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Vol. 1: Antibodies

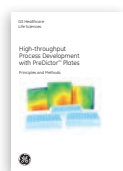


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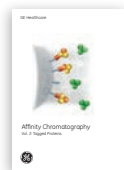
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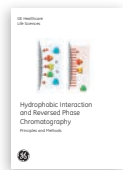
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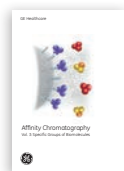
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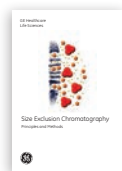
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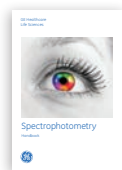
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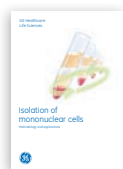
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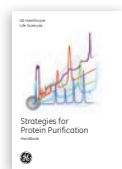
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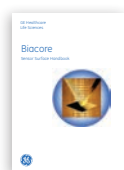
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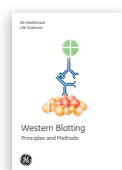
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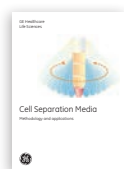
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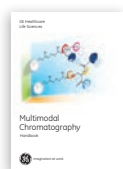
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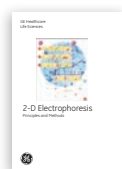
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Affinity Chromatography

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Introduction

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

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

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

Purity required for final application

- Purity check and functional analysis
- Importance and properties of remaining impurities

Physicochemical characteristics

- Size
- Charge
- pI
- Stability

Scale of purification

- Microgram
- Milligram
- Gram

Source

- Sample preparation

Economy

- Time and expense

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

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

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

This handbook is volume 1 in a series of three volumes covering aspects of the purification of proteins by affinity chromatography (AC). The three volumes are:

Affinity Chromatography, Vol. 1: Antibodies

Affinity Chromatography, Vol. 2: Tagged Proteins

Affinity Chromatography, Vol. 3: Specific Groups of Biomolecules

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
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
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
GST	glutathione S-transferase
HCP	host cell protein
HIC	hydrophobic interaction chromatography
HMW	high molecular weight
HSA	human serum albumin
IEX	ion exchange chromatography
IgA, IgG etc.	different classes of immunoglobulin
IMAC	Immobilized metal ion affinity chromatography
LC-MS	liquid chromatography-mass spectrometry
LMW	low molecular weight

MAB	monoclonal antibody
MPa	megaPascal
M_r	relative molecular weight
MS	mass spectrometry
n	native, as in nProtein A
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/v	weight to volume

Chromatography terminology

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

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

Design of experiments (DoE)	DoE allows use of a minimum number of experiments, in which several experimental parameters can be varied simultaneously. Based on the obtained data, a mathematical model of the studied process (e.g., a protein purification protocol or a chromatography step) is created. The model can be used to understand the influence of the experimental parameters on the outcome and to find an optimum for the process.
Dynamic binding capacity	The binding capacity determined by applying the target using flow through a column, as opposed to equilibrium binding capacity determined by batch experiment.
Efficiency	Measured as number of theoretical plates. High efficiency means that sharp peaks will be obtained.
Effluent	The mobile phase leaving the column (= eluate).
Eluate	The mobile phase leaving the column (= effluent).
Eluent	The buffer/solution used during chromatography (= mobile phase).
Elution buffer	Buffer/solution used for elution (desorption) of bound solutes (for example, proteins) from a column.
Elution volume	The volume of buffer/solution (eluent) required to elute the solute for example, a protein (= retention volume).
Elution time	The time required for elution of a solute (protein) (= retention time).
Flow rate	Volumetric flow (ml/min) or linear flow rate (cm/h). Measurement of flow through a column and/or chromatography system.
Flowthrough	Material passing the column during sample loading (without being bound).
Frit	Type of deep filter often used at top and bottom of columns.
Gradient elution	Continuous increased or decreased concentration of a substance (in the eluent) that causes elution of bound solutes (for example, proteins).
Hydrophobic interaction chromatography (HIC)	Method based on the hydrophobic interaction between solutes (for example, proteins) and the chromatography medium in the presence of high salt concentration.
Hydroxyapatite chromatography	Mixed-mode ion exchange chromatography method.
Immobilized metal ion affinity chromatography (IMAC)	Method based on the affinity of proteins with His, Cys, or Trp amino residues on their surface and metal ions on the chromatography medium.
Ion exchange chromatography (IEX)	Method based on electrostatic interactions between solutes (for example, proteins) and chromatography medium.
Isocratic elution	Elution of the solutes without changing the composition of the buffer/solution (eluent).
Ligand	The specific molecular group that is coupled to the matrix to give some decided function to the chromatography medium.
Ligand density	Related to ligand concentration. The distribution of ligands on the surfaces (also surfaces inside pores) of the chromatography matrix.
Linear velocity	The flow rate normalized by the column cross section (cm/h).
Mass transfer	Movement of a solute (for example, a protein) in and out of the stationary phase. Important factor for column efficiency.
Matrix	The matrix is the nonfunctional base for the chromatography medium. The matrix has a porous structure that provides a large surface that can be modified with ligands that introduce possibilities for protein binding.

Mobile phase	The fluid (buffer/solution) carrying the solutes during chromatography (= eluent).
Peak broadening	Same as band broadening.
Peak capacity	The number of peaks that can be separated using a chromatography column.
Peak tailing	Broadening at the end of a peak due to additional delay of a fraction of the solute. Results in increased asymmetry factor.
Pore	Cavity in a chromatography matrix.
Pore volume	The total volume of the pores in a chromatography medium.
Pressure over the packed bed	The pressure drop across the packed bed upon passage of solution through the column. Caused by flow resistance in the packed bed.
Recovery	The relative amount of target protein that is retrieved after purification compared with amount loaded on the column.
Resin	The term is sometimes used instead of the more generic term, chromatography medium.
Resolution	Measurement of the ability of a packed column to separate two solutes (peaks).
Retention volume	Same as elution volume.
Retention time	Same as elution time.
Reversed phase chromatography (RPC)	Method based on hydrophobic interactions between solutes (sample components) and ligands coupled to the chromatography medium. Organic modifiers (for example, acetonitrile) in the eluent are used for elution.
Sample	The material loaded on the chromatography column/medium, or to be analyzed.
Sample application	Applying/loading sample on the column.
Sample loading	Loading/applying sample on the column.
Sample volume	Usually the volume of the sample loaded on the chromatography column/medium.
Selectivity	Measure of the relative retention of two solutes in a column. Related to the distance between two peaks.
Size exclusion chromatography (SEC)	Separates solutes (for example, proteins) according to size. Also called gel filtration (GF).
Solute	The dissolved substance (for example, a protein) in, for example, the mobile phase.
Stationary phase	Often called resin, chromatography beads, chromatography material, chromatography medium or media.
Step gradient elution	Stepwise increase in concentration of the substance that affects elution of bound solutes.
Void volume	The elution volume of solutes that do not enter the pores or interact with the chromatography medium, thus passing between the beads in the packed bed.
Wash	Wash step. Removal of unbound or weakly bound material from a column after the sample loading.
Wash buffer	Buffer/solution used for washing the column after sample loading.
Wash volume	Volume of buffer/solution used for the wash step.
Yield	Amount of target protein (or other solute) obtained after a purification step, or after the entire purification (multiple steps).
Zone broadening	Same as peak broadening.

Chapter 1

Antibody structure, classification, and production

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

Native sources

Immunoglobulins

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

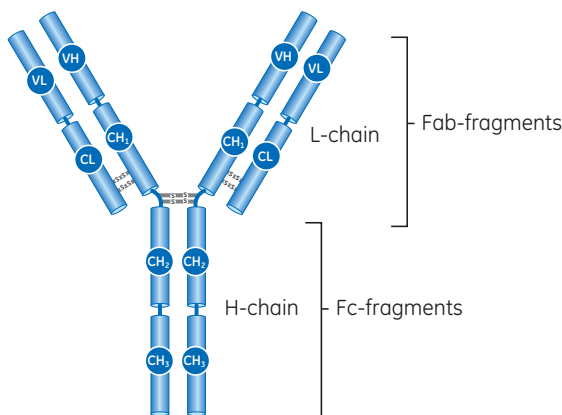


Fig 1.1. Basic H2L2 structure of a typical immunoglobulin.

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






Characteristic	Antibody classes				
	IgG	IgM	IgA	IgE	IgD
Heavy chain	γ	μ	α	ϵ	δ
Light chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Y structure					

Fig 1.2. The five major classes of immunoglobulin.

1. Antibodies of classes G, D, and E are of monomeric type H2L2.
2. IgA in serum is mainly monomeric, but in secretions, such as saliva and tears, IgA is found as a dimer held together by the secretory piece and the J-polypeptide chain (H2L2)-SC-J-(H2L2). The dimer has four antigen binding sites.
3. IgM is composed of five monomeric units (H2L2)₅ and has 10 antigen binding sites.
4. IgG and IgA are further divided into subclasses that result from minor differences in the amino acid sequence within each class. In humans, the four IgG subclasses IgG₁, IgG₂, IgG₃, and IgG₄ have γ_1 , γ_2 , γ_3 , and γ_4 heavy chains, respectively. Mouse IgG has four IgG subclasses: IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃, with heavy chains γ_1 , γ_{2a} , γ_{2b} , and γ_3 . These heavy chains have virtually the same size and similar electrophoretic properties, but their amino acid sequences differ considerably. Human IgA has two subclasses; IgA₁ and IgA₂, while mouse IgA has only one subclass.

Table 1.1a. Physicochemical properties of human immunoglobulins

Immunoglobulin	Heavy chain	Light chain	Sedimentation coefficient	Relative molecular weight (M _r)	M _r , heavy chain	Carbohydrate content (%)	Isoelectric point (pI)
IgG ₁	γ_1	κ, λ	7S	146 000	50 000	2 to 3	5.0 to 9.5
IgG ₂	γ_2	κ, λ	7S	146 000	50 000	2 to 3	5.0 to 8.5
IgG ₃	γ_3	κ, λ	7S	170 000	60 000	2 to 3	8.2 to 9.0
IgG ₄	γ_4	κ, λ	7S	146 000	50 000	2 to 3	5.0 to 6.0
IgM	μ	κ, λ	19S	900 000	68 000	12	5.1 to 7.8
IgA ₁	α_1	κ, λ	7S	160 000	56 000	7 to 11	5.2 to 6.6
IgA ₂	α_2	κ, λ	7S	160 000	52 000	7 to 11	5.2 to 6.6
IgD	δ	κ, λ	7S	184 000	68 000	12	–
IgE	ϵ	κ, λ	8S	190 000	72 000	12	–

Table 1.1b. Physicochemical properties of mouse immunoglobulins

Immunoglobulin	Heavy chain	Light chain	Sedimentation coefficient	Relative molecular weight (M_r)	M_r , heavy chain	Carbohydrate content (%)	pI
IgG ₁	γ_1	κ, λ	7S	150 000	50 000	2 to 3	7.0 to 8.5
IgG _{2a}	γ_{2a}	κ, λ	7S	150 000	50 000	2 to 3	6.5 to 7.5
IgG _{2b}	γ_{2b}	κ, λ	7S	150 000	50 000	2 to 3	5.5 to 7.0
IgG ₃	γ_3	κ, λ	7S	150 000	50 000	2 to 3	–
IgM	μ	κ, λ	19S	900 000	80 000	12	4.5 to 7.0
IgA	α	κ, λ	7S	170 000	70 000	7 to 11	4.0 to 7.0
IgD	δ	κ, λ	7S	180 000	68 000	12 to 14	–
IgE	ϵ	κ, λ	8S	190 000	80 000	12	–

IgY immunoglobulin

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

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

Antibody fragments

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

Fragmentation of immunoglobulins has created the potential for new applications. For example, chimeric, nonimmunogenic ‘humanized’ mouse Fab, Fab’, and F(ab)₂ fragments are of great interest in tumor therapy since they penetrate tumors more rapidly and are also cleared from the circulation more rapidly than full-size antibodies.

Figure 1.3 shows the fragments created by enzymatic cleavage.

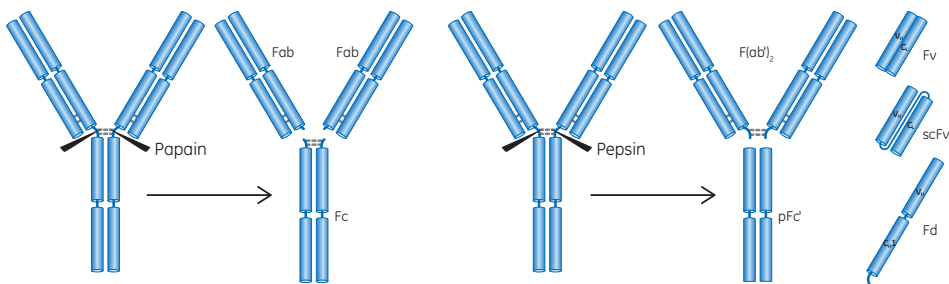


Fig 1.3. Antibody fragments are created by enzymatic cleavage.

The most common types of antibody fragments are:

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

F(ab')₂ fragment: pepsin digestion creates a fragment containing two antigen binding sites and comprises two Fab units and the hinge.

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

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

Dabs: domain antibodies, the smallest functional entity of antibodies.

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

Polyclonal antibodies

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

Monoclonal antibodies

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

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

Genetically engineered sources

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

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

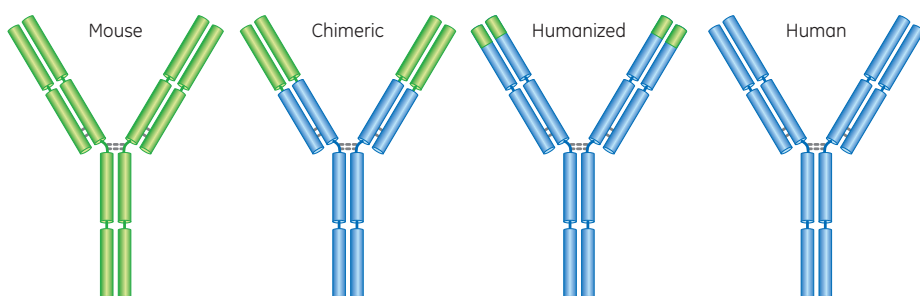


Fig 1.4. Various modifications of both native and recombinant antibodies are now possible.

Antibody fragments

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

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

Recombinant antibodies

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

Antibody fusion proteins are divided into two groups:

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

References

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

Chapter 2

Sample preparation

Sources and their associated contaminants

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

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

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

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



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

Extraction of recombinant antibodies and antibody fragments

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

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




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

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

	Typical conditions for use	Purpose
Buffer components		
Tris	20 mM, pH 7.4	Stabilize pH
NaCl	100 mM	Maintain ionic strength of medium
EDTA	10 mM	Reduce oxidation damage, chelate metal ions
Sucrose or glucose	25 mM	Stabilize lysosomal membranes, reduce protease release
Ionic or nonionic detergent		Solubilize poorly soluble proteins For details on handling inclusion bodies, please see <i>Recombinant Protein Purification Handbook</i> , 18-1142-75
DNAse and RNAse	1 µg/ml	Degradation of nucleic acids in order to reduce viscosity of sample solution
Protease inhibitors¹		Inhibits
PMSF	0.5 to 1 mM	Serine proteases
APMSF	0.4 to 4 mM	Serine proteases
Benzamidine-HCl	0.2 mM	Serine proteases
Pepstatin	1 µM	Aspartic proteases
Leupeptin	10 to 100 µM	Cysteine and serine proteases
Chymostatin	10 to 100 µM	Chymotrypsin, papain, cysteine proteases
Antipain-HCl	1 to 100 µM	Papain, cysteine and serine proteases
EDTA	2 to 10 mM	Metal-dependent proteases (zinc and iron)
EGTA	2 to 10 mM	Metal-dependent proteases (calcium)

Table continues on next page.

Table 2.2 *cont.*

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

PMSF – Phenylmethylsulfonyl fluoride.

APMSF – 4-Aminophenyl-methylsulfonyl fluoride.

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

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



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

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

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



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

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

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

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



Centrifuge and filter any sample immediately before chromatographic purification.

Lipids and lipoproteins can clog chromatographic columns and should be removed prior to purification. Ascites have a particularly high lipid content. See removal of specific impurities on *Removal of specific impurities before purification* in this chapter.

Phenol red is often added to cell culture supernatants as a pH indicator. Since phenol red can bind to certain chromatographic media, it is advisable to remove it prior to purification. See removal of specific impurities on *Removal of gross impurities by precipitation* in this chapter.

Centrifugation and filtration

Centrifugation removes most particulate matter, such as cell debris. If the sample is still not clear after centrifugation, use a 5 µm filter as a first step and one of the filters listed in Table 2.4 as a second step.



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



For cell lysates, centrifuge at 40000 to 50000 × g for 30 min (may be reduced to 10 to 15 min if a short handling time is required).



Use the cooling function of the centrifuge and precool the rotor by storing it in the cold room or by starting to cool the centrifuge well in advance with the rotor in place.



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

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

Table 2.4. Selecting filter pore sizes

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



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



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

Sample preparation before purification

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

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

Removal of specific impurities before purification

Lipoproteins

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

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



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

Alternative 1:

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

1. Add 0.04 ml 10% dextran sulfate solution and 1 ml of 1 M calcium chloride/ml of sample.
2. Mix for 15 min.
3. Centrifuge 10 000 \times g for 10 min.
4. Discard the precipitate.
5. Transfer the sample into a buffer suitable for purification using a desalting column (see *Desalting and buffer exchange* later in this chapter).


Alternative 2:

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

1. Add solid PVP to the sample solution to a final concentration of 3% (w/v).
2. Stir for 4 h at 4°C.
3. Centrifuge at 17 000 \times g.
4. Discard the precipitate.
5. Transfer the sample into a buffer suitable for purification using a desalting column (see *Desalting and buffer exchange* later in this chapter).

Phenol red

Phenol red is used as a pH indicator in laboratory-scale cell culture. Although not directly interfering with purification, phenol red binds to certain purification media and should be removed as early as possible. Phenol red is known to bind to anion exchange chromatography (AEX) media at pH > 7.0.

 Use a desalting column to simultaneously remove the low molecular weight phenol red and transfer sample to the correct buffer conditions for further purification (see *Desalting and buffer exchange* later in this chapter.).

Removal of gross impurities by precipitation

Low molecular weight contaminants

If samples contain a high level of low molecular weight contaminants, use a desalting column as described further in *Desalting and buffer exchange* to prepare the sample for the first chromatography step.

Fractional precipitation

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

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

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

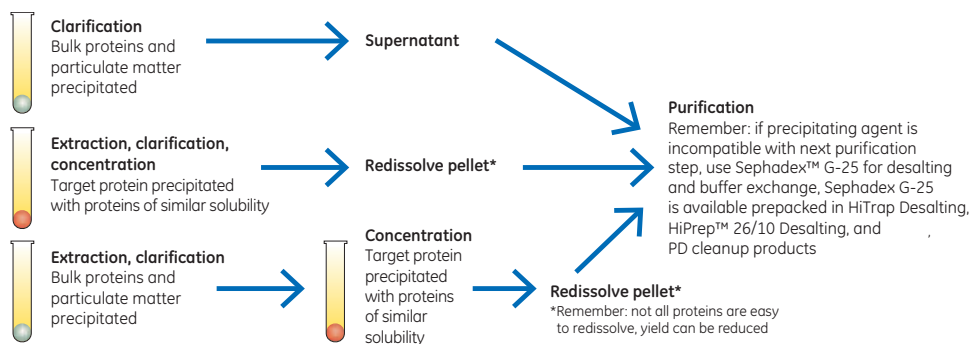




Fig 2.1. Three ways to use precipitation.

