Percentage of acrylamide used in SDS gels for proteins of different molecular weights

Acrylamide in resolving gel (%)		Mol. weight range
Homogeneous:	5	36 000 to 200 000
	7.5	24 000 to 200 000
	10	14 000 to 200 000
	12.5	14 000 to 100 000
	15	14 000 to 60 000[1]
Gradient:	5 to 15	14 000 to 200 000
	5 to 20	10 000 to 200 000
	10 to 20	10 000 to 150 000

[1] The larger proteins fail to move significantly into the gel.

The gel is usually stained after electrophoresis in order to make the protein bands visible by, for example, Coomassie Blue or silver staining. A more recent way of making protein visible is by prelabeling the proteins by fluorescent dye (Amersham™ WB Cy™5 dye reagent) before loading the sample in the gel. By doing in this way the gel image can be acquired directly after finished electrophoresis by laser scanner or CCD camera and the result is obtained much faster. This workflow is outlined on the following page.

Protein prelabeling with CyDye™

1. Prepare samples by prelabeling with Amersham WB Cy5 dye reagent.
2. Vortex briefly and heat for 5 min at 90°C to 100°C.
3. Load the samples and, optionally, a MW marker onto a SDS-polyacrylamide gel.
4. Run the gel and proceed directly to image capture.

☞ For information and advice on electrophoresis techniques, refer to the handbook *2-D Electrophoresis, Principles and Methods,* 80642960. For information on the Amersham WB system and accessories including Amersham WB Cy5 prelabeling reagents, visit www.cytiva.com/westernblotting.

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 to confirm protein identity and quantitate the level of target molecule

1. Separate the protein samples by SDS-PAGE.
2. Transfer the separated proteins from the gel to an appropriate membrane, depending on the choice of detection reagents. Amersham Protran™ (NC) or Amersham Hybond™ P (PVDF) membranes are recommended for chemiluminescent detection using Amersham ECL™ start, Amersham ECL, Amersham ECL Prime, or Amersham ECL Select™ Western blotting detection reagents. Amersham Protran Premium (NC) or Amersham Hybond LFP (PVDF) membranes are recommended for fluorescent detection with Amersham ECL Plex™ Western blotting detection system.
3. Develop the membrane with the appropriate specified reagents.

☞ Electrophoresis, protein transfer, and probing may be accomplished using a variety of equipment and reagents. The Amersham WB system is an automated system that can be used for all these steps including software evaluation. For more information, visit www.cytiva.com/westernblotting. For further information on the basic principles and methods used in Western blotting, refer to the *Western Blotting Handbook*, 28999897 and the instruction manuals supplied with the detection kits.

- ELISAs are most commonly used as activity assays
- Functional assays using the phenomenon of surface plasmon resonance (SPR) to detect immunospecific interactions (e.g., using Biacore™ systems) enable the determination of active concentration, epitope mapping, and studies of interaction kinetics

☞ The *Biacore Assay Handbook*, 29019400 gives a general overview of the different types of SPR-based applications. The handbook also provides advice on sample preparation, design, and optimization of different assays.

Detection and assay of tagged proteins

SDS-PAGE, Western blotting, and ELISA 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, for example, the GST Detection Module for enzymatic detection and quantitation of GST-tagged proteins. Further details on the detection and quantitation of GST and his-tagged proteins are available in the *Affinity Chromatography, Vol. 2: Tagged Proteins*, 18114275 and the *GST Gene Fusion System Handbook*, 18115758 from Cytiva.

Appendix 8
Storage of biological samples

✋ The advice given here is of a general nature and cannot be applied to every biological sample. Always consider the properties of the specific sample and its intended use before following any of these recommendations.

General recommendations

- Add stabilizing agents, when necessary. Stabilizing agents are often required for storage of purified proteins
- Serum, culture supernatants, and ascitic fluid should be kept frozen at -20°C or -70°C, in small aliquots
- Avoid repeated freeze/thawing or freeze drying/redissolving that can 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 h 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 Appendix 1, *Sample preparation*).