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

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

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

Table 2.5. Examples of precipitation techniques

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulfate	As described below.	> 1 mg/ml proteins especially immunoglobulins.	Stabilizes proteins, no denaturation; supernatant can go directly to hydrophobic interaction chromatography (HIC). Reduces lipid content.
Dextran sulfate	Add 0.04 ml of 10% dextran sulfate and 1 ml of 1 M CaCl_2 /ml of sample, mix 15 min, centrifuge at $10\,000 \times g$, discard pellet.	Samples with high levels of lipoprotein, e.g., ascites.	Precipitates lipoproteins.
Polyvinylpyrrolidone	Add 3% (w/v), stir 4 h, centrifuge at $17\,000 \times g$, discard pellet.	Samples with high levels of lipoprotein, e.g., ascites.	Alternative to dextran sulfate.
Polyethylene glycol (PEG, $M_r > 4000$)	Up to 20% (w/v).	Plasma proteins.	No denaturation; supernatant goes directly to ion exchange chromatography (IEX) or affinity chromatography (AC); complete removal can 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)		Precipitates nucleic acids.
Caprylic acid	1:15 (w/w)	Antibody concentration should be > 1 mg/ml.	Precipitates bulk of proteins from sera or ascites, leaving immunoglobulins in solution.

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

Ammonium sulfate precipitation

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



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

The principle of the method is to increase the concentration of ammonium sulfate to a level where antibodies will begin to “salt out.” Different antibodies salt out at different concentrations, a process that can be used to advantage to remove contaminating proteins from the crude extract. The salt concentration needs to be optimized to remove contaminants and not the desired antibody. An additional step with increased salt concentration should then precipitate the antibody. If the antibody cannot be safely precipitated and redissolved, only the first step should be employed.

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

Solutions needed for precipitation:

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

1 M Tris-HCl, pH 8.0

Buffer for the first purification step

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



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

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

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



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



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



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



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



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

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

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

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

Caprylic acid precipitation

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



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



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



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



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

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

Solutions needed for precipitation:

- Caprylic (octanoic) acid
- 2 M hydrochloric acid
- 2 M sodium hydroxide
- Buffer for the first purification step

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

Resolubilization of antibody precipitates

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

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

Denaturing agent	Typical conditions for use	Removal/comment
Urea	2 to 8 M	Remove using Sephadex G-25
Guanidine hydrochloride	3 to 6 M	Remove using Sephadex G-25 or IEX
Sarcosyl	1.5%	Remove using Sephadex G-25 or IEX
N-Octyl glucoside	2%	Remove using Sephadex G-25 or IEX
Sodium dodecyl sulfate	0.1% to 0.5%	Exchange for nonionic detergent during first chromatographic step; avoid ALEX
Alkaline pH	> pH 9.0, sodium hydroxide	Might need to adjust pH during chromatography to maintain solubility


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

Desalting and buffer exchange

General considerations

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

GE offers a range of prepacked chromatography columns and 96-well filter plates that can be used manually, together with a chromatography system, or in high-throughput applications (Table 2.8). These products contain Sephadex G-25, a size exclusion chromatography (SEC, also known as gel filtration) medium that allows effective removal of low molecular weight substances from antibodies with a molecular weight > 5000.

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


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

Table 2.8. Selection guide for desalting/buffer exchange columns

Columns and 96-well plates	Medium	Loaded volume (ml)	Eluted volume (ml)	Dilution factor	Operation
HiTrap Desalting	Sephadex G-25 Superfine	0.25	1.0	4	Syringe/pump/ chromatography system
		0.5	1.5	3	Syringe/pump/ chromatography system
		1.0	2.0	2	Syringe/pump/ chromatography system
		1.5 (max.)	2.0	1.3	Syringe/pump/ chromatography system
2× HiTrap Desalting	Sephadex G-25 Superfine	3.0 (max.)	4.0 to 5.0	1.3 to 1.7	Syringe/pump/ chromatography system
3× HiTrap Desalting	Sephadex G-25 Superfine	4.5 (max.)	6.0 to 7.0	1.3 to 1.7	Syringe/pump/ chromatography system
HiPrep 26/10 Desalting	Sephadex G-25 Fine	10	10 to 15	1.0 to 1.5	Pump/chromatography system
		15 (max.)	15 to 20	1.0 to 1.3	Pump/chromatography system
2× HiPrep 26/10 Desalting	Sephadex G-25 Fine	30 (max.)	30 to 40	1.0 to 1.3	Pump/chromatography system
3× HiPrep 26/10 Desalting	Sephadex G-25 Fine	45 (max.)	45 to 55	1.0 to 1.2	Pump/chromatography system
4× HiPrep 26/10 Desalting	Sephadex G-25 Fine	60 (max.)	60 to 70	1.0 to 1.2	Pump/chromatography system
PD SpinTrap™ G-25	Sephadex G-25 Medium	0.1 to 0.18	0.1 to 0.18	No dilution	Centrifuge
PD MultiTrap™ G-25	Sephadex G-25 Medium	0.07 to 0.13	0.07 to 0.13	No dilution	Centrifuge
PD MiniTrap™ G-25	Sephadex G-25 Medium	0.2 to 0.5	0.1 to 0.5	No dilution	Centrifuge
		0.1 to 0.5	1.0	2	Gravity flow
PD MidiTrap™ G-25	Sephadex G-25 Medium	0.5 to 1.0	0.5 to 1.0	No dilution	Centrifuge
		0.1 to 0.5	1.5	1.5	Gravity flow
PD-10 Desalting Columns	Sephadex G-25 Medium	1.0 to 2.5	1.0 to 2.5	No dilution	Centrifuge
		0.1 to 0.5	3.5	1 to 1.5	Gravity flow

Sample volumes of up to 30% of the total volume of the desalting column can be processed. The high speed and capacity of the separation allows even relatively large sample volumes to be processed rapidly and efficiently in the laboratory. Sample concentration does not influence the separation as long as the concentration of antibodies does not exceed approximately 70 mg/ml when using normal aqueous buffers, and provided that the antibody is stable and soluble at the concentration used.

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

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

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

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

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

Small-scale desalting of samples

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

For smaller sample volumes in the range of 100 to 180 μ l, multiple samples can be run on PD SpinTrap G-25 spin columns together with a microcentrifuge or PD MultiTrap G-25 96-well plate using centrifugation for extraction (Fig 2.2).

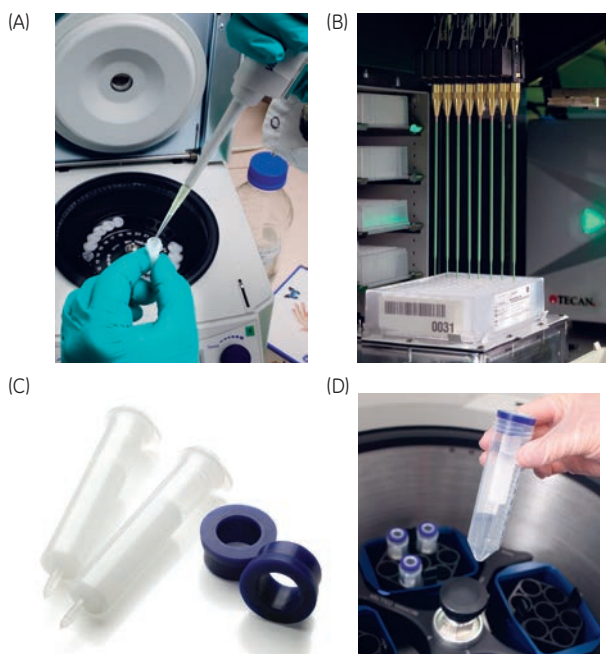


Fig 2.2. (A) PD SpinTrap G-25 sample preparation. (B) PD MultiTrap G-25 sample automated preparation in a robotic system. (C and D) Spin Adapters are used together with PD-10 Desalting Columns, PD MidiTrap G-25, and PD MiniTrap G-25 in a standard centrifuge.

Desalting larger sample volumes using HiTrap and HiPrep columns

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

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

Buffer preparation

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

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

Sample preparation

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

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

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

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

Manual desalting with HiTrap columns



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

HiTrap Desalting is a 5 ml column (Fig 2.3) packed with the tried-and-tested SEC medium, Sephadex G-25 Superfine. The medium is based on cross-linked dextran beads that allow excellent resolution and high flow rates. The fractionation range for globular proteins is between M_r 1 000 and 5 000, with an exclusion limit of approximately M_r 5 000. This ensures group separations of proteins/peptides larger than M_r 5 000 from molecules with a molecular weight less than M_r 1 000.

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

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

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

Column: HiTrap Desalting
Sample: 2 mg/ml bovine serum albumin (BSA) in 50 mM sodium phosphate, 500 mM sodium chloride, pH 7.0
Sample volume: 1.4 ml
Buffer: 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0
Flow rate: 10 ml/min

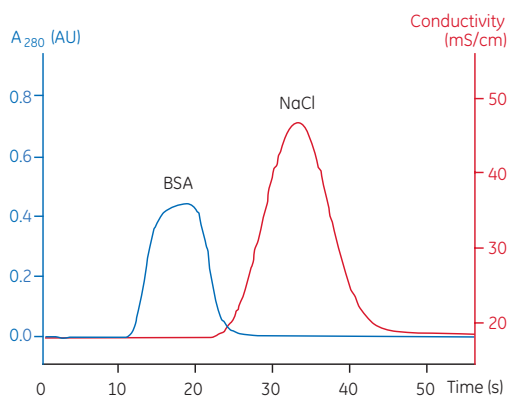


Fig 2.4. Highly efficient desalting in 30 s using HiTrap Desalting.

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

Column equilibration

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

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

Manual desalting using a syringe

1. To operate the column with a syringe, connect the syringe to the column using the supplied connector.
2. Equilibrate the column, see previous page *Column equilibration*.
3. Apply the sample using a 2 to 5 ml syringe at a flow rate between 1 and 10 ml/min. Discard the liquid eluted from the column. If the sample volume is less than 1.5 ml, change to buffer and proceed with the injection until a total of 1.5 ml has been eluted. Discard the eluted liquid.
4. Elute the protein with the appropriate volume selected from Table 2.9. Collect the desalted protein.



The maximum recommended sample volume when using one HiTrap Desalting 5 ml column is 1.5 ml, see Table 2.9. See Table 2.8 for information on application of smaller sample volumes.

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

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



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



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

Desalting using a pump


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

Automated desalting with HiTrap Desalting columns on ÄKTAprime plus



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

Buffer preparation

Equilibration buffer (port A1): Prepare at least 500 ml of the required buffer

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


Sample preparation

-  Pass the sample through a 0.45 µm filter.
-  The maximum recommended sample volume is 1.5 ml.

Preparing ÄKTAprime plus

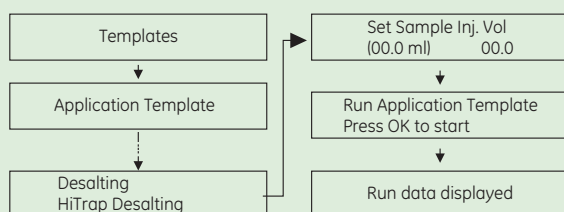
1. Place the inlet tubing from port A (port valve) and port B (2-port valve) in to the buffer.
2. Place the three brown waste tubings in the waste flask.
3. Connect the column between port 1 on the injection valve (7-port valve) and the UV flow cell.
4. Fill the fraction collector rack with 18 mm tubes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

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

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

Selecting Application Template and starting the method

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



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

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

Column: HiTrap Desalting
Sample: Normal sized globular protein in 20 mM sodium phosphate, 500 mM sodium chloride, 500 mM imidazole, pH 7.4
Buffer port (A1): 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.0

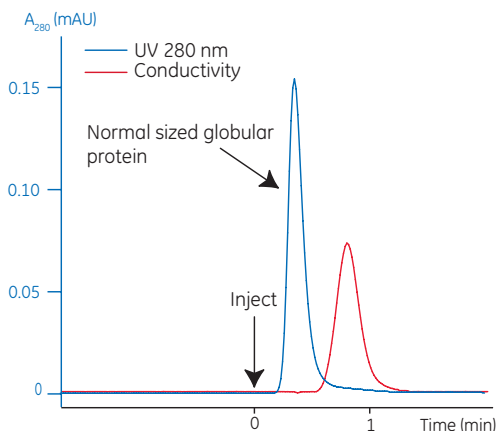


Fig 2.5. Typical desalting of a normal sized globular protein using a chromatography system.

Scaling up desalting from HiTrap to HiPrep

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

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



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

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

Buffer preparation

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

Sample preparation



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



Pass the sample through a 0.45 µm filter.



The maximum recommended sample volume is 15 ml.

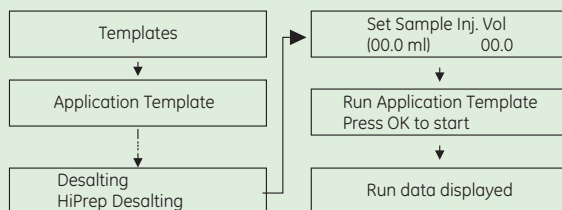
1. Place the inlet tubing from port A (8-port valve) and port B (2-port valve) in the buffer.
2. Place the three brown waste tubings in the waste flask.
3. Connect the column between port 1 on the injection valve (7-port valve) and the UV flow cell.
4. Fill the fraction collector rack with 18 mm tubes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

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

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

Selecting Application Template and starting the method

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



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

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

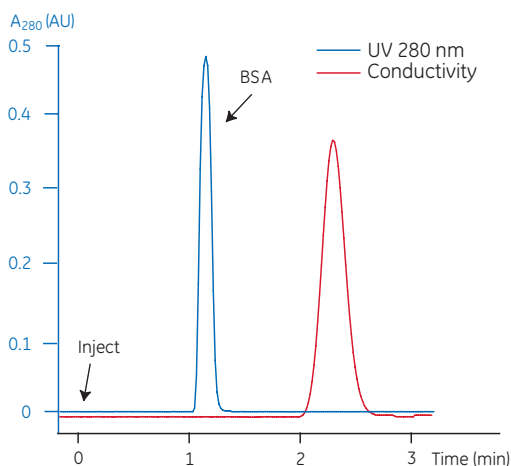


Fig 2.7. A typical desalting of BSA using a chromatography system.

Small-scale desalting and buffer exchange with PD desalting columns

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

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

PD SpinTrap G-25



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

PD SpinTrap G-25 is a single-use spin column that is designed for rapid, highly reproducible desalting and buffer exchange of 100 to 180 μ l sample using a standard microcentrifuge (Fig 2.2 A and 2.8). The columns provide highly reproducible, parallel desalting/buffer exchange and cleanup of protein samples without sample dilution.

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

Buffer

Equilibration buffer: Appropriate for the application

Desalting procedure

1. Suspend the medium by vortexing. Loosen screw cap lid and remove bottom closure using the plastic bottom cap removal tool.
2. Place the column in an appropriately sized collection tube and remove the storage solution by centrifugation for 1 min at $800 \times g$.
3. Equilibrate by adding 400 μ l equilibration buffer and centrifuge for 1 min at $800 \times g$. Discard the flowthrough and replace the collection tube. Repeat this procedure four times.



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

4. Replace the used collection tube with a new clean collection tube for sample collection.
5. Apply 100 to 180 μ l sample slowly to the middle of the prepacked column.
6. Elute by centrifugation at $800 \times g$ for 2 min.



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



Recovery is dependent on type of protein or other biomolecule. Typically, recovery is in the range of 70% to 90%. Concentration of the sample can improve recovery. Recovery can be improved for sample volumes less than 140 μ l by adding 40 μ l equilibration buffer after the sample has fully absorbed into the column bed.

PD MultiTrap G-25



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

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

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

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

96-well plate:	PD MultiTrap G-25
Sample:	1 mg/ml of BSA in 1 M sodium chloride
Sample volume:	130 μ l in each well
Equilibration buffer:	Ultrapure water

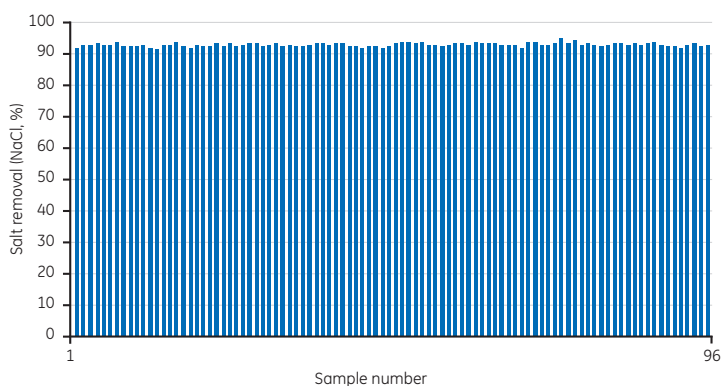


Fig 2.10. Removal of sodium chloride from BSA on a PD MultiTrap G-25 96-well plate showed highly reproducible results. The average desalting capacity was 93% and the well-to-well variation was 1% (relative standard deviation).

Centrifugation protocol

Buffer

Equilibration buffer: Appropriate for the application

Desalting procedure

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



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

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



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



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

PD MiniTrap G-25



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

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

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

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

Gravity protocol

Buffer

Equilibration buffer: Appropriate for the application



Desalting procedure

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



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

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

-  For desalting larger sample volumes, use HiTrap and HiPrep columns, see Table 2.8.
-  Recovery is dependent on type of protein. Typically, recovery is in the range of 70% to 90%. Concentration of the sample can improve recovery. Recovery and desalting capacity are higher when using gravity flow compared with centrifugation.

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

Column: PD MiniTrap G-25
 Sample: 1 mg/ml BSA in 1 M sodium chloride
 Sample volume: 500 μ l
 Equilibration buffer: Ultrapure water

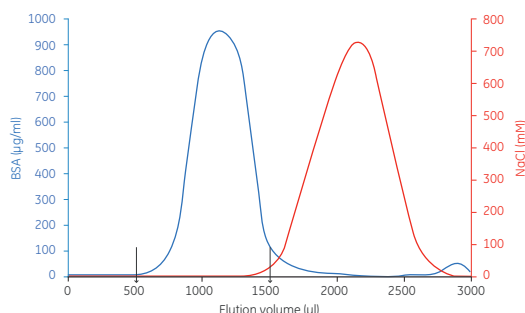


Fig 2.12. Removal of sodium chloride from BSA using the gravity protocol. The protein recovery was 95%.


Centrifugation protocol

Buffer

Equilibration buffer: Appropriate for the application

Desalting procedure

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

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

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

- 👉 For desalting larger sample volumes, use HiTrap and HiPrep columns, see Table 2.8.
- 👉 Recovery is dependent on type of protein. Typically, recovery is in the range of 70% to 90%. Concentration of the sample can improve recovery. Recovery and desalting capacity are higher using gravity flow compared with centrifugation.

PD MidiTrap G-25



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

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

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

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

Gravity protocol

Buffer

Equilibration buffer: Appropriate for the application

Desalting procedure

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

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

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



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



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

Centrifugation protocol

Buffer

Equilibration buffer: Appropriate for the application

Desalting procedure

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



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

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



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



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

Disposable PD-10 Desalting Columns

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

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

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

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

Column: Disposable PD-10 Desalting Column
Sample: Human serum albumin (HSA),
25 mg in 2.5 ml of 500 mM sodium chloride
Equilibration: Distilled water

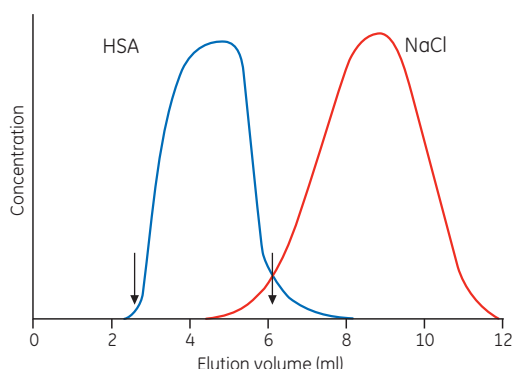


Fig 2.14. Removal of sodium chloride from albumin solution. A PD-10 Desalting column was equilibrated with distilled water. The sample contained human serum albumin (25 mg) dissolved in 2.5 ml of 500 mM sodium chloride solution. A total of 23.8 mg albumin was recovered in 3.5 ml eluent corresponding to a yield of 95.3% (between arrows). Initial total salt content of sample before desalting was 2%.


Gravity protocol

Buffer

Equilibration buffer: Appropriate for the application


Desalting procedure

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

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

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

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

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


Centrifugation protocol

Buffer

Equilibration buffer: Appropriate for the application


Desalting procedure

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

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

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

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

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

Chapter 3

Small-scale purification by affinity chromatography

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

When there is an immunospecific interaction, affinity chromatography (AC) is often the first and sometimes the only step required. However, to achieve satisfactory sample homogeneity, a further polishing step, often size exclusion chromatography, might be required. Affinity purification offers high selectivity, and usually, high capacity for the target protein(s). The target molecule is concentrated into a smaller volume and purity levels often above 95% are possible in one step.