Specific recommendations for purified proteins

- Store as a precipitate in high concentration of ammonium sulfate, 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. Store samples in small aliquots and keep frozen
- Sterile filter to prolong storage time
- Add stabilizing agents such as glycerol (5% to 20%) or serum albumin (10 mg/mL) to help maintain biological activity. Remember that any additive will reduce the purity of the protein and might need to be removed at a later stage
- Avoid repeated freeze/thawing or freeze drying/redissolving that can reduce biological activity

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

Related literature

	Code number
Purification handbooks	
Affinity Chromatography, Vol. 1: Antibodies	18103746
Affinity Chromatography, Vol. 2: Tagged Proteins	18114275
Affinity Chromatography, Vol. 3: Specific Groups of Biomolecules	18102229
Ion Exchange Chromatography	11000421
Hydrophobic Interaction and Reversed Phase Chromatography	11001269
Multimodal Chromatography	29054808
Protein Sample Preparation	28988741
Purifying Challenging Proteins	28909531
Size Exclusion Chromatography	18102218
Strategies for Protein Purification	28983331
ÄKTA Laboratory-scale Chromatography Systems	29010831
Protein analysis handbooks	
Biacore Assay	29019400
Biacore Sensor Surface	BR100571
Western Blotting	28999897
Selection guides	
Affinity chromatography columns and media, Selection guide	18112186
Prepacked chromatography columns for ÄKTA systems, Selection guide	28931778

Ordering information

Product	Quantity	Code number
Prepacked AC columns		
Albumin and IgG Depletion SpinTrap	10 columns	28948020
HiScreen Blue FF	1 × 4.7 mL	28978243
HiScreen Capto Blue	1 × 4.7 mL	28992474
HiTrap Benzamidine FF (high sub)	2 × 1 mL	17514302
	5 × 1 mL	17514301
	1 × 5 mL	17514401
HiScreen Capto Chelating	1 × 4.7 mL	17548510
HiScreen Capto Lentil Lectin	1 × 4.7 mL	29157958
HiScreen IXSelect	1 × 4.7 mL	17371410
HiTrap IXSelect	5 × 1 mL	17371411
	1 × 5 mL	17371412
HiTrap Albumin and IgG Depletion	2 × 1 mL	28946603
HiTrap AVB Sepharose HP	5 × 1 mL	28411211
	1 × 5 mL	28411212
HiTrap Blue HP	5 × 1 mL	17041201
	1 × 5 mL	17041301
HiTrap Capto Chelating	5 × 1 mL	17548511
	5 × 5 mL	17548512
HiTrap Capto Lentil Lectin	5 × 1 mL	17548911
	5 × 5 mL	17548912
HiTrap Chelating HP	5 × 1 mL	17040801
	1 × 5 mL	17040901
HiTrap GCSFSelect	5 × 1 mL	17548311
	5 × 5 mL	17548312
HiTrap Heparin HP	5 × 1 mL	17040601
	1 × 5 mL	17040701
HiTrap Streptavidin HP	5 × 1 mL	17511201
HiPrep Heparin FF 16/10	1 × 20 mL	28936549
Streptavidin HP SpinTrap	16 columns	28903130
Streptavidin HP SpinTrap Buffer Kit	1	28913568
Prepacked 96-well plates		
Streptavidin HP MultiTrap	4 × 96-well plates	28903131
Magnetic beads		
Sera-Mag Streptavidin-Coated (low biotin binding)	1 mL	30152103011150
	5 mL	30152103010150
	100 mL	30152103010350
Sera-Mag Streptavidin-Coated (med. biotin binding)	1 mL	30152104011150
	5 mL	30152104010150
	100 mL	30152104010350
Sera-Mag Streptavidin-Coated (high biotin binding)	1 mL	30152105011150
	5 mL	30152105010150
	100 mL	30152105010350
Sera-Mag SpeedBead Streptavidin-Coated (med. biotin binding)	1 mL	66152104011150
	5 mL	66152104010150
	100 mL	66152104010350
Sera-Mag SpeedBeads Streptavidin-Blocked	1 mL	21152104011150
	5 mL	21152104010150
	100 mL	21152104010350
Sera-Mag SpeedBeads Neutravidin-Coated	1 mL	78152104011150
	5 mL	78152104010150
	100 mL	78152104010350
Sera-Mag Carboxylate-Modified (hydrophilic)	15 mL	24152105050250
	100 mL	24152105050350
Sera-Mag Carboxylate-Modified (hydrophobic)	15 mL	44152105050250
	100 mL	44152105050350
Sera-Mag SpeedBeads Carboxylate-Modified (hydrophilic)	15 mL	45152105050250
	100 mL	45152105050350
Sera-Mag SpeedBeads Carboxylate-Modified (hydrophobic)	15 mL	65152105050250
	100 mL	65152105050350
Streptavidin Mag Sepharose	2 × 1 mL, 10% slurry	28985738
	5 × 1 mL, 10% slurry	28985799
TiO_2 Mag Sepharose	1 × 500 µL	28944010
	4 × 500 µL	28951377
NHS Mag Sepharose	1 × 500 µL	28944009
	4 × 500 µL	28951380