This chapter describes the AC media and prepacked formats available from GE for small-scale purification of antibodies using bulk chromatography media prepacked in columns, as well as in 96-well plates and spin columns. Advice on handling of the different formats is provided and purification protocols for each format are described. Chromatography media developed for large-scale purification of antibodies, such as the latest addition to the MabSelect™ product range, MabSelect SuRe™ LX, is also described, while the purification strategies in this scale are briefly described in Chapter 7.

Affinity ligands for antibody purification

Protein G and protein A bind to different IgG

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

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

Table 3.1 shows a comparison of the relative binding strengths of protein G and protein A to different immunoglobulins. The information has been compiled from various publications. Binding strengths are tested with free protein G or protein A and can be used as guidelines to predict the binding behavior to a protein G or protein A purification medium. However, when coupled to an affinity matrix, the interaction can be altered. For example, rat IgG₁ binds to Protein G Sepharose, but not to Protein A Sepharose.

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

Table 3.1. Relative binding strengths of antibodies from various species to protein G and protein A as measured in a competitive ELISA test. The amount of IgG required to give a 50% inhibition of binding of rabbit IgG conjugated with alkaline phosphatase was determined

Species	Subclass	Protein G binding	Protein A binding
Human	IgA	—	variable
	IgD	—	—
	IgE	—	—
	IgG ₁	++++	++++
	IgG ₂	++++	++++
	IgG ₃	++++	—
	IgG ₄	++++	++++
	IgM*	—	variable
Avian egg yolk	IgY†	—	—
Cow		++++	++
Dog		+	++
Goat		++	—
Guinea pig	IgG ₁	++	++++
Hamster		++	+
Horse		++++	++
Koala		+	—
Llama		+	—
Monkey (rhesus)		++++	++++
Mouse	IgG ₁	++++	+
	IgG _{2a}	++++	++++
	IgG ₂	+++	+++
	IgG ₃	+++	++
	IgM*	—	variable
Pig		+++	+++
Rabbit		+++	++++
Rat	IgG ₁	+	—
	IgG _{2a}	++++	—
	IgG _{2b}	++	—
	IgG ₃	++	+
Sheep		++	+/—

* Purified using HiTrap IgM Purification HP columns.

† Purified using HiTrap IgY Purification HP columns.

++++ = strong binding.

++ = medium binding.

— = weak or no binding.

Protein L binds to the variable region of the kappa light chain

Protein L was first isolated from the surface of bacterial species *Peptostreptococcus magnus* and was found to bind immunoglobulins through immunoglobulin light chain interaction, from which the name was suggested. Since no part of the heavy chain is involved in the binding interaction, protein L binds a wider range of antibody classes than protein A or G. Protein L binds to representatives of all antibody classes, including IgG, IgM, IgA, IgE, and IgD. Recombinant protein L has four binding domains and binds to the variable region of the kappa light chain of immunoglobulins and immunoglobulin fragments. Protein L binds to three of four kappa light chain subtypes in humans (1, 3, and 4) and kappa 1 in mice. Table 3.2 maps the full recombinant protein L binding affinity.

Table 3.2. Protein L binding affinities

Species	Antibody class	Affinity [†]
General	Kappa light chain (subtypes 1,3,4)	Strong
	Lambda light chain	No binding
	Heavy chain	No binding
	Fab	Strong
	ScFv	Strong
	Dab	Strong
Human	IgG ₁	Strong
	IgG ₂	Strong
	IgG ₃	Strong
	IgG ₄	Strong
	IgA	Strong
	IgD	Strong
	IgE	Strong
	IgM	Strong
Mouse	IgG ₁	Strong
	IgG _{2a}	Strong
	IgG _{2b}	Strong
	IgG ₃	Strong
	IgM	Strong
Rat	IgG ₁	Strong
	IgG _{2a}	Strong
	IgG _{2b}	Strong
	IgG _{2c}	Strong
Pig	Total IgG	Strong
Dog	Total IgG	Weak
Cow	IgG ₁	No binding
	IgG ₂	No binding
Goat	IgG ₁	No binding
	IgG ₂	No binding
Sheep	IgG ₁	No binding
	IgG ₂	No binding
Chicken	Total IgG	No binding

* De Chateau, M. et al. *On the interaction between protein L and immunoglobulins of various mammalian species*. Scand. J. Immunol. **37**, 399-405 (1993).

[†] Binding to protein L occurs only if the immunoglobulin has the appropriate kappa light chains. Stated binding affinity refers only to species and subtypes with appropriate kappa light chains. Lambda light chains and some kappa light chains will not bind.

Ligands that bind to the constant region of Fab kappa or lambda light chain

GE also offers two ligands that bind to the constant region of the kappa or the lambda light chain, respectively. The ligands are recombinant proteins (M_r 13 000) and produced in *S. cerevisiae*. Coupled to chromatography media, the ligands are the first choice for purification of kappa and lambda Fab fragments (KappaSelect and Lambda FabSelect, respectively).

Types of AC media and prepacked formats

Chromatography media

GE offers a recombinant form of protein G grown in *E. coli* from which the albumin-binding region of the native protein has been genetically deleted. This recombinant protein G ligand is coupled to both Protein G Sepharose 4 Fast Flow and Protein G Sepharose High Performance.

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

Native protein A (nProtein A) is also available coupled to Sepharose 4 Fast Flow and Sepharose High Performance while recombinant protein A (rProtein A) only is available coupled to Sepharose 4 Fast Flow. Recombinant protein A bound to Sepharose offers several potential advantages compared to native protein A. rProtein A has been engineered to favor single point oriented immobilization via thioether coupling, which results in enhanced binding capacity for IgG. Furthermore, rProtein A is produced in *E. coli* and no human IgG affinity step is used during validated fermentation and purification processes, minimizing risk of human IgG contamination.

The MabSelect family of chromatography media is designed for capturing MABs from large sample volumes. The recombinant protein A ligand of MabSelect is engineered to favor an oriented coupling that delivers enhanced binding capacity.

MabSelect SuRe uses an alkali-tolerant recombinant protein A ligand that is resistant to harsh cleaning agents (e.g., 100 to 500 mM sodium hydroxide).

MabSelect Xtra uses the same recombinant protein A ligand as MabSelect, but the medium has a smaller particle size and greater porosity for increased dynamic binding capacity at higher flow rates.

MabSelect SuRe LX uses the same recombinant protein A ligand as MabSelect SuRe, has very high dynamic binding capacity at extended residence times, and is developed for high-titer antibody processes. Alkali tolerance, high capacity, and low ligand leakage in combination with the rigid base matrix makes MabSelect SuRe LX an excellent purification choice for manufacturers of MABs.

Capto L is an affinity chromatography medium for capture of antibodies and antibody fragments. It combines a rigid, high-flow agarose matrix with the immunoglobulin-binding recombinant protein L ligand, which has strong affinity for the variable region of an antibody's kappa light chain. Capto L is therefore suitable for capture of a wide range of antibody fragments such as Fabs, single-chain variable fragments (scFv), and domain antibodies (Dabs).

Binding capacity and other characteristics of the various chromatography media described above are summarized in Appendix 1.

Magnetic bead media

Protein G and Protein A (nProtein A) are also available as ligands to Mag Sepharose, a paramagnetic bead media based on highly cross-linked agarose particles. Protein G Mag Sepharose and Protein A Mag Sepharose are designed to simplify enrichment of target proteins by immunoprecipitation or pull-down applications. Protein G Mag Sepharose Xtra and Protein A Mag Sepharose Xtra magnetic beads are designed for rapid, small-scale purification and screening of monoclonal and polyclonal antibodies from serum and cell supernatants. Mag Sepharose is conveniently handled together with MagRack 6, a separation tool for microcentrifuge tubes where up to six samples can be processed in parallel or MagRack Maxi for enrichment of up to 50 ml of sample.

Reuse and storage

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

Affinity media are stored in 20% ethanol at 2°C to 8°C.

Prepacked formats

Sepharose based chromatography media are available in large variety of prepacked formats from small-scale spin columns to larger columns, as well as columns operated by gravity flow and in 96-well plates (Fig 3.1). All prepacked columns and 96-well plates are supplied with a detailed protocol that outlines the buffers and steps required for optimal results.

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

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

Fast and efficient manual purification of monoclonal and polyclonal antibodies can be performed by gravity flow using 1 ml prepacked GraviTrap™ columns .

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

MabSelect and Capto L chromatography media are available in bulk (lab packs), prepacked HiTrap for small-scale purification, HiScreen columns for use in process development, and 96-well PreDicator plates for high-throughput screening.

For purification scale-up, chromatography media can be packed in XK, Tricorn, or HiScale columns.



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

Optimization of parameters

Some parameters for antibody purification can require optimization to obtain the optimal result.

Examples of parameters that can require optimization are:

- Choice of buffers
- Sample pretreatment
- Amount of antibody to be purified
- Number of washes

Purification using Protein G Sepharose chromatography media

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

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

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

Protein G Sepharose chromatography media bind IgG over a wide pH range with a strong affinity at physiological pH and ionic strength. For the optimum binding conditions for IgG from a particular species, consult the most recent literature.

Avoid excessive washing if the interaction between antibody and ligand is weak, since this might decrease yield.

To elute the IgG, it is necessary to lower the pH to between 2.5 and 3.0 depending on the antibody. If biological activity of the antibody or antibody fragment is lost due to the low pH required for elution, try Protein A Sepharose; the elution conditions used are generally milder (see Table 3.5).

Table 3.3 shows the options for purification of antibodies using Protein G Sepharose chromatography media.

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

Product	Format or column size	Binding capacity (mg IgG/ml medium)	Description
Protein G Sepharose 4 Fast Flow	5 ml 25 ml	> 20 (human) 23 (cow) 19 (goat) 17 (guinea pig) 10 (mouse) 7 (rat)	Supplied as suspension for packing in, e.g., XK and Tricorn columns; used for scaling up purification.
Protein G GraviTrap	1 ml gravity columns	Approx. 20 (human)	Fast and efficient manual purification of monoclonal and polyclonal antibodies.
rProtein A/ Protein G GraviTrap	1 ml gravity columns	Approx. 35 mg IgG/ column (human)	Fast and efficient manual purification of monoclonal and polyclonal antibodies.
Protein G HP MultiTrap	96-well plates	> 25 (human)	For small-scale purification, screening of antibody constructs, optimization of buffer conditions, protein enrichment by immunoprecipitation.
Ab SpinTrap	100 µl spin columns	> 25 (human)	For small-scale purification of IgG and fragments, including human IgG ₃ using a microcentrifuge. Strong affinity to mouse monoclonal IgG ₃ and rat IgG.
Protein G HP SpinTrap	100 µl spin columns	> 25 (human)	For small-scale purification of IgG and fragments, including human IgG ₃ and protein enrichment of target antigens by immunoprecipitation using a microcentrifuge. Strong affinity to mouse monoclonal IgG ₃ and rat IgG. Supplied with a protocol for antibody purification and for capture of target antigens by immunoprecipitation.
HiTrap Protein G HP	1 ml, 5 ml columns	> 25 (human)	Laboratory-scale purification of IgG and fragments, including human IgG ₃ . Strong affinity to monoclonal mouse IgG ₁ and rat IgG. Prepacked 1 ml or 5 ml columns can be connected in series to facilitate scale-up.
MABTrap Kit	1 Kit	> 25 (human)	Purification of IgG and fragments, including human IgG ₃ . Strong affinity to mouse monoclonal IgG ₁ and rat IgG. Kit contains HiTrap Protein G HP (1 × 1 ml), accessories, premade buffers for 10 purifications, and detailed experimental protocols.
Companion product			
Ab Buffer Kit	1 Kit		Premade buffers recommended for antibody purification using Ab SpinTrap, Protein G HP SpinTrap, HiTrap Protein G HP, and Protein G HP MultiTrap.

Protein G Sepharose 4 Fast Flow

Protein G Sepharose 4 Fast Flow consists of 90 μm beads of highly cross-linked agarose, which provide a robust and stable chromatography matrix that allows high flow rates. The medium is a good choice for general-purpose capture of antibodies and scale-up in the laboratory.

Protein G Sepharose 4 Fast Flow is available as a bulk medium (Fig 3.2) for packing in XK, HiScale, and Tricorn columns (Fig 3.3) at laboratory scale. Furthermore, Protein G Sepharose 4 Fast Flow can be packed in larger columns for industrial-scale purification of monoclonal and polyclonal antibodies.



Fig 3.2. Protein G Sepharose 4 Fast Flow is available in various bulk (lab packs) sizes for laboratory and process-scale applications.

Column packing

Refer to Appendix 5 for general column packing guidelines.

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



Fig 3.3. Empty Tricorn columns for packing. Once packed, these columns can be used for purification using either a pump or chromatography system.

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



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

Sample preparation



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



The sample should have a pH around 7.0 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 0.02 M sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralizing buffer: 1 M Tris-HCl, pH 9.0




Water and chemicals used for buffer preparation should be of high purity.




Filter buffers through a 0.45 µm filter before use.

Purification


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

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

2. If the column contains 20% ethanol, wash it with 5 column volumes of distilled water. Use a linear flow rate of 50 to 100 cm/h.

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

3. Equilibrate the column with 5 to 10 column volumes of binding buffer at a linear flow rate of 150 cm/h.
4. Apply the pretreated sample.
5. Wash with binding buffer until the absorbance reaches the baseline.
6. Elute with elution buffer using a step or linear gradient. For step elution, 5 column volumes of elution buffer are usually sufficient. For linear gradient elution, a shallow gradient over 20 column volumes allows separation of proteins with similar binding strengths.
7. After elution, regenerate the column by washing it with 5 to 10 column volumes of binding buffer. The column is now ready for a new purification.

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

Storage

Store in 20% ethanol at 2°C to 8°C.

Protein G GraviTrap

Protein G GraviTrap are gravity-flow columns prepacked with 1 ml of Protein G Sepharose 4 Fast Flow. The columns are designed for fast and efficient manual purification of monoclonal and polyclonal antibodies and antibody fragments from cell culture supernatant and biological fluids. The antibodies are simply captured with high specificity on protein G ligands in the gravity-flow columns. You do not need any other instrument with this protocol because the entire process relies on the flow of gravity. Together with the packaging that can be converted into a column stand (Workmate), parallel purification is made simple (Fig 3.4). The columns are reusable up to five times.



Fig 3.4. Protein G GraviTrap are prepacked gravity-flow columns that provide simple, manual purification of antibodies from cell culture.

rProtein A Sepharose and a mix of Protein G Sepharose 4 Fast Flow and rProtein A Sepharose 4 Fast Flow are also available in GraviTrap format. The simple four-step procedure for purification is shown in Figure 3.5.

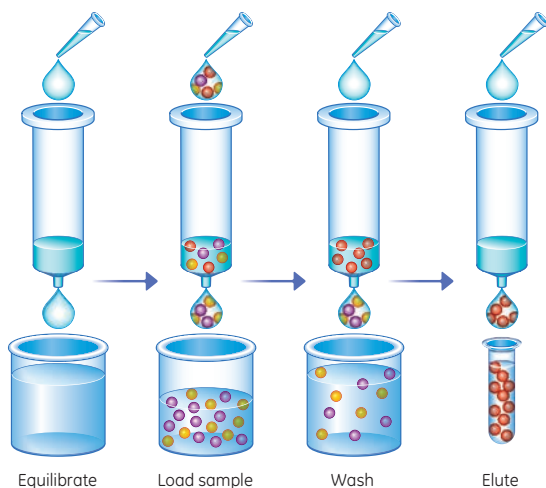


Fig 3.5. Purifying antibodies with Protein G GraviTrap is a simple four-step procedure.

Sample preparation

Refer to Chapter 2 for general considerations.



The sample should have a pH around 7.0 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 0.02 M sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralizing buffer: 1 M Tris-HCl, pH 9.0



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



Buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28903059).

Purification

1. Cut off the bottom tip and remove the top cap. Pour off the column storage solution and place the column in the Workmate column stand. If needed, mount LabMate (PD-10 Buffer Reservoir) on top of the column.
2. Equilibrate the column with 10 ml of binding buffer.
3. After equilibration, add the sample. A volume of 1 to 20 ml is recommended. If the sample volume is less than 1 ml, dilute to 1 ml with binding buffer.
4. Add 15 ml binding buffer.
5. Add 3 to 5 ml of elution buffer. Collect the elution fraction. The collected elution fraction contains the purified protein.



As a safety measure to preserve the activity of acid-labile IgG, addition of 1 M Tris-HCl, pH 9.0 to the tubes used for collecting antibody-containing fractions (60 to 200 µl/ml eluted fraction) is recommended. In this way, the final pH of the sample will be approximately neutral.



The eluted fractions can be buffer exchanged using PD-10 Desalting columns or HiTrap Desalting columns (see Chapter 2).

6. After elution, regenerate the column by washing it with 5 to 10 ml of binding buffer. The column is now ready for a new purification.



Depending on the nature of the sample, Protein G GraviTrap columns may be reused up to five times consecutively. Reuse of the columns should only be considered when processing identical samples to avoid cross-contamination.

Protein G Sepharose High Performance

Protein G Sepharose High Performance consists of 34 µm highly cross-linked agarose beads for high-performance purification of antibodies (Table 3.4). This medium offers the highest possible binding capacity and is compatible with additives commonly used in antibody purification. Protein G Sepharose High Performance is stable over a broad pH range. The high chemical and physical stability, as well as broad operating pH range of the medium preserves the biological activity of the antibody while ensuring a highly pure product.

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

Protein G HP MultiTrap

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

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

The procedure for small-scale screening and purification of antibodies is described below. For a description of the immunoprecipitation procedure, download Instructions 28906773 at www.gelifesciences.com.



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

Sample preparation

Refer to Chapter 2 for general considerations.

- ☞ The sample should have a pH around 7.0 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 0.02 M sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

- ☞ Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.45 µm filter before use.
- ☞ Buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28903059).

Purification

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

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

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

6. Replace the collection plate with a fresh collection plate prepared in step 1.
7. Add 200 μ l of elution buffer to elute the antibody and centrifuge for 30 s at 70 \times g. Collect the eluate. Repeat this step. Most of the bound antibody is eluted after two elution steps.



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

Storage

Store in 20% ethanol at 2°C to 8°C.

Protein G HP SpinTrap/Ab SpinTrap

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

The 16 columns supplied in each package can be used with a standard microcentrifuge and one purification run takes less than 20 min.

Each package of Protein G HP SpinTrap contains a protocol for protein enrichment of target antibody-antigen complexes by immunoprecipitation and a protocol for antibody purification.

Ab SpinTrap is another pack size of Protein G HP SpinTrap columns (Fig 3.8); 50 columns are provided with Ab SpinTrap.

For a description of the immunoprecipitation procedure for both products, download Instructions 28906772 at www.gelifesciences.com.



Fig 3.7. Protein G HP SpinTrap columns are used for protein enrichment of target proteins using antibodies bound to the protein G ligand and are also used for small-scale purification of antibodies.



Fig 3.8. Ab SpinTrap columns and Ab Buffer Kit combine to allow rapid, small-scale purification of antibodies using a microcentrifuge.

Purification of antibodies from serum without sample clarification, dilution, or filtration is possible with Protein G HP SpinTrap column and Ab SpinTrap columns. Using these columns, loss of target protein caused by manual operations such as sample centrifugation, transfer of sample to centrifuge tubes, and collecting supernatant is minimized. Figure 3.9 shows the result of purification of anti-HSA (human serum albumin, lane 2 of SDS gel) using Ab SpinTrap column from the undiluted serum of an immunized rabbit.

<i>Column:</i>	Ab SpinTrap
<i>Sample:</i>	600 μ l of undiluted serum from rabbit containing anti-HSA
<i>Equilibration:</i>	600 μ l of 20 mM sodium phosphate, pH 7.0
<i>Incubation:</i>	4 min
<i>Wash:</i>	2 \times 600 μ l of 20 mM sodium phosphate, pH 7.0
<i>Elution:</i>	2 \times 400 μ l of 100 mM glycine-HCl, pH 2.7

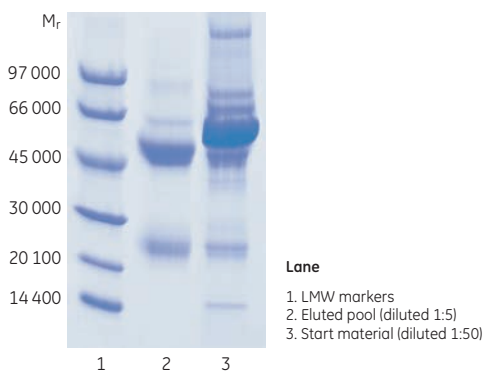


Fig 3.9. SDS-PAGE (reducing conditions; ExcelGel™ SDS Gradient 8–18; Coomassie™ Blue staining) of eluted pool of purified anti-HSA in undiluted serum from an immunized rabbit.

General handling of SpinTrap columns

Lids and bottom caps: Lids and bottom caps 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).

Bottom cap removal: Twist the bottom cap off the SpinTrap column before dispensing liquid into the column. Remember to save the bottom cap.

Incubation: Make sure that the medium is fully suspended before incubating with end-over-end mixing. All incubations should normally be performed at room temperature. However, incubations may be performed at lower temperatures when a slower process is preferable.

After centrifugation: Immediately after centrifugation, re-insert the bottom cap into the bottom of the SpinTrap column (before the incubation and elution steps).

Liquid collection: After each step, place the SpinTrap column in a fresh 2 ml microcentrifuge tube (not included) for liquid collection.

Elution: For the elution steps, mix by manually inverting the SpinTrap column.

Sample preparation

Refer to Chapter 2 for general considerations.



The sample should have a pH around 7 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 0.02 M sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralizing buffer: 1 M Tris-HCl, pH 9.0



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



Buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28903059).

Purification

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



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

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



Several sample applications can be made subsequently as long as the capacity of the column is not exceeded.

5. Wash by adding 600 μ l binding buffer, centrifuge for 30 s at 70 to 100 \times g.
6. Add 400 μ l of elution buffer and mix by inversion. Place the column in a 2 ml microcentrifuge tube containing 30 μ l neutralizing buffer (see step 1). Elute by centrifugation for 30 s at 70 \times g and collect the eluate.
7. Place the column in a new 2 ml microcentrifuge tube containing 30 μ l neutralizing buffer (see step 1). Centrifuge for 30 s at 70 \times g and collect the second eluate. Most of the bound antibody is eluted after two elution steps.



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

HiTrap Protein G HP columns

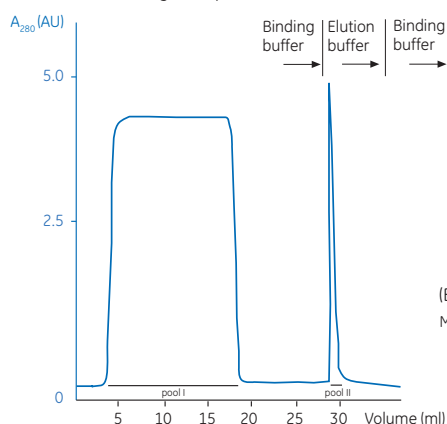
HiTrap Protein G HP (Fig 3.10) is a convenient, ready-to-use column prepacked with Protein G Sepharose High Performance. The columns are available in 1 ml and 5 ml sizes. In common with all HiTrap columns, HiTrap Protein G HP can be used for rapid antibody purification with a syringe, pump, or chromatography system. Furthermore, purification capacity can be greatly increased by connecting columns in series.



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

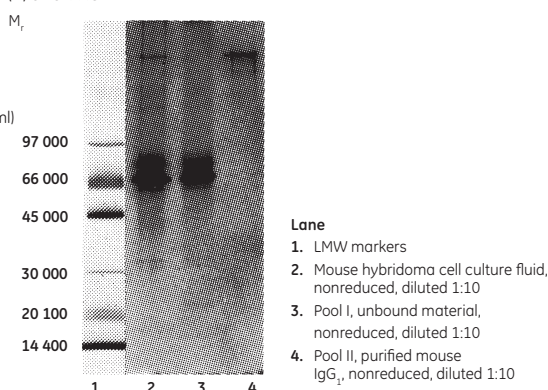
Figure 3.11 shows the purification of mouse monoclonal IgG₁ on HiTrap Protein G HP. The monoclonal antibody was purified from a hybridoma cell culture supernatant.

(A) Purification using HiTrap Protein G HP



Column: HiTrap Protein G HP 1 ml
Sample: 12 ml hybridoma cell culture fluid containing mouse IgG₁
Binding buffer: 20 mM sodium phosphate, pH 7.0
Elution buffer: 100 mM glycine-HCl, pH 2.7
Flow rate: 1 ml/min
Electrophoresis: SDS-PAGE, PhastSystem™, PhastGel™ Gradient 10–15, 1 µl sample, silver stained
Immunodiffusion: 1% Agarose A in 750 mM Tris, 250 mM 5,5-diethylbarbituric acid, 5 mM calcium lactate, 0.02% sodium azide, pH 8.6

(B) SDS-PAGE



(C) Immunodiffusion

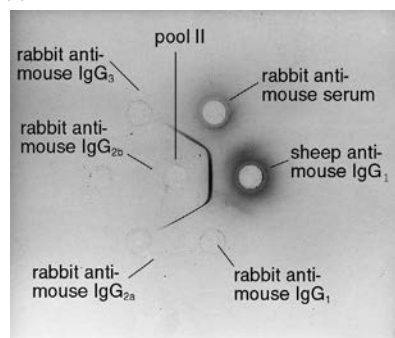


Fig 3.11. (A) Purification of mouse monoclonal IgG₁ from cell culture supernatant on HiTrap Protein G HP 1 ml column. Purity of mouse IgG₁ was confirmed by (B) nonreducing SDS-PAGE on PhastSystem using PhastGel 10-15 (silver stained), and (C) agarose-gel immunodiffusion.

Sample preparation

Refer to Chapter 2 for general considerations.

- The sample should be adjusted to the composition of the binding buffer. This can be done by either diluting the sample with binding buffer or by buffer exchange using HiTrap Desalting, HiPrep 26/10 Desalting or PD-10 Desalting columns, see Chapter 2.
- The sample should be fully solubilized. We recommend centrifugation or filtration immediately before loading on the column to remove particulate material (0.45 µm filter).
- Never apply turbid solution to the column.

Buffer preparation

Binding buffer: 0.02 M sodium phosphate, pH 7.0
 Elution buffer: 0.1 M glycine-HCl, pH 2.7
 Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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



For purification using a syringe on HiTrap Protein G HP 1 ml or 5 ml columns, buffers can be prepared from the 10× stock solutions of binding and elution buffers supplied with Ab Buffer Kit (28903059).

Purification

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



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

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

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



IgG from most species and subclasses bind to protein G at near physiological pH and ionic strength. For the optimum binding conditions for IgG from a particular species, consult the most recent literature.






Avoid excessive washing if the interaction between the antibody and ligand is weak, since this might decrease yield.

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



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

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

-  Desalt and/or transfer purified IgG fractions to a suitable buffer using a desalting column (see Chapter 2).
-  To increase capacity, connect several HiTrap Protein G HP columns (1 ml or 5 ml) in series. HiTrap columns can be used with a syringe, a peristaltic pump or connected to a liquid chromatography system (see Chapter 5 for details of ÄKTA chromatography systems).
-  Reuse of HiTrap Protein G Sepharose HP columns depends on the nature of the sample and should only be considered when processing identical samples to avoid cross-contamination.

Storage

Before storage we recommend to wash the column with 5 column volumes of 20% ethanol to prevent microbial growth. Store the column in 20% ethanol at 2°C to 8°C.

MAbTrap Kit

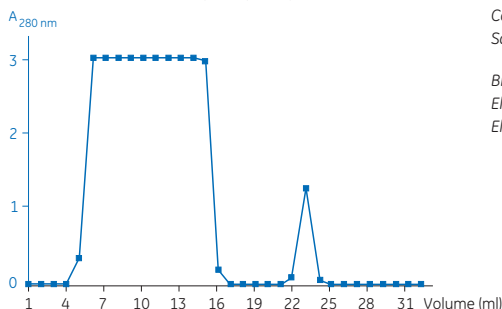
MAbTrap™ Kit contains one HiTrap Protein G HP 1 ml column, binding, elution, and neutralization buffers, a syringe with fittings, and an optimized purification protocol (Fig 3.12). The kit contains sufficient material for up to 20 purifications of monoclonal or polyclonal IgG from serum, cell culture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



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

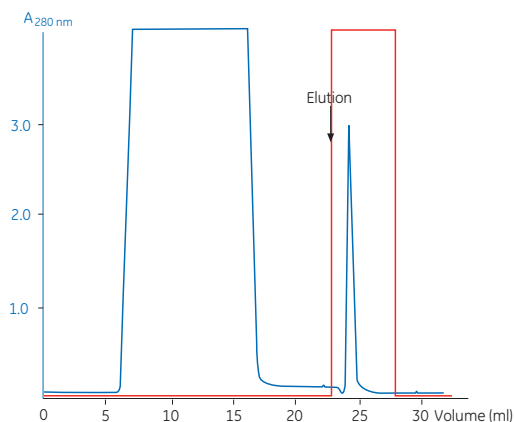
Figure 3.13 shows the purification of mouse monoclonal IgG₁ from cell culture supernatant using a syringe operation and a similar purification with pump operation. Operating HiTrap Protein G HP 1 ml with a syringe resulted in an IgG₁ pool of 3 ml with an absorbance of 0.44 (A_{280}) and a corresponding yield of 0.9 mg pure mouse monoclonal IgG₁. A similar experiment in which the column was operated with a P-1 pump resulted in an IgG₁ pool of 2 ml with an absorbance of 0.60 (A_{280}), corresponding also to a total yield of 0.9 mg pure mouse monoclonal IgG₁.

(A) Purification using a syringe

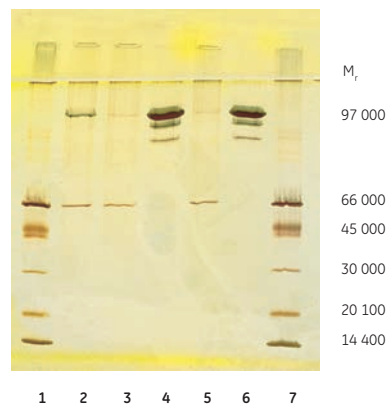


Column: HiTrap Protein G HP 1 ml
 Sample: 10 ml cell supernatant containing mouse monoclonal IgG₁, anti-transferrin
 Binding buffer: 20 mM sodium phosphate, pH 7.0
 Elution buffer: 100 mM glycine-HCl, pH 2.7
 Electrophoresis: SDS-PAGE, PhastSystem, PhastGel Gradient 10-15, 1 µl sample, silver stained

(B) Purification using a pump



(C) SDS-PAGE analysis



Lane
 1. LMW markers
 2. Cell culture supernatant, mouse monoclonal IgG₁, diluted 1:1
 3. Flowthrough, using peristaltic pump, diluted 1:10
 4. Eluted mouse monoclonal IgG₁, using a peristaltic pump
 5. Flowthrough, using a syringe, diluted 1:10
 6. Eluted mouse monoclonal IgG₁, using a syringe
 7. LMW markers

Fig 3.13. Purification of mouse monoclonal IgG₁ from cell culture supernatant; (A) using a syringe and (B) a peristaltic pump. (C) Analysis of eluted fractions by nonreducing SDS-PAGE on PhastSystem using PhastGel 10-15, silver stained.

Sample preparation, purification, and storage

Refer to *HiTrap Protein G HP columns* earlier in this chapter regarding sample preparation, purification protocol, and storage recommendations.

Buffer preparation

Binding buffer: Dilute binding buffer concentrate 10-fold
 Elution buffer: Dilute elution buffer concentrate 10-fold
 Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

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

Purification using protein A-based chromatography media

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

Both native protein A (nProtein A) and recombinant protein A (rProtein A) ligands are available from GE. These molecules share similar specificity for the Fc region of IgG, but the recombinant protein A has been engineered to include a C-terminal cysteine that enables a single-point coupling when the protein is coupled to Sepharose, which ensures higher binding capacity. Besides the well-known affinity for the Fc region of IgG, protein A also has affinity for certain variants of the Fab region, and consequently, protein A chromatography media can in some cases be used for the purification of Fab and F(ab')₂ fragments. Protein A Sepharose chromatography media from GE also possess a considerably higher binding capacity than Protein G Sepharose chromatography media and therefore the preferred choice for capture of monoclonal antibodies in industrial-scale processes (see Chapter 7).

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

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



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



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



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






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

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

Product	Format or column size	Binding capacity (mg IgG/ml medium)	Description
nProtein A Sepharose 4 Fast Flow	5 ml 25 ml	Approx. 30 (human)	Supplied as suspension for packing in, e.g., XK and Tricorn columns; used for scaling up purification.
Protein A HP MultiTrap	96-well plates	Approx. 20 (human)	For small-scale purification of IgG, screening of different protein constructs, optimization of buffer conditions, protein enrichment by immunoprecipitation.
Protein A HP SpinTrap	100 µl spin columns	Approx. 20 (human)	For small-scale purification of IgG and fragments, protein enrichment by immunoprecipitation.
HiTrap Protein A HP	1 ml, 5 ml columns	Approx. 20 (human)	Laboratory-scale purification of IgG and fragments. Prepacked 1 ml or 5 ml columns can be connected in series to facilitate scale-up.
rProtein A Sepharose Fast Flow	5 ml 25 ml	Approx. 50 (human)	Supplied as suspension for packing in, e.g., XK and Tricorn columns; used for scaling up purification. Higher binding capacity than nProtein A Sepharose 4 Fast Flow.
rProtein A GraviTrap	1 ml gravity columns	Approx. 50 (human)	Fast and efficient manual purification of monoclonal and polyclonal antibodies.
rProtein A/Protein G GraviTrap	1 ml gravity columns	Approx. 35 mg IgG/column (human)	Fast and efficient manual purification of monoclonal and polyclonal antibodies.
HiTrap rProtein A FF	1 ml, 5 ml columns	Approx. 50 (human)	Laboratory-scale purification of IgG, fragments, and subclasses. Prepacked 1 ml or 5 ml columns can be connected in series to scale up. Enhanced binding capacity.
Companion product			
Ab Buffer Kit	1 Kit		Premade buffers recommended for antibody purification using Protein A HP MultiTrap, Protein A HP SpinTrap, HiTrap Protein A HP, and HiTrap rProtein A FF.

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

Species	Subclass	Protein A binding pH	Protein A elution pH
Human	IgG ₁	6.0 to 7.0	3.5 to 4.5
	IgG ₂	6.0 to 7.0	3.5 to 4.5
	IgG ₃	8.0 to 9.0	≤ 7.0
	IgG ₄	7.0 to 8.0	3.0 to 6.0
Cow	IgG ₂	n.a.	2.0
Goat	IgG ₂	n.a.	5.8
Guinea pig	IgG ₁	n.a.	4.8
	IgG ₂	n.a.	4.3
Mouse	IgG ₁	8.0 to 9.0	4.5 to 6.0
	IgG _{2a}	7.0 to 8.0	3.5 to 5.5
	IgG _{2b}	Approx. 7.0	3.0 to 4.0
	IgG ₃	Approx. 7.0	3.5 to 5.5
Rat	IgG ₁	≥ 9.0	7.0 to 8.0
	IgG _{2a}	≥ 9.0	≤ 8.0
	IgG _{2b}	≥ 9.0	≤ 8.0
	IgG ₃	8.0 to 9.0	3.0 to 4.0 (using 3 M potassium isothiocyanate)

-  Binding strengths are tested with free protein A and can be used as guidelines to predict the binding behavior to a protein A purification medium. However, when coupled to an affinity matrix, the interaction can be altered. For example, rat IgG₁ does not bind to protein A, but does bind to Protein A Sepharose.
-  With some antibodies, for example mouse IgG₁, a high concentration of sodium chloride in the binding buffer might be necessary to achieve efficient binding. Recommended binding buffers are 1.5 M glycine, 3 M sodium chloride, pH 8.9 or 0.02 M sodium phosphate, 3 M sodium chloride, pH 7.0.
-  Most antibodies and subclasses bind protein A close to physiological pH and ionic strength. Avoid excessive washing if the interaction between the protein of interest and the ligand is weak since this might decrease yield.
-  Use a mild elution method when labile antibodies are isolated. Reverse the flow of the wash buffer and elute with 0.1 M glycyltyrosine in 2 M sodium chloride, pH 7.0 at room temperature, applied in pulses. (Note: glycyltyrosine absorbs strongly at wavelengths used for detecting proteins). The specific elution is so mild that the purified IgG is unlikely to be denatured. Alternative elution buffers include: 1 M acetic acid pH 3.0, 0.1 M glycine-HCl pH 3.0, or 3 M potassium isothiocyanate. Note: potassium isothiocyanate can severely affect structure and immunological activity.
-  Desalt and/or transfer purified IgG fractions into a suitable buffer using a desalting column (see Chapter 2).



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



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

nProtein A Sepharose 4 Fast Flow



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

nProtein A Sepharose 4 Fast Flow (Fig 3.14) is native protein A coupled to Sepharose 4 Fast Flow. The medium is designed for recovery and purification of monoclonal antibodies from cell culture supernatants, serum, and ascites at both laboratory and process scale. Leakage of ligand from the Sepharose Fast Flow matrix is low. Moreover, nProtein A Sepharose 4 Fast Flow has high mechanical and chemical stability, withstanding high concentrations of hydrogen bond disrupting agents such as urea, guanidine hydrochloride, and sodium thiocyanate. The low ligand leakage and high flow rate of the Sepharose Fast Flow medium allow the use of nProtein A Sepharose 4 Fast Flow for scale-up of monoclonal and polyclonal antibody purification.

Column packing

Refer to Appendix 5 for general guidelines column packing.

A suitable packing method for nProtein A Sepharose 4 Fast Flow is described in the *Protein G Sepharose 4 Fast Flow* section earlier in this chapter.

Sample preparation

Refer to Chapter 2 for general considerations.



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

☞ IgG from many species has a medium to strong affinity for protein A at physiological pH. Sample pH should be between 6.0 and 9.0 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 0.02 M sodium phosphate, pH 7.0

Elution buffer: 0.1 M citric acid, pH 3.0

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Purification

Follow the protocol described in *Protein G Sepharose 4 Fast Flow* earlier in this chapter.

Protein A HP MultiTrap

Protein A HP MultiTrap is a 96-well plate prepacked with Protein A Sepharose High Performance for the preparation of protein samples, optimization of buffer conditions, enrichment of proteins of interest from clarified cell lysates and biological fluids, and purification of antibodies (Fig 3.15). Purification runs are performed in parallel, which ensures fast and reliable capture of antibodies from a large number of complex samples. Each package of Protein A HP MultiTrap contains four prepacked multiwell plates and alternative protocols for protein enrichment of target antibody-antigen complexes by immunoprecipitation and antibody purification. Collection plates (see *Ordering information*) for collecting eluted, purified antibody are available separately.

The protocol for screening and purification of antibodies is the same as described for Protein G Sepharose HP MultiTrap earlier in this chapter. For a description of immunoprecipitation using this product, download Instructions 28906771 at www.gelifesciences.com.



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

Sample preparation, buffer preparation, and purification

Protein A HP MultiTrap is used with the same protocol as Protein G Sepharose HP MultiTrap, see earlier in this chapter.

Protein A HP SpinTrap columns

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

The 16 columns supplied in each package can be used with a standard microcentrifuge. Each package of Protein A HP SpinTrap contains alternative protocols for protein enrichment of target antibody-antigen complexes by immunoprecipitation and for antibody purification.

The same procedure as described for Protein G Sepharose HP earlier in this chapter can be used for screening and purification of antibodies. For a description of immunoprecipitation using this product, download Instructions 28906770 at www.gelifesciences.com.



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

Sample preparation, buffer preparation, and purification

Protein A HP SpinTrap is used with the same protocol as Protein G Sepharose HP SpinTrap, see earlier in this chapter.