Product	Quantity	Code number
Chromatography media		
2´5´ ADP Sepharose 4B	5 g	17070001
IXSelect	25 mL	17371401
	200 mL	17371402
VIISelect	25 mL	17547701
	100 mL	17547702
VIIISelect	25 mL	17545001
AVB Sepharose High Performance	25 mL	28411210
	75 mL	28411201
Benzamidine Sepharose 4 Fast Flow (high sub)	25 mL	17512310
	100 mL	17512301
Benzamidine Sepharose 4 Fast Flow (low sub)	25 mL	28410899
	100 mL	28410801
Blue Sepharose 6 Fast Flow	50 mL	17094801
Capto Blue	25 mL	17544801
Capto Blue (high sub)	25 mL	17545201
Capto DeVirS	25 mL	17546601
	100 mL	17546602
Capto Heparin	25 mL	17546201
	200 mL	17546202
Capto Lentil Lectin	25 mL	17548901
	100 mL	17548902
Calmodulin Sepharose 4B	10 mL	17052901
Chelating Sepharose Fast Flow	50 mL	17057501

Product	Quantity	Code number
Con A Sepharose 4B	5 mL	17044003
	100 mL	17044001
GCSFSelect	25 mL	17548301
	200 mL	17548302
Gelatin Sepharose 4B	25 mL	17095601
Heparin Sepharose 6 Fast Flow	50 mL	17099801
	250 mL	17099825
Heparin Sepharose High Performance	25 mL	90100019
	100 mL	90100401
Lentil Lectin Sepharose 4B	25 mL	17044401
Streptavidin Sepharose High Performance	5 mL	17511301
Preactivated media and columns for ligand coupling		
HiTrap NHS-activated HP	5 × 1 mL	17071601
	1 × 5 mL	17071701
NHS-activated Sepharose 4 Fast Flow	25 mL	17090601
CNBr-activated Sepharose 4 Fast Flow	10 g	17098101
CNBr-activated Sepharose 4B	15 g	17043001
Epoxy-activated Sepharose 6B	15 g	17048001
EAH Sepharose 4B	50 mL	17056901
Activated Thiol Sepharose 4B	15 g	17064001

Product	Quantity	Code number
Prepacked desalting columns		
HiTrap Desalting	1 × 5 mL	29048684
	5 × 5 mL	17140801
HiPrep 26/10 Desalting	1 × 53 mL	17508701
	4 × 53 mL	17508702
PD-10 Desalting Column	30	17085101

Product index

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