HiTrap Protein A HP columns

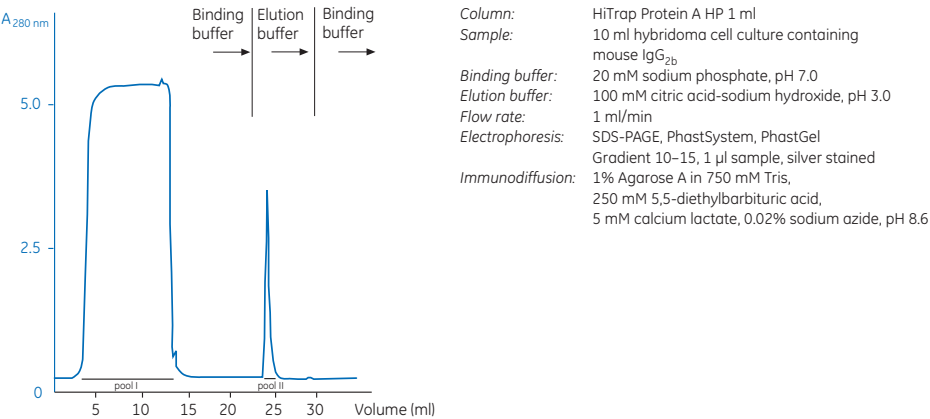
HiTrap Protein A HP are 1 ml and 5 ml ready-to-use columns prepacked with Protein A Sepharose High Performance (Fig 3.17). The columns are used for convenient purification of antibodies from cell culture supernatants, serum, and ascites. Purification can be performed using a syringe, pump, or chromatography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



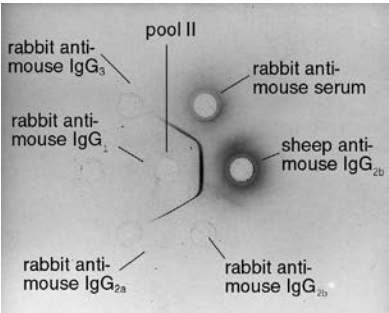
Fig 3.17. HiTrap Protein A HP columns are designed for rapid purification of antibodies using a syringe, pump, or chromatography system.

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

(A) Purification using HiTrap Protein A HP



(C) Immunodiffusion



(B) SDS-PAGE analysis

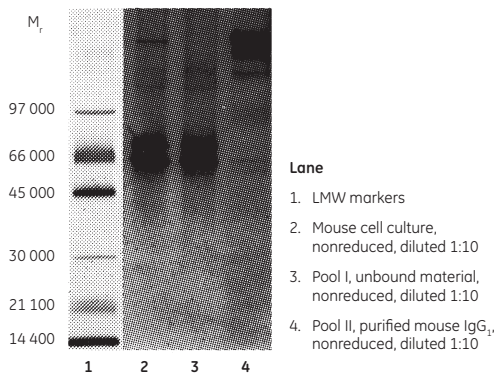


Fig 3.18. (A) Purification of mouse monoclonal IgG_{2b} from cell culture supernatant on HiTrap Protein A HP, 1 ml column. Purity of mouse IgG_{2b} was confirmed by (B) nonreducing SDS-PAGE on PhastSystem using PhastGel 10-15 (silver stained), and (C) agarose-gel immunodiffusion.

Sample preparation/Buffer preparation/Purification

Use the same protocol as for HiTrap Protein G HP, see earlier in this chapter.

rProtein A Sepharose Fast Flow

rProtein A Sepharose Fast Flow (Fig 3.19) is an affinity medium with high binding capacity for monoclonal and polyclonal antibodies. The binding capacity of rProtein A Sepharose Fast Flow is considerably higher than for nProtein A Sepharose 4 Fast Flow. The recombinant protein A ligand of rProtein A Sepharose Fast Flow has been specially engineered to favor an oriented coupling giving a matrix with enhanced binding capacity. Ligand leakage is low. rProtein A Sepharose Fast Flow is produced in *E. coli* and no human IgG affinity step is used during validated fermentation and purification processes, minimizing risk of human IgG contamination.

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



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

Column packing

Refer to Appendix 5 for general column packing guidelines.

A suitable packing method for Sepharose 4 Fast Flow is described in the *Protein G Sepharose 4 Fast Flow* section earlier in this chapter.

Sample preparation

Refer to Chapter 2 for general considerations.



Centrifuge samples ($10\,000 \times g$ for 10 min) to remove cells and debris. Filter through a $0.45\ \mu\text{m}$ filter.



IgG from many species has a medium to strong affinity for protein A at physiological pH. Sample pH should be between 6.0 and 9.0 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (see Chapter 2) or dilution and pH adjustment.

Buffer preparation

Binding buffer: 0.02 M sodium phosphate, pH 7.0

Elution buffer: 0.1 M sodium citrate, pH 3.0 to 6.0

Neutralizing buffer: 1 M Tris-HCl, pH 9.0



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

Purification

Use the protocol for *Protein G Sepharose 4 Fast Flow* described earlier in this chapter.

rProtein A GraviTrap and rProtein A/Protein G GraviTrap

rProtein A GraviTrap are gravity-flow columns prepacked with 1 ml of rProtein A Sepharose 4 Fast Flow. The columns are designed for fast and efficient manual purification of monoclonal and polyclonal antibodies, antibody fragments from cell culture supernatant, and biological fluids. The antibodies are simply captured with high specificity on protein A ligands in the gravity-flow columns. You do not need any other instrument with this protocol because the entire process relies on the flow of gravity. Together with the package that can be converted into a column stand (Workmate), parallel purification is made simple (see Fig 3.4). The columns are reusable up to five times.

A mix of rProtein A Sepharose 4 Fast Flow and Protein G Sepharose 4 Fast Flow is also available in GraviTrap format. The simple four-step procedure for purification using these GraviTrap columns is shown in Figure 3.5.

Sample preparation, buffer preparation, and purification

rProtein A GraviTrap as well as rProtein A/Protein G GraviTrap use the same protocol as Protein G GraviTrap, see earlier in this chapter.

HiTrap rProtein A FF columns

HiTrap rProtein A FF is a ready-to-use column prepacked with rProtein A Sepharose Fast Flow (Fig 3.20) for the convenient purification of monoclonal antibodies from cell culture supernatants, serum, and ascites. The column is suitable for small-scale purification of monoclonal antibodies from multiple species, screening, and process development. The columns are available in 1 ml and 5 ml sizes. In common with all HiTrap columns, HiTrap rProtein A FF can be used for rapid purification using a syringe, pump, or chromatography system. Furthermore, purification capacity can be greatly increased by connecting columns in series.

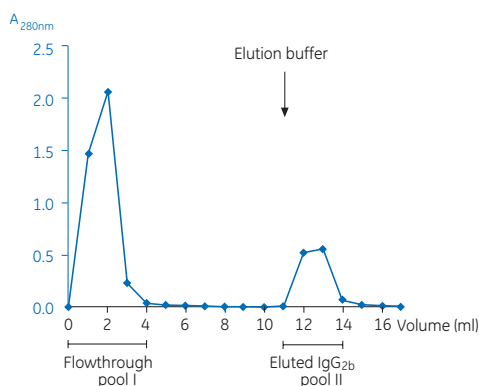


Fig 3.20. HiTrap rProtein A FF are columns prepacked with rProtein A Sepharose Fast Flow, which has very high binding capacity for monoclonal antibodies.

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

(A) Purification using HiTrap rProtein A FF

Column: HiTrap rProtein A FF, 1 ml
Sample: 1 ml mouse ascites containing IgG_{2b}
filtered through a 0.45 µm filter.
The sample was a kind gift from Dr. N. Linde,
EC Diagnostics, Sweden
Binding buffer: 20 mM sodium phosphate, pH 7.0
Elution buffer: 100 mM sodium citrate, pH 3.0
Flow rate: Approx. 1 ml/min
Instrumentation: Syringe



(B) SDS-PAGE analysis

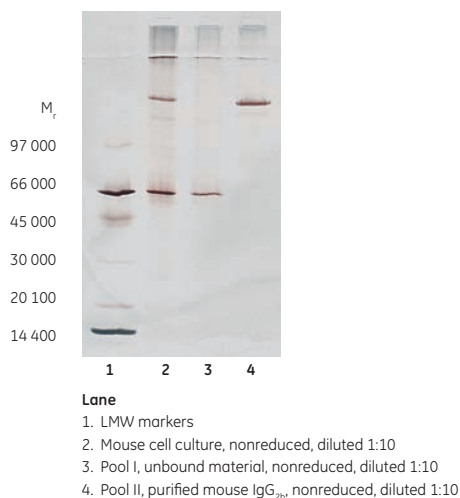


Fig 3.21. (A) Purification of mouse IgG_{2b} from ascites on a 1 ml HiTrap rProtein A FF column using a syringe. (B) Analysis of eluted IgG_{2b} by SDS-PAGE on PhastSystem and a PhastGel Gradient 10-15 precast gel revealed a purity of 95% (silver staining).

Sample preparation, buffer preparation, and purification

HiTrap Protein A columns use the same protocol as HiTrap Protein G HP, see earlier in this chapter.

Refer to Table 3.5 regarding binding and elution conditions commonly used with Protein A Sepharose chromatography media for purification of IgG from different species

MabSelect chromatography media and prepacked columns

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

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

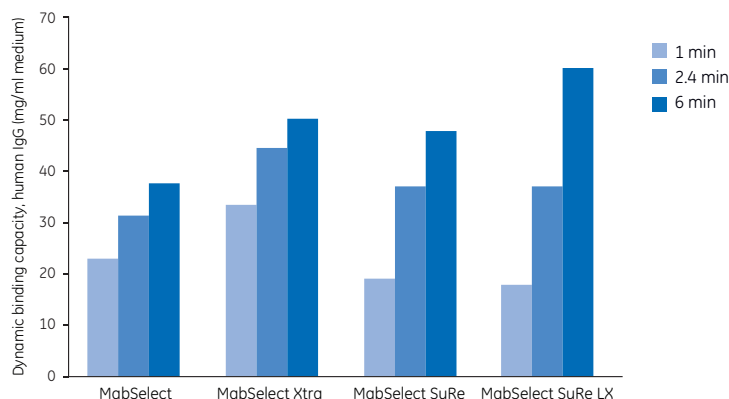


Fig 3.22. Dynamic binding capacity of MAb at various residence times of chromatography media from the MabSelect family. MabSelect SuRe LX shows excellent binding capacity for human IgG at the longest residence time (6 min).

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

MabSelect SuRe LX has been further developed from MabSelect SuRe to give even higher binding capacity from high-expression cell cultures with increased antibody titers. As an example, at 6 min residence time, the dynamic binding capacity of MabSelect SuRe LX for human IgG is approximately 60 g/l (Fig 3.22). This special combination of high binding capacity plus alkaline stability gives manufacturers of MAbs many opportunities to improve process economics and product quality. HiTrap MabSelect SuRe columns are prepacked with MabSelect SuRe. These columns can be used to develop an effective cleaning-in-place (CIP) protocol for purification of MAbs at industrial scale.



Fig 3.23. HiTrap MabSelect, HiTrap MabSelect SuRe, and HiTrap MabSelect Xtra columns for laboratory-scale purification of monoclonal antibodies with optimized binding capacity at high flow rates.

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

MabSelect chromatography media are also available prepacked in HiScreen columns which are part of the process development platform available from GE. The small column volume of 4.7 ml and bed height of 10 cm make HiScreen columns excellent tools for method optimization, parameter screening, robustness testing, and convenient scale-up. Process fluid velocities can be applied, since the 10 cm bed height gives enough residence time and the results can then serve as basis for linear process scale-up.

Finally, MabSelect chromatography media are available in miniaturized formats for parallel experiments, such as PreDicator™ 96-well filter plates and PreDicator RoboColumn™ units, which allow for screening of a variety of chromatography conditions such as buffer conditions for binding and elution. Data generated using the PreDicator product platform show good correlation with data obtained running packed chromatography columns, making them excellent tools for initial screenings of process conditions.

Table 3.6 shows the options for purification of antibodies using the family of MabSelect chromatography media.

Table 3.6. Options in selection for purification of antibodies using chromatography media from MabSelect family, a complete list is found in Appendix 1

	MabSelect	MabSelect Xtra	MabSelect SuRe	MabSelect SuRe LX
	Suitable for scale-up applications and large-scale purification of antibodies.	For capture of high titer feeds	Withstands rigorous and cost-effective CIP protocols	Outstanding capacity at longer residence times
Dynamic binding capacity	~ 30 mg hlgG/mL medium ¹	~ 40 mg hlgG/mL medium ²	~ 30 mg hlgG/mL medium ¹	~ 60 mg hlgG/mL medium ³
Recommended operating mobile velocity ⁴	500 cm/h	300 cm/h	500 cm/h	500 cm/h
Recommended CIP reagents	Reducing agent (e.g., 100 mM 1-thioglycerol) + 15 mM sodium hydroxide	Reducing agent (e.g., 100 mM 1-thioglycerol) + 15 mM sodium hydroxide	100 to 500 mM sodium hydroxide	100 to 500 mM sodium hydroxide
Ligand	Recombinant protein A	Recombinant protein A	Alkali-tolerant variant of protein A	Alkali-tolerant variant of protein A
Particle size ⁵	d _{50v} ~ 85 µm	d _{50v} ~ 75 µm	d _{50v} ~ 85 µm	d _{50v} ~ 85 µm
Available formats	Bulk packs from 25 ml to 10 l; HiTrap, HiScreen, PreDicator, Predictor RoboColumn	Bulk packs from 25 ml to 10 l; HiTrap, HiScreen, PreDicator, Predictor RoboColumn	Bulk packs from 25 ml to 10 l; HiTrap, HiScreen, PreDicator, Predictor RoboColumn	Bulk packs from 25 ml to 10 l; HiTrap, HiScreen, PreDicator, Predictor RoboColumn

¹ Determined at 10% breakthrough by frontal analysis at a mobile phase velocity of 500 cm/h at a 20 cm bed height (residence time: 2.4 min).

² Determined at 10% breakthrough by frontal analysis at a mobile phase velocity of 250 cm/h at a 10 cm bed height (residence time: 2.4 min).

³ Determined at 10% breakthrough by frontal analysis at a mobile phase velocity of 100 cm/h at a 10 cm bed height (residence time: 6 min).

⁴ In AxiChrom 300 column, 20 cm bed height operating at < 0.2 Mpa (2 bar, 29 psi), 25°C.

⁵ d_{50v} is the median particle size of the cumulative volume distribution.

Packing Tricorn 10/100 columns with MabSelect or MabSelect SuRe

Refer to Appendix 5 for general column packing guidelines.

Preparing the suspension

Suspension solution: 50 mM phosphate buffer, 150 mM sodium chloride, pH 7.0
Packing solution: 150 mM sodium chloride
10 ml MabSelect or MabSelect SuRe (corresponds to 14 ml MabSelect or MabSelect SuRe/20% ethanol suspension)
Sintered glass filter funnel (medium grade, G3 type)
Filtering flask

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

Assembling and packing the column

Equipment needed:

Tricorn 10/100 column
Tricorn 10 Coarse Filter Kit
Tricorn Packing Connector 10-10
Tricorn Glass Tube 10/100
Tricorn 10 bottom unit with a 10 mm filter mounted
Pump P-901 or similar
20 ml pipettes

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



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

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



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

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

Packing Tricorn 10/100 columns with MabSelect Xtra

Refer to Appendix 5 for general column packing guidelines.

Preparing the suspension

Suspension solution: 0.05 M phosphate buffer, 0.15 M sodium chloride, pH 7.0

Packing solution: 0.15 M sodium chloride

4 M sodium chloride

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

Sintered glass filter funnel (medium grade, G3 type)

Filtering flask

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

Assembling and packing the column

Equipment needed:

Tricorn 10/100 column
Tricorn 10 Coarse Filter Kit
Tricorn Packing Connector 10-10
Tricorn Glass Tube 10/300
Tricorn 10 bottom unit with a 10 mm filter mounted
Pump P-901 or similar
25 ml pipettes

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



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



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

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



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

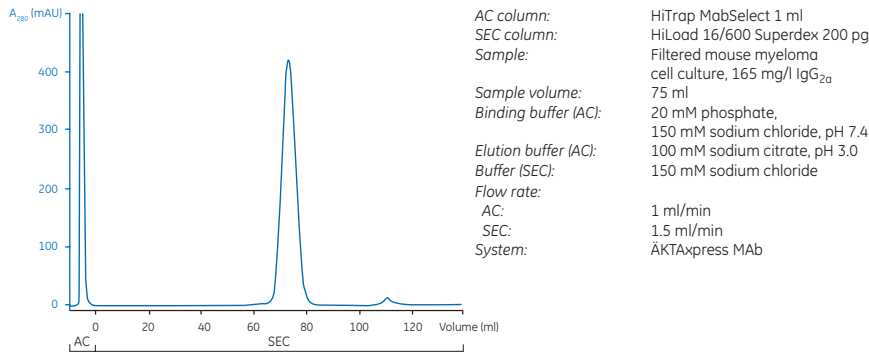
11. Switch off and disconnect the pump, re-fit the stop plug into Tricorn 10 Bottom Unit. Take the column from the stand and remove Tricorn 10/300 Glass Tube and packing connector over a sink. Remount the column vertically.
12. If necessary, remove excess medium by resuspending the top of the packed bed and remove with a Pasteur pipette or spatula. For a 10 cm bed height, the surface of the packed bed should be leveled with the lower end of the glass tube threads. Top-off the column with packing solution.
13. Wet a top coarse filter from the Tricorn 10 Coarse Filter Kit, the bottom of the adapter, and O-ring in 70% ethanol. Place the top coarse filter at the center of the adapter with the glossy side towards the adapter. Mount the adapter unit onto the column tube. Connect the pump.
14. Remove the stop plug and continue to pack at 10 ml/min (764 cm/h) for 1 min.
15. Mark the position of the bed surface on the column. Stop the pump, re-fit the stop plug into the bottom of the column. Reposition the adapter to approximately 1 mm below the marked position.
16. Wash the column with 15 ml of distilled water at 5 ml/min (382 cm/h) before checking packing quality.

HiTrap MabSelect and HiTrap MabSelect Xtra

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

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

(A) Purification using HiTrap MabSelect on ÄKTExpress MAB



(B) SDS-PAGE analysis

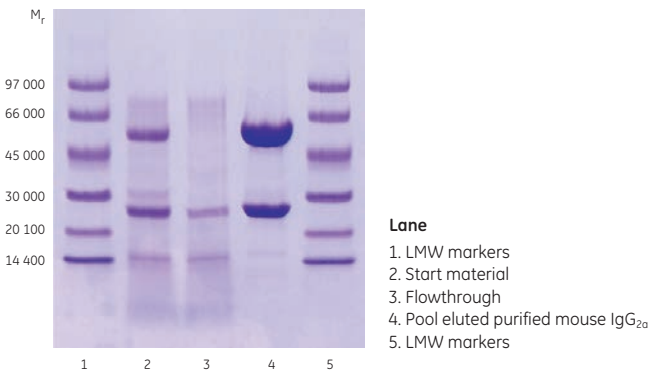
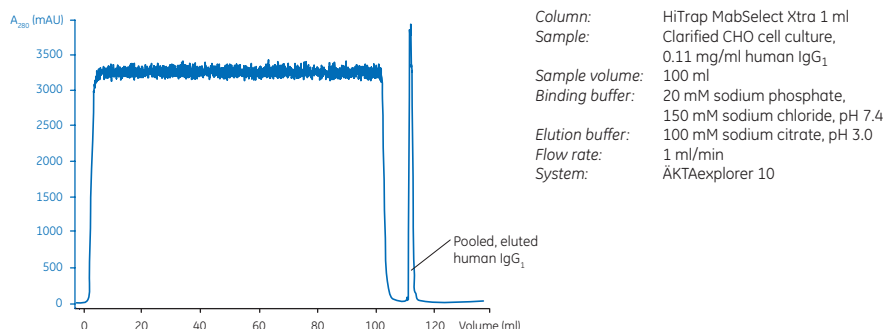


Fig 3.24. (A) Purification of IgG_{2a} using HiTrap MabSelect 1 ml in an automated, two-step purification on ÄKTExpress MAB (B) SDS-PAGE analysis on ExcelGel SDS Gradient 8-18, reducing conditions.

Successful purification of human monoclonal IgG₁ in one step using HiTrap MabSelect Xtra is shown in Figure 3.25. Highly pure MAb is seen in lane 4 of the SDS-PAGE gel.

(A) Purification using HiTrap MabSelect Xtra on ÄKTAexplorer 10



(B) SDS-PAGE analysis

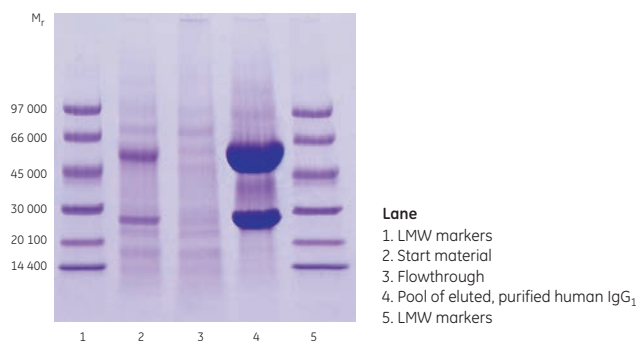


Fig 3.25. (A) Purification of human IgG₁ in one step on HiTrap MabSelect Xtra 1 ml on ÄKTAexplorer 10. (B) SDS-PAGE analysis on ExcelGel SDS Gradient 8-18, reducing conditions.

Sample preparation

Refer to Chapter 2 for general considerations.



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



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

Buffer preparation

Binding buffer: 0.02 M sodium phosphate, 0.15 M sodium chloride, pH 7.2

Elution buffer: 0.1 M sodium citrate, pH 3.0 to 3.6

Neutralizing buffer: 1 M Tris-HCl, pH 9.0



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

Purification

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

Binding buffer: 0.02 M sodium phosphate, 0.15 M sodium chloride, pH 7.2

Elution buffer: 0.1 M sodium citrate, pH 3.0 to 3.6

Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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



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

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

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



Stepwise elution allows the target antibody to be eluted in a more concentrated form, thus decreasing buffer consumption and shortening cycle times. Decreasing the flow rate might be necessary due to the high concentrations of protein in the eluted pool. When optimizing elution conditions, determine the highest pH that allows efficient elution of antibody from the column. This will prevent denaturing sensitive antibodies due to low pH.

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



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



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

Cleaning-in-place (CIP)

For cleaning-in-place protocols for the removal of unwanted precipitated or denatured contaminants and hydrophobically bound substances, see Appendix 5 and the Instructions for HiTrap MabSelect/HiTrap MabSelect Xtra (28408414), which are available for download at www.gelifesciences.com.

Storage

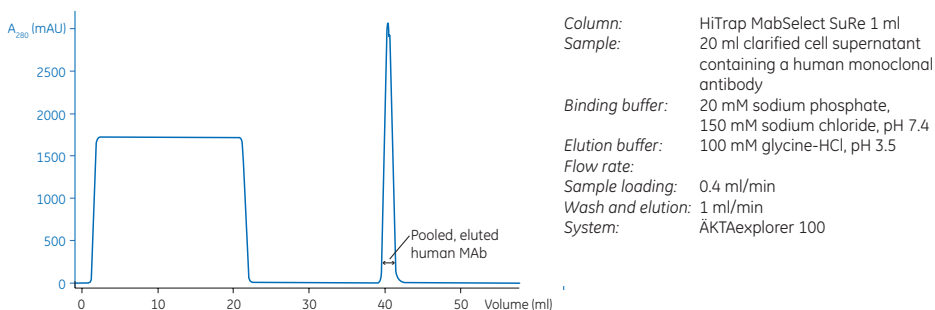
Store in 20% ethanol at 2°C to 8°C.

HiTrap MabSelect SuRe

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

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

(A) Purification using HiTrap MabSelect SuRe on ÄKTAexplorer 100



(B) SDS-PAGE analysis

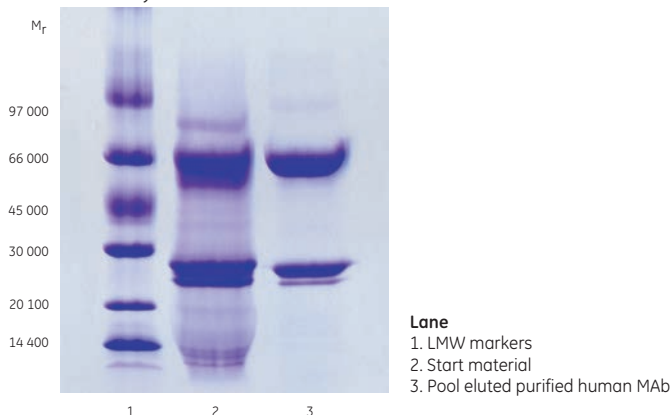




Fig 3.26. (A) One-step purification of a human monoclonal antibody on HiTrap MabSelect SuRe 1 ml on ÄKTAexplorer 100. (B) SDS-PAGE analysis on ExcelGel SDS Gradient 8-18, reducing conditions.


Sample preparation

Refer to Chapter 2 for general considerations.

-  Centrifuge samples (10 000 × g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter.
-  IgG from many species has a medium to strong affinity for protein A at physiological pH. Sample pH should be between 6 and 9 before applying to the column. If required, adjust sample conditions to the pH and ionic strength of the binding buffer by either buffer exchange on a desalting column (Chapter 2) or dilution and pH adjustment.

Buffer preparation


Binding buffer: 0.02 M sodium phosphate, 0.15 M sodium chloride, pH 7.2
 Elution buffer: 0.1 M sodium citrate, pH 3.0 to 3.6
 Neutralizing buffer: 1 M Tris-HCl, pH 9.0

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

Purification

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

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

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

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

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



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



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

9. If no cleaning-in-place is planned after elution, regenerate the column with 5 column volumes of elution buffer or and wash with 3 column volumes of binding buffer. If required, perform cleaning-in-place directly after elution with at least 2 column volumes of 0.1 to 0.5 M sodium hydroxide ensuring a contact time of 10 to 15 min. Wash with 5 column volumes of binding buffer.

10. Re-equilibrate the column with 5 to 10 column volumes binding buffer (or until the column has reached the same pH as the binding buffer).

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



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

Cleaning-in-place (CIP)

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



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

For more information on CIP for HiTrap MabSelect SuRe, see Appendix 5 and Instructions 11003489 at www.gelifesciences.com.

Storage

Store in 20% ethanol at 2°C to 8°C.

MabSelect SuRe LX

For this medium, we refer to the product instructions (28976500 at www.gelifesciences.com) for advice on handling, column packing, and protocol.

Purification by magnetic beads

Protein A Mag Sepharose Xtra/Protein G Mag Sepharose Xtra

Protein A Mag Sepharose Xtra and Protein G Mag Sepharose Xtra products are magnetic beads designed for high capacity small-scale purification/screening of monoclonal and polyclonal antibodies from various species.

Protein A Mag Sepharose Xtra and Protein G Mag Sepharose Xtra provide flexible purification allowing a wide range of sample volumes and easy scaling up by varying the bead quantity.

The magnetic bead format has excellent properties for small-scale experiments. The high density of the beads allows rapid capture by magnetic devices while the visibility of the beads ensures reliable collection of the bound antibodies in the purification procedure. The products are provided with protocols optimized for antibody purification.

MagRack 6 enables preparation of up to six samples captured in 1.5 ml microcentrifuge tubes (Fig 3.27). When the tubes are placed in the rack, the magnetic beads are attracted to the magnet within a few seconds. This allows easy removal of the supernatant whereas the magnetic beads are left in the tube. MagRack Maxi is suitable for volumes up to 50 ml, for example, capture of antibodies in low titer from a larger volume.



Fig 3.27. The high density of Mag Sepharose beads allows rapid capture by MagRack 6 magnetic device.

☞ For immunoprecipitation, it is recommended to use the Protein A Mag Sepharose and Protein G Mag Sepharose. These products have optimized capacities for immunoprecipitation applications, see Appendix 3 for more information.

Advice on handling magnetic bead medium

☞ Protein A Mag Sepharose Xtra and Protein G Mag Sepharose Xtra are intended for single use only.

☞ 1.5 ml Eppendorf tubes and MagRack 6 should be used in the protocol. When using volumes above 1.5 ml, for example larger volumes up to 50 ml, MagRack maxi is recommended.

General magnetic separation step

- Remove the magnet before adding liquid.
- Insert the magnet before removing liquid.

Dispensing the medium slurry

- Prior to dispensing the medium slurry, make sure it is homogeneous by vortexing.
- When the medium slurry is resuspended, immediately pipette the required amount of medium slurry into the desired tube.
- Repeat the resuspension step between each pipetting from the medium slurry vial.

Handling of liquid

- Use the magnetic rack with the magnet in place for each liquid removal step.
- Before application of liquid, remove the magnet from the magnetic rack.
- After addition of liquid, allow resuspension of the beads by vortexing or manual inversion of the tube. When processing multiple samples, manual inversion of the magnetic rack is recommended.

Incubation steps

- During incubation steps, make sure the magnetic beads are well resuspended and kept in solution by end-over-end mixing or by using a benchtop shaker.
- Incubation steps generally take place at room temperature. However, incubation can take place at +4°C over night if this is the recommended storage condition for the specific sample.
- When purifying samples of low concentrations or large volumes, an increase of the incubation time might be necessary.
- If needed, a pipette can be used to remove liquid from the lid.

Sample preparation

Refer to Chapter 2 for general considerations.



Check the pH of the sample, and adjust if necessary before applying the sample to the beads. The pH of the sample should equal the pH of the binding buffer. Adjusting the pH could be done by either diluting the sample with binding buffer or by buffer exchange using PD MiniTrap G-25 or HiTrap Desalting.



Clarification of sample might be needed before applying it to the beads.

Buffer preparation

Binding buffer: PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 100 mM sodium hydrogen phosphate, 2 mM potassium hydrogen phosphate), pH 7.4
Elution buffer: 100 mM glycine-HCl, pH 2.8
Neutralizing buffer: 1 M Tris-HCl, pH 9.0



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

Purification

This protocol is suitable for most antibody purifications.

Magnetic bead preparation

1. Mix the medium slurry thoroughly by vortexing. Dispense 100 µl homogenous medium slurry into an Eppendorf tube.
2. Place the Eppendorf tube in the magnetic rack, for example, MagRack 6.
3. Remove the storage solution.

Equilibration

4. Add 500 µl binding buffer.
5. Resuspend the medium.
6. Remove the liquid.

Sample application

7. Immediately after equilibration, add 300 µl of sample. If the sample volume is less than 300 µl, dilute to 300 µl with binding buffer.
8. Resuspend the medium and incubate for 30 min with slow end-over-end mixing or by using a benchtop shaker.
9. Remove the liquid.

Washing (perform this step two times totally)

10. Add 500 µl binding buffer.
11. Resuspend the medium.
12. Remove the liquid.

Elution

13. Add 100 µl of elution buffer.
14. Resuspend the medium.
15. Remove and collect the elution fraction. The collected elution fraction contains the main part of the purified antibody. If desired, repeat the elution.



As a safety measure to preserve the activity of acid-labile antibodies, we recommend the addition of 1 M Tris-HCl, pH 9.0 to tubes used for collecting antibody-containing fractions.

Protein A Mag Sepharose/Protein G Sepharose

Protein A Mag Sepharose and Protein G Sepharose products are magnetic beads designed for coupling of antibodies enabling enrichment of target protein for further downstream analyses such as mass spectrometry (MS and LC-MS) and electrophoresis techniques.

A generic procedure for immunoprecipitation using Protein A Mag Sepharose and Protein G Mag Sepharose magnetic beads is found in the *Protein Sample Preparation Handbook* (28988741) or the instructions for use (28953763).

Purifying antibody fragments

Monoclonal antibodies are typically purified using a platform approach where capture by protein A affinity chromatography has become the industry standard. However, a corresponding solution for antibody fragments has until now been lacking. One reason is the high diversity of the antibody fragments; and another is that chromatography media currently available on the market do not meet the demands for industrial-scale purification. The introduction of Capto L provides the foundation for a purification platform approach for this class of biomolecules.

Capto L products

Capto L is designed for capture of a wide range of antibody fragments such as Fabs, single-chain variable fragments (scFv), and domain antibodies (Dabs). Capto L is available in bulk sizes (5 ml to 10 l) as well as prepacked formats to support screening and optimization of binding and elution conditions. Prepacked formats include PreDicator 96-well plates and PreDicator RoboColumn units, as well as HiTrap and HiScreen prepacked columns (Fig 3.28).



Fig 3.28 Capto L for capture of antibody fragments and is available in bulk sizes (5 ml to 10 l) as well as prepacked formats.

In cases where Capto L lacks affinity for the antibody fragment of interest, there are a number of complementary affinity chromatography media available from GE to enable a comprehensive capture toolkit. KappaSelect binds kappa Fabs (constant region) and Lambda FabSelect binds lambda Fabs (constant region). In addition MabSelect can be used to capture antibody fragments due to its affinity for the heavy chain subtype VH₃. Figure 3.29 is a general guide for chromatography media selection.

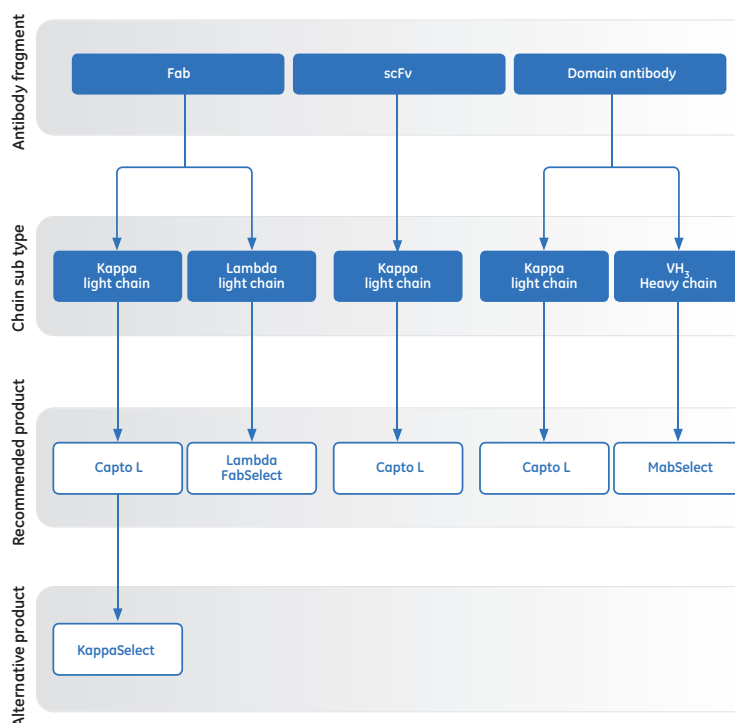


Fig 3.29. AC media for purification of antibody fragments.

Due to the high affinity binding of protein L to the variable region of the kappa light chain, Capto L purifies conventional Fabs as well as the smallest functional entity of antibodies, known as domain antibodies (Dabs). Figure 3.30 shows the dynamic binding capacity at 10% breakthrough (Qb10%) of Capto L for a number of different antibody fragments. Note that because dynamic binding capacity is normally measured in mg/ml, the molecular weight of the target molecule is an important factor to consider. Table 3.7 presents the dynamic binding capacity in relation to the molecular weight and the corresponding molar binding capacity.

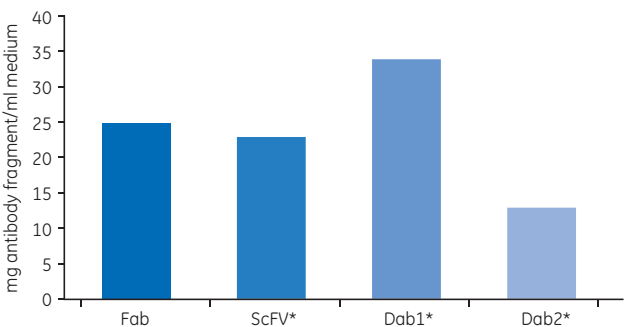


Fig 3.30. Capto L dynamic binding capacity at 10% breakthrough (Qb10%) of four human antibody fragments. Fab fragment kindly provided by UCB Celltech.

Table 3.7. Dynamic binding capacity for different antibody fragments

Molecule	DBC (mg/ml)	M _r	Molar equivalence*
Fab	25	50 000	0.5 µmol Fab/ml medium
scFv fusion protein	23	57 000	0.5 µmol scFv/ml medium
Dab1	34	25 000	1.4 µmol Dab/ml medium
Dab2	13	10 000	1.3 µmol Dab/ml medium

* Note as a comparison that typical Protein A chromatography media capture app. 0.3 µmol IgG/ml medium.

Antibody fragments are often expressed in microbial systems and the homogenate entering downstream purification is often crude and challenging. In the following example, a domain antibody was purified from clarified *E. coli* fermentation broth using Capto L.

Figure 3.31A shows approximately 11.4 mg Dab/ml medium loaded at pH 7.0 at a flow rate of 300 cm/h and a residence time of 4 min. A wash step followed at pH 5.0 to remove weakly bound impurities. Elution of bound material was performed with a step gradient using sodium acetate buffer, pH 3.0. Flowthrough and eluted fractions were collected and analyzed by SDS-PAGE. The elution pool contained highly enriched Dab protein, Figure 3.31B, lane 4. Product recovery was 87% and the *E. coli* host cell protein (HCP) levels were reduced from ~ 28 million ppm to ~ 6000 ppm. This single capture step resulted in a HCP level clearance log reduction of 3.6 and a final purity of Dab protein of 93.2%, see Table 3.8.

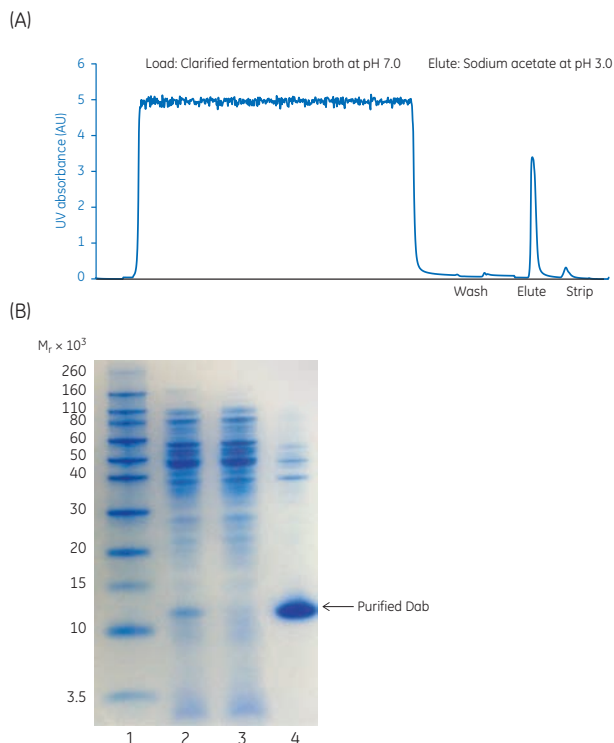


Fig 3.31. (A) Purification of Dab fragments from *E. coli* using Capto L. (B) SDS-PAGE under reducing conditions of sample load, flowthrough, and eluted fractions from the purification of Dab fragments on Capto L. SDS-PAGE was run under reducing conditions, Coomassie staining.

Table 3.8. Results from purification of Dab protein from clarified *E. coli* broth using Capto L medium. The results presented were obtained through customer collaboration

Parameter	Result
<i>E. coli</i> HCP clearance log	3.6 log reduction
Purity (evaluated by SEC)	93.2% monomer 6.5% aggregates
Yield	87%

Protein G also has an affinity binding site for certain Fab regions, and consequently, Protein G AC media can in some cases be used for the purification of Fab and F(ab')₂ fragments as well. Figure 3.32 shows the purification of recombinant mouse Fab fragments, expressed in *E. coli*, in a single affinity purification step using Protein G Sepharose 4 Fast Flow.

Medium: Protein G Sepharose 4 Fast Flow
Sample: 15 ml recombinant mouse Fab, expressed in *E. coli*.
Binding buffer: 50 mM Tris-HCl, 150 mM sodium chloride, 0.05% Tween™, pH 7.4
Elution buffer: 200 mM acetic acid, pH 2.8
Flow rate: 0.8 ml/min

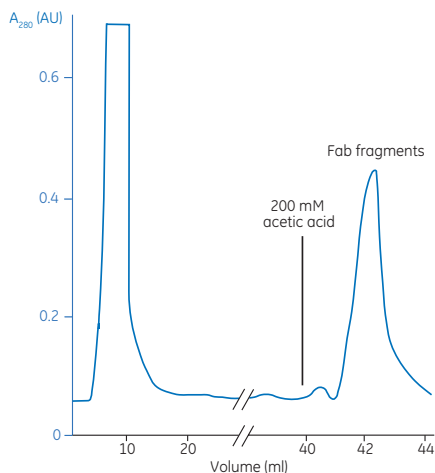


Fig. 3.32. Purification of recombinant mouse Fab fragments, expressed in *E. coli* using Protein G Sepharose 4 Fast Flow.

Purification of other classes of antibodies

IgA

Protein A can interact with human colostral IgA as well as human myeloma IgA2 but not IgA₁. Polyclonal IgA from pig, dog, and cat and monoclonal canine IgA have also exhibited binding affinity for protein A.

Protein L has strong affinity for human IgA (see Table 3.2) and Capto L is suitable for purification of this type of antibody.

IgD

Protein L has strong affinity for human IgD (see Table 3.2) and Capto L is suitable for purification of this type of antibody.

IgE

Protein L has strong affinity for human IgE (see Table 3.2) and Capto L is suitable for purification of this type of antibody.

IgM

IgM present in human and mouse serum binds weakly to protein A. However protein L has strong affinity for human and mouse IgM (see Table 3.2) and Capto L is hence suitable for purification of this type of antibody.

Another alternative is capture of IgM on thiophilic adsorption ligands, a tried-and-tested method for purification of this subspecies. The technique using HiTrap IgM Purification HP described below is optimized for purification of monoclonal IgM from hybridoma cell culture, but it can be used as a starting point to determine the binding and elution conditions required for IgM from other species.

Purifying IgM using HiTrap IgM Purification HP

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

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

Figure 3.33A shows the results from the purification of monoclonal α -Shigella IgM from hybridoma cell culture supernatant. Analysis by SDS-PAGE (Fig 3.33B) demonstrated a purity level of over 80%. Results from an ELISA (not shown) indicated high activity in the purified fraction.

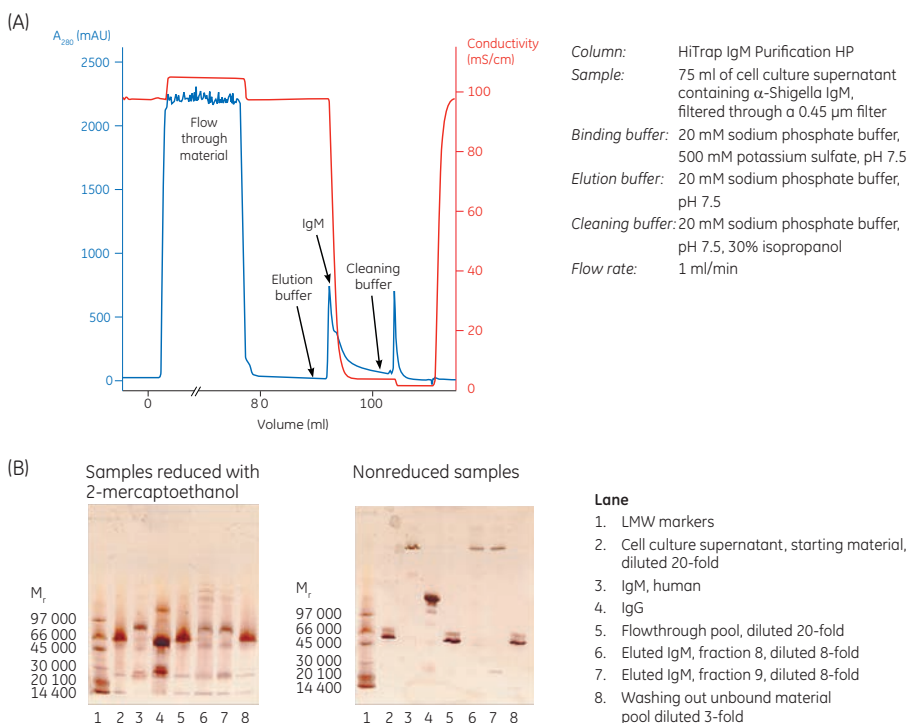



Fig 3.33. (A) Purification of α -Shigella IgM on HiTrap IgM Purification HP. (B) SDS-PAGE of sample, flowthrough, and eluted pools was performed under reducing (left gel) and nonreducing (right gel) conditions. SDS-PAGE was performed on PhastSystem using PhastGel 4–15, silver staining.


Sample preparation


Refer to Chapter 2 for general considerations.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 800 mM ammonium sulfate, pH 7.5
Elution buffer: 20 mM sodium phosphate, pH 7.5
Wash buffer: 20 mM sodium phosphate, pH 7.5 with 30% isopropanol

 The sample must have the same concentration of ammonium sulfate as in the binding buffer (0.8 M). Slowly add small amounts of solid ammonium sulfate to the sample from the hybridoma cell culture until the final concentration is 800 mM. Stir slowly and continuously. Pass the sample through a 0.45 µm filter immediately before applying it to the column. Some monoclonal IgM might not bind to the column at a concentration of 800 mM ammonium sulfate. Binding can be improved by increasing the ammonium sulfate concentration to 1 M.

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


 Ammonium sulfate can be replaced by 500 mM potassium sulfate. Most monoclonal IgM binds to the column in the presence of 500 mM potassium sulfate and the purity of IgM is comparable to the purity achieved with 800 mM ammonium sulfate.


Purification


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

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

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

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

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

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

Storage

Store in 20% ethanol at 2°C to 8°C.

IgY

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

Purifying IgY using HiTrap IgY Purification HP

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

Figure 3.34 shows the purification of α -Hb IgY from 45 ml of egg yolk extract (corresponding to one quarter of a yolk) and the SDS-PAGE analysis indicating a purity of over 70%.

Column: HiTrap IgY Purification HP
Sample: 45 ml of egg yolk extract (corresponding to 1/4 of an egg yolk) containing α -Hb IgY, filtered through a 0.45 μ m filter
Binding buffer: 20 mM sodium phosphate buffer, 500 mM potassium sulfate, pH 7.5
Elution buffer: 20 mM sodium phosphate buffer, pH 7.5
Cleaning buffer: 20 mM sodium phosphate buffer, pH 7.5, 30% isopropanol
Flow rate: 5 ml/min

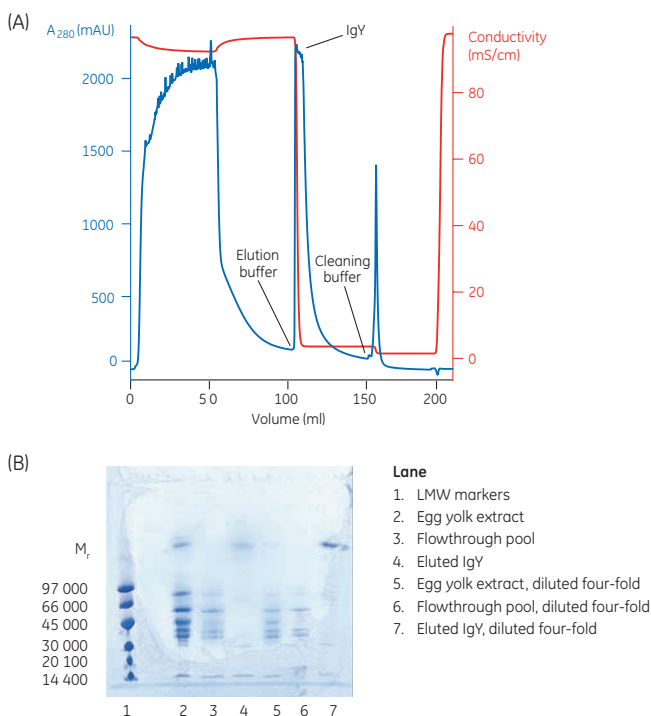


Fig 3.34. (A) Purification of IgY on HiTrap IgY Purification HP. (B) SDS-PAGE of nonreduced samples on PhastSystem using PhastGel 4–15, Coomassie staining.

Sample preparation

Refer to Chapter 2 for general considerations.



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

Buffer preparation

Binding buffer: 20 mM sodium phosphate, 500 mM potassium sulfate, pH 7.5

Elution buffer: 20 mM sodium phosphate, pH 7.5

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

To improve recovery of total IgY or a specific IgY antibody, replace 500 mM potassium sulfate with 600 to 800 mM sodium sulfate in the binding buffer. The sample should have the same concentration of sodium sulfate as the binding buffer. Using more than the recommended salt concentration in the binding buffer will reduce the purity of the eluted IgY.




1. Separate the egg yolk from the egg white.
2. Add nine parts of distilled water to one part egg yolk.
3. Mix and stir slowly for 6 h at 4°C.
4. Centrifuge at 10 000 × g, at 4°C for 25 min to precipitate the lipids.
5. Collect the supernatant containing the IgY.
6. Slowly add potassium sulfate to the sample, stirring constantly, to a final concentration of 500 mM.
7. Adjust pH to 7.5.
8. Pass the sample through a 0.45 µm filter immediately before applying it to the column.

Purification

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

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

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

-  The purity of the eluted IgY can be improved by using gradient elution with, for example, a linear gradient 0% to 100% elution buffer over 10 column volumes, followed by 100% elution buffer for several column volumes.
-  To increase binding capacity, connect several HiTrap IgY Purification HP columns in series. A HiTrap column can be used with a syringe, a peristaltic pump, or connected to a liquid chromatography system.
-  Reuse of HiTrap IgY Purification HP depends on the nature of the sample. To prevent cross-contamination, it should only be reused when processing identical samples.

Storage

Store in 20% ethanol at 2°C to 8°C.

Making immunospecific AC media with custom ligands

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

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

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

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




-  If no primary amine group on the ligand to be coupled is available, ligand attachment via carboxyl, thiol, or hydroxyl groups can be considered. These procedures are described in the handbook *Affinity Chromatography: Principles and Methods* (18102229).
-  A pure ligand is required that has a proven reversible high affinity for the target molecule. Using an antigen or an anti-antibody as a ligand will give a high degree of purification. If possible, test the affinity of the interaction.
-  Immunospecific interactions often require harsh elution conditions. Collect fractions into a neutralizing buffer, such as 60 to 200 µl 1 M Tris-HCl, pH 9.0 per milliliter fraction.

Figure 3.35 shows the partial purification of an IgE-stimulating factor from a human T-cell line, using IgE as the specific affinity ligand coupled to HiTrap NHS-activated HP 1 ml column. Figure 3.36 shows purification of anti-mouse Fc-IgG from sheep serum using mouse IgG₁ coupled to HiTrap NHS-activated HP 1 ml column.

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

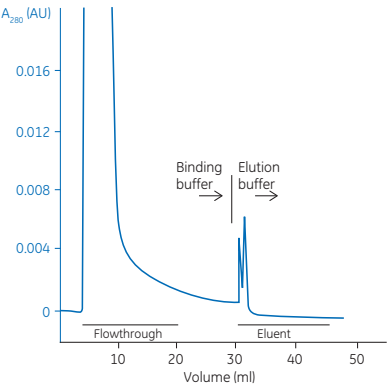


Fig 3.35. Purification of an IgE-stimulating factor from a human T-cell line.

Column: HiTrap NHS-activated HP 1 ml. Mouse IgG, (10 mg, 3.2 ml) was coupled in 200 mM sodium hydrogen carbonate, 500 mM sodium chloride, pH 8.3, room temp., recycled with a peristaltic pump for 1 h. Yield was 95% (9.5 mg).
Sample: 50 ml sheep anti-mouse Fc serum filtered 0.45 µm
Binding buffer: 75 mM Tris-HCl, pH 8.0
Elution buffer: 100 mM glycine-HCl, 500 mM sodium chloride, pH 2.7
Flow rate: 1.0 ml/min
Electro-phoresis: SDS-PAGE. PhastSystem. PhastGel Gradient 8–25 1 µl sample, Coomassie stained

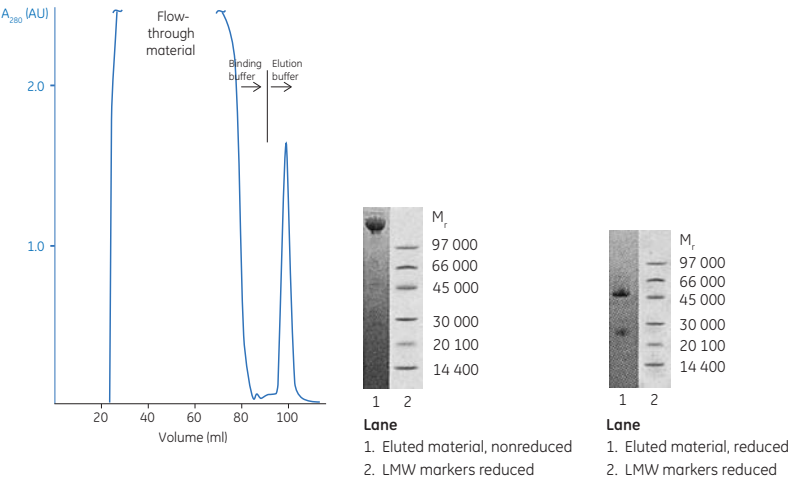


Fig 3.36. Purification of anti-mouse Fc-IgG from sheep antiserum.

Coupling ligands to HiTrap NHS-activated HP columns

The protocol below describes the preparation of a prepacked HiTrap NHS-activated HP column and a recommendation for a preliminary purification protocol. Many of these details are generally applicable to NHS-activated Sepharose chromatography media. Coupling can take place within the pH range of 6.5 to 9.0 with a maximum yield achieved at around pH 8.0.

A general column packing procedure is described in Appendix 5.

Buffer preparation

Acidification solution: 1 mM HCl (kept on ice)

Coupling buffer: 200 mM sodium hydrogen carbonate, 500 mM sodium chloride, pH 8.3



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



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

Ligand and HiTrap column preparation

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

Ligand coupling

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



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

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

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



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

Washing and deactivation

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

Buffer A: 500 mM ethanolamine, 500 mM sodium chloride, pH 8.3

Buffer B: 100 mM acetate, 500 mM sodium chloride, pH 4.0

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

Storage

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



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



Sodium azide is carcinogenic, handle with care.

Performing a purification on a coupled HiTrap NHS-activated column



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



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



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

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

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



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

Elution buffers

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

- Low pH (below pH 2.5)
- High pH (up to pH 11.0)
- Substances that reduce the polarity of a buffer can facilitate elution without affecting protein activity: dioxane (up to 10%), ethylene glycol (up to 50%)

Adding a polishing step after initial purification

One-step affinity purification generally achieves satisfactory purity of the target antibody. To achieve adequate homogeneity of the purified antibody, however, an additional polishing step using size exclusion chromatography (SEC) is recommended. This is described in section Dimers and aggregates in Chapter 4. This chapter also describes methods for removal of specific contaminants remaining from purification of IgG from native source or serum as well as cell culture.

Multistep purification strategies are described in Chapter 6.

Chapter 4

Removal of specific contaminants after initial purification

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

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

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



For some cell culture preparations, it is possible to decrease the level of serum during growth, thereby reducing or eliminating many of these impurities before purification. An alternative solution is to consider the use of a different host that does not require these supplements.



Select chromatography techniques that utilize differences in the characteristics of the contaminant and target molecule: ion exchange chromatography (IEX) for separation by differences in charge; hydrophobic interaction chromatography (HIC) for separation by differences in hydrophobicity; and size exclusion chromatography (SEC) for separation by size. See Appendix 9 for an overview of the principles of the chromatography techniques used at laboratory scale.



If the pI value of the antibody is sufficiently different from the contaminants, a cation exchange medium (negatively charged) can be used for removal of the contaminants at a pH above the pI of the impurities and below that of the antibody. This will ensure that the antibody (positively charged) binds to the column while the impurities, including negatively charged nucleic acids, pass through.

Bovine immunoglobulins

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

Difficulties have also been encountered when murine monoclonal antibodies are the target molecule. The similarities between the physical characteristics of the target antibody and the contaminants require careful selection and optimization to find the most suitable chromatography technique for purification. Both HIC and IEX can be used.



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

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

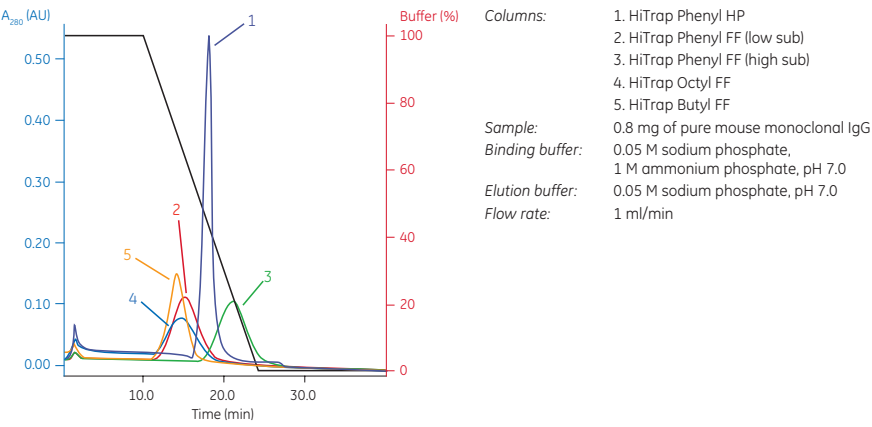


Fig 4.1. Screening of a mouse monoclonal antibody for optimal purification conditions using HiTrap HIC columns.

Albumin and transferrin

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



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



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

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

Removal of albumin using Blue Sepharose chromatography media

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



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



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



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

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

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

¹ Protein binding capacity varies for different proteins

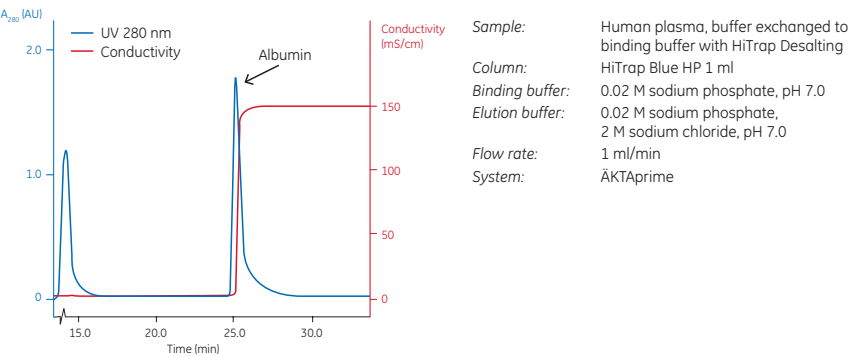


Fig 4.3. Effective removal of albumin from human plasma using a HiTrap Blue HP 1 ml column.

Figure 4.3 shows the removal of human serum albumin from plasma using HiTrap Blue HP 1 ml. The protocol for removal of albumin using HiTrap Blue HP 1 ml and 5 ml columns is described below.

Buffer preparation

Binding buffer: 20 mM sodium phosphate, pH 7.0, or 50 mM potassium hydrogen phosphate (KH_2PO_4), pH 7.0

Elution buffer: 0.02 M sodium phosphate, 2 M sodium chloride, pH 7.0, or 0.05 M potassium hydrogen phosphate, 1.5 M potassium chloride, pH 7.0

Albumin removal

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

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

Storage

Store in 20% ethanol at 2°C to 8°C.

α_2 -macroglobulin and haptoglobulin

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

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



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

Dimers and aggregates

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

SEC is one of the techniques for removing aggregates at laboratory scale and is used as the final polishing step in many purification strategies. A medium such as Superdex 200 Increase will give optimal separation between monomer and dimer.

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



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



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



For purification with larger sample volumes, use HiLoad™ 16/600 Superdex 200 pg or HiLoad 26/600 Superdex 200 pg prepacked columns.

Table 4.1 presents SEC products recommended for analysis and polishing of antibodies at lab scale.

Table 4.1. SEC products recommended for analysis and polishing of antibodies and antibody fragments at laboratory scale

Column	Bed dimensions diam. × height (mm)	Recommended sample volume	Suitable for
Superdex 200 Increase 10/300 GL	10 × 300	25 to 500 µl	High-resolution analysis/ small-scale polishing
Superdex 200 Increase 5/150 GL	5 × 150	4 to 50 µl	Purity check/rapid screening
Superdex 200 Increase 3.2/300	3.2 × 300	4 to 50 µl	High-resolution analysis/ microscale polishing
Superdex 75 10/300 GL	10 × 300	25 to 250 µl	High-resolution analysis/ small-scale polishing
Superdex 75 5/150 GL	5 × 150	4 to 50 µl	Purity check/rapid screening
Superdex 75 3.2/300	3.2 × 300	4 to 50 µl	High-resolution analysis/ microscale polishing
HiLoad 16/600 Superdex 200 pg	16 × 600	Up to 5 ml	Preparative scale (mg)
HiLoad 26/600 Superdex 200 pg	26 × 600	Up to 13 ml	Preparative scale (mg)
HiLoad 16/600 Superdex 75 pg	16 × 600	Up to 5 ml	Preparative scale (mg)
HiLoad 26/600 Superdex 75 pg	26 × 600	Up to 13 ml	Preparative scale (mg)

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

Column: Superdex 200 Increase 10/300 GL
Sample: Monoclonal antibody
Sample volume: 50 µl
Buffer: PBS (10 mM phosphate buffer, 140 mM sodium chloride, pH 7.4)
Flow rate: 0.5 ml/min
System: ÄKTA pure

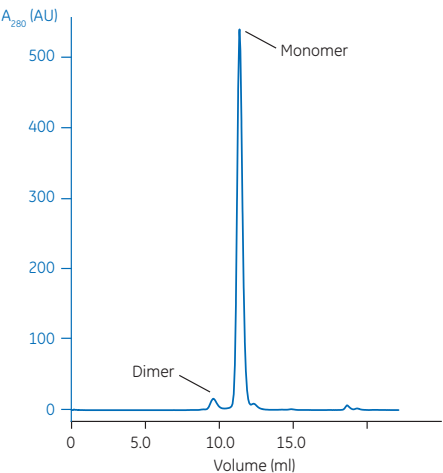


Fig 4.4. Separation of the monomer and dimer of a monoclonal antibody on Superdex 200 Increase 10/300 GL.

DNA and endotoxins

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



Nucleic acids often dissociate from proteins at high salt concentrations. This makes HIC a suitable technique for capturing the target protein and removing nucleic acids.



Since DNA and endotoxins are negatively charged over a wide pH interval, a cation exchange chromatography step at a pH below the isoelectric point of the antibody will bind the target protein and allow the negatively charged molecules to wash through the column. Consequently, if anion exchange is used as the initial capture step, these contaminants will be removed at an early stage in purification.



If endotoxins or DNA need to be removed from a purified product, anion exchange chromatography at using Canto Q or Canto adhere a pH value slightly below the isoelectric point of the antibody will bind the endotoxins and DNA while the antibody will wash through the column. Alternatively, use a pH that binds both molecular species, but allows them to be clearly separated during gradient elution from the column.

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

Affinity ligands

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

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

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

Column: HiTrap SP HP 1 ml
Sample: Purified antibody (0.61 mg) spiked with recombinant protein A (1.8 mg)
Binding buffer: 0.02 M sodium citrate, pH 5.2
Elution buffer: 0.02 M sodium citrate, 1 M sodium chloride, pH 5.2
Flow rate: 4 ml/min
Gradient: 0% to 45% elution buffer in 15 CV

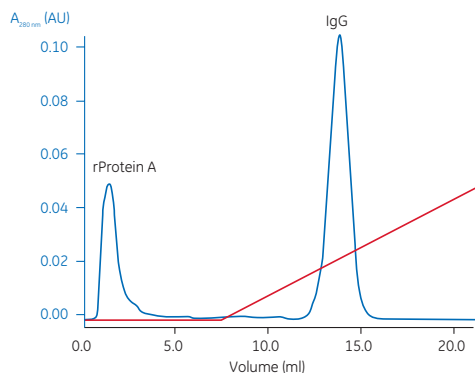


Fig 4.5. Removal of protein A from mouse IgG_{2b} by cation exchange chromatography on HiTrap SP HP. Recombinant protein A was spiked into mouse IgG_{2b} previously purified on rProtein A Sepharose Fast Flow.

Host cell proteins (HCP)

Proteins from the host cell can co-purify with proteins of interest, for example, Chinese Hamster Ovary (CHO) cell proteins in the manufacture of recombinant MAbs. The residual amount of HCP after the affinity chromatography step needs to be minimized to avoid potential immunogenicity reactions. GE offers several chromatography media suitable for HCP removal. Capto adhere and Capto adhere ImpRes are based on multimodal anion exchange ligands and can remove key contaminants such as DNA, HCP, leached protein A, dimers, larger aggregates, and viruses in a single step. See Chapter 7 for more information on large-scale purification.

Chapter 5

Automated purification of antibodies using ÄKTA chromatography systems

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

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

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



Fig 5.1. ÄKTA start.



Fig 5.2. ÄKTA avant.

Purification of antibodies can also be performed on more advanced chromatography systems. ÄKTA avant (Fig 5.2) is a system designed for fast and secure development of scalable methods and processes. It is the system of choice for developing large-scale antibody purification processes.

When a single affinity step does not yield the purity required for a specific application or when a buffer exchange or polishing step is required after the affinity step, multiple chromatography steps are needed. ÄKTApure or ÄKTApure (Fig. 5.3) are the systems to choose from when a higher level of automation is required. ÄKTApure has the smallest footprint, and 1 to 12 systems can be controlled in parallel by one computer. ÄKTApure delivers the highest possible throughput for purification of monoclonal antibodies, with no user intervention needed. Single or two-step purifications of up to four different antibodies can be performed automatically per run and system. ÄKTApure is a highly flexible system. Here multistep purifications can be performed in different ways and purification progress monitored using detectors such as multiple UV wavelengths, conductivity, and pH, as preferred. ÄKTApure also has larger and different fractionation options compared to ÄKTApure.

Other ÄKTA systems are also available for the purification of antibodies, see Table 5.1.



Fig 5.3. ÄKTApure single (left) and ÄKTApure chromatography systems.

Table 5.1. Standard ÄKTA protein purification systems

Way of working	ÄKTA start	ÄKTAprime plus	ÄKTAxpress	ÄKTA pure	ÄKTA avant	ÄKTApilot™	ÄKTA ready	ÄKTAprocess™
Scale								
Laboratory scale	•	•	•	•	•	-	-	-
Process development	-	-	-	(•)	•	•	-	-
Manufacturing and production	-	-	-	-	-	-	•	•
Type of work								
Template methods	•	•	•	•	•	w	-	-
Method development and scale-up	-	-	-	•	•	•	-	-
Automation								
Buffer preparation function	-	-	-	-	•	-	-	-
pH scouting	-	-	-	(•)	•	-	-	-
Chromatography media and column scouting	-	-	-	•	•	•	-	-
Multistep purification	-	(•)	•	•	(•)	-	-	-
Regulatory demands								
System control and data handling for regulatory requirement	-	-	•	•	•	•	•	•
Software								
UNICORN™	•	-	•	•	•	•	•	•
UNICORN start	•	-	-	-	-	-	-	-
PrimeView	-	•	-	-	-	-	-	-

- Fully supported
- (•) Partly supported
- Not recommended or not applicable
- w wizard

Chapter 6

Multistep purification strategies

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

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

Table 6.1. Characteristics of native IgG and IgM

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

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

Examples of multistep purification

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

Example 1: Two-step purification of mouse monoclonal IgG₁ using HiTrap rProtein A FF for the capture step

This example demonstrates the effectiveness of using a high selectivity affinity purification step for initial capture. In common with most antibody preparations, IgG aggregates and/or dimers can be present, which would therefore require a second purification step. To achieve highest purity, it is therefore essential to include an SEC polishing step.

Target molecule: Mouse monoclonal IgG₁.

Source material: Cell culture supernatant.

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

Capture

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

In contrast to other IgG subclasses, most mouse monoclonal antibodies of the IgG₁ subclass require a high salt concentration to bind to rProtein A. Figure 6.1 shows the results of a scouting experiment performed to define the optimal salt concentration for binding. Scouting is also used to select the optimal pH for elution of the monoclonal antibody (pH 4.5 was selected in this example, results not shown).



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

Column: HiTrap rProtein A FF 1 ml
Sample: Cell culture supernatant containing monoclonal IgG₁, 90 µl
Binding buffer: 0.1 M sodium phosphate, 0 to 3.5 M sodium chloride, pH 7.4
Elution buffer: 0.1 M sodium citrate, pH 3.0
Flow rate: 1 ml/min
System: ÄKTA system

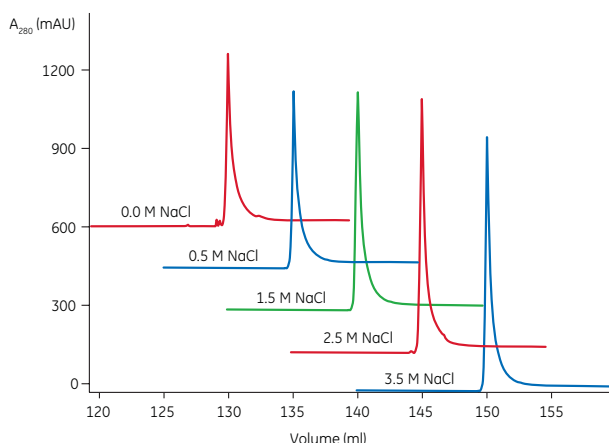


Fig 6.1. Automatic scouting of optimal sodium chloride concentration in the binding buffer on HiTrap rProtein A FF.

Optimization of binding and elution conditions gave a well-resolved peak containing IgG₁, as shown in Figure 6.2.

Column: HiTrap rProtein A FF 1 ml
Sample: Cell culture supernatant containing monoclonal IgG₁
(46 ml cell culture supernatant diluted to 100 ml with binding buffer)
Binding buffer: 100 mM sodium phosphate, 2.5 M sodium chloride, pH 7.4
Elution buffer: 100 mM sodium citrate, pH 4.5
Flow: 1 ml/min
System: ÄKTA system

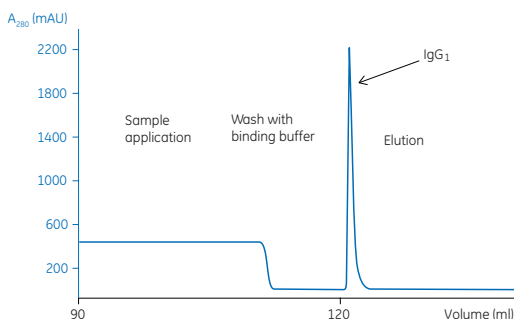


Fig 6.2. Optimized capture step on HiTrap rProtein A FF.

Intermediate purification

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

Polishing

HiLoad 16/600 Superdex 200 pg was used for the SEC polishing step to remove low or trace levels of contaminants, which in this case were IgG aggregates and/or dimers (Fig 6.3).

Column: HiLoad 16/600 Superdex 200 pg
Sample: Fraction from HiTrap rProtein A FF column containing monoclonal IgG₁ (3 ml)
Buffer: 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.4
Flow: 1 ml/min
System: ÄKTA system

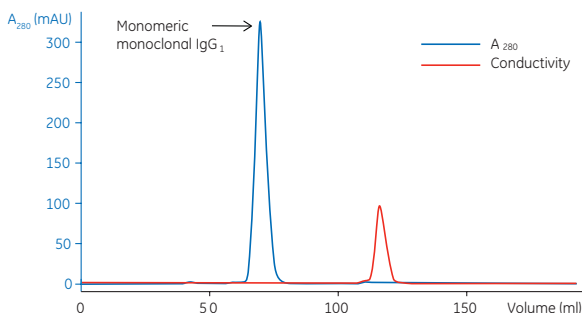


Fig 6.3. Polishing on HiLoad 16/600 Superdex 200 pg.



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

Yield and analysis

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

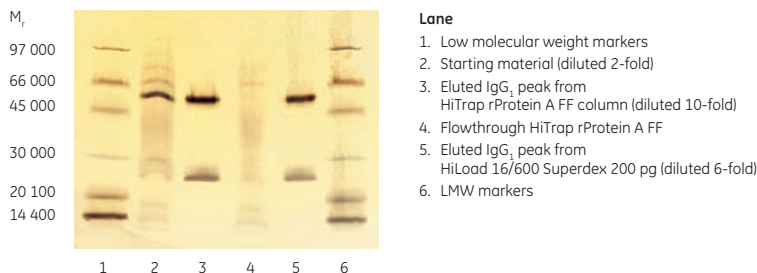


Fig 6.4. Purity analysis by SDS-PAGE, reducing conditions, using a PhastGel Gradient 10–15 gel on PhastSystem.

Example 2: Two-step purification of mouse monoclonal IgG₁ using HiTrap Protein G HP for the capture step

This case shows a purification method for mouse monoclonal IgG from cell culture supernatant using HiTrap Protein G HP for the initial capture. Polishing was performed in the second, SEC step on HiLoad 16/600 Superdex 200 pg. The capture and polishing steps were performed on ÄKTAprime plus. Monoclonal mouse IgG₁ was captured in the first step and eluted using a low pH buffer.

Target molecule: Mouse monoclonal IgG₁.

Source material: Cell culture supernatant.

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

Capture

Binding buffer: 20 mM potassium phosphate, pH 7.0

Elution buffer: 100 mM glycine-HCl, pH 2.7

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

Column: HiTrap Protein G HP 1 ml
Sample: 10 ml cell culture supernatant containing mouse monoclonal IgG₁
Binding buffer: 20 mM potassium phosphate, pH 7.0.
Elution buffer: 100 mM glycine-HCl, pH 2.7
Flow rate: 1 ml/min
System: ÄKTAprime plus

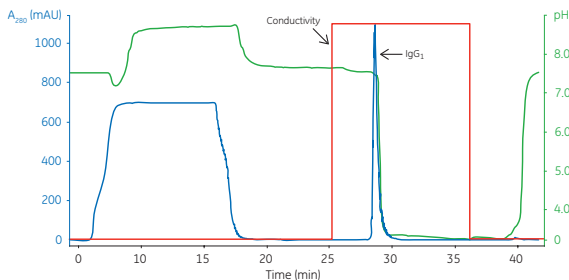


Fig 6.5. Capture step in a two-step purification of mouse monoclonal IgG₁ using HiTrap Protein G HP. The curves shown are absorbance (blue), pH (green), and conductivity (red).

Intermediate purification

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

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

Column: HiLoad 16/600 Superdex 200 pg
Sample: Pooled fractions from the capture step, 2 ml
Buffer: Phosphate buffered saline, pH 7.4
Flow rate: 1 ml/min
System: ÄKTAprime plus

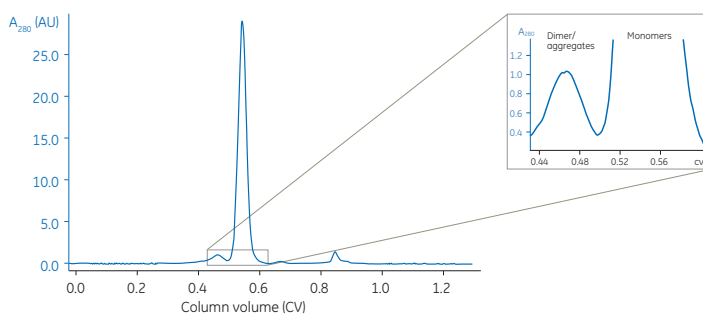


Fig 6.6. Note the separation between dimers and monomers (magnified).



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

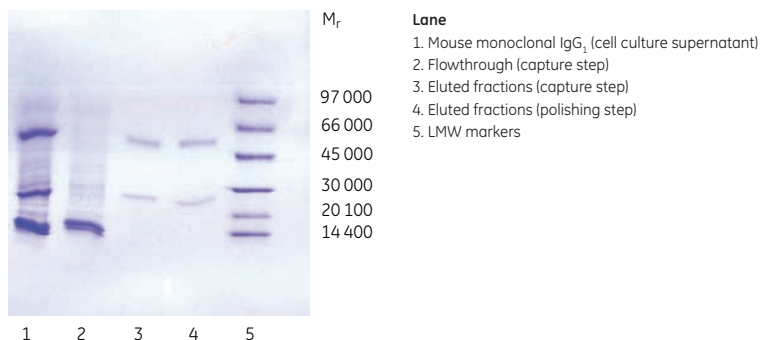


Fig 6.7. Purity analysis of mouse monoclonal IgG₁ by SDS-PAGE, reducing conditions.

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

Example 3: Unattended two-step purification of antibodies

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

Target molecule: Human monoclonal antibody.

Source material: Cell culture supernatant.

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

Binding buffer: 20 mM phosphate, 150 mM sodium chloride, pH 7.0

Elution buffer: 100 mM sodium citrate, pH 3.0

Desalting (DS) buffer: 50 mM phosphate buffer, 150 mM sodium chloride, pH 7.2

Columns: AC: HiTrap MabSelect SuRe 1 ml
DS: HiPrep 26/10 Desalting

Sample: 20 ml human monoclonal antibody in culture supernatant, ~ 0.5 mg/ml

Sample volume: 20 ml

Flow rate: 1 ml/min

System: ÄKTExpress

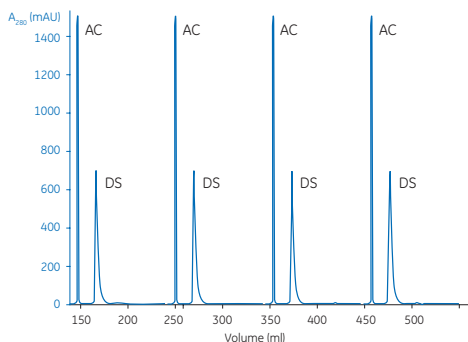


Fig 6.8. Chromatogram of four repetitive runs showing purification of human monoclonal antibody from cell culture by affinity chromatography (AC) and desalting (DS).

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

Example 4: Two-step purification of a mouse monoclonal IgG₁ for diagnostic use

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

Target molecule: Mouse monoclonal IgG₁ anti-IgE.

Source material: Hybridoma cell culture.

Clarification: Sample was filtered and ammonium sulfate added to 50 mM. This was to enhance binding to the HIC column, not to precipitate the monoclonal antibody.

Capture

HIC purification was chosen for the capture step because the antibody binds very strongly to the medium (Phenyl Sepharose High Performance) and most fetal calf serum proteins pass through the column as shown in Figure 6.9. The sample was concentrated into a smaller volume for polishing.



Screening of HIC media using HiTrap HIC Selection Kit is recommended to select the medium that gives optimal results. See *Ordering information* or refer to *Hydrophobic Interaction and Reversed Phase Chromatography: Principles and Methods*, 11001269 for more information.



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

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

Elution buffer: 20 mM potassium phosphate, pH 7.0

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

Column: HiLoad 16/10 Phenyl Sepharose HP
Sample: Hybridoma cell culture supernatant, mouse IgG₁, anti-IgE.
 Ammonium sulfate added to 500 mM final concentration
Binding buffer: 20 mM potassium phosphate, 500 mM ammonium sulfate, pH 7.0
Elution buffer: 20 mM potassium phosphate, pH 7.0
Flow rate: 3.3 ml/min
Gradient: 0% to 100% elution buffer, 10 CV

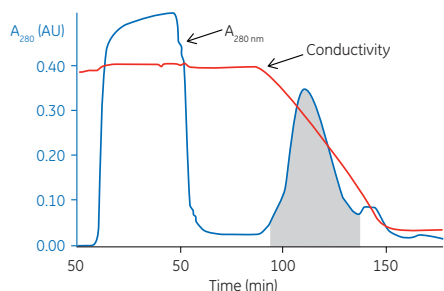


Fig 6.9. Capture of mouse IgG₁ on HiLoad 16/10 Phenyl Sepharose HP.

Intermediate purification

No intermediate step is required as the capture step gives a purity level > 95%.

Polishing

A final purity of > 99% was achieved using Superdex 200 prep grade (Fig 6.10).

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

Medium: Superdex 200 prep grade, 60 cm bed height

Sample: Fraction from HIC capture step (Fig 6.9)

Buffer: Phosphate buffered saline (PBS), pH 7.5

Flow rate: 15 ml/min

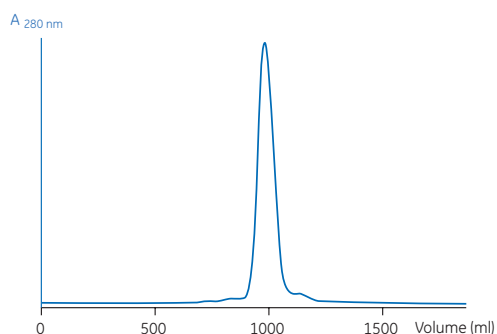


Fig 6.10. Polishing of mouse monoclonal IgG₁ anti-IgE using Superdex 200 prep grade.

Chapter 7

Large-scale purification

The clinical success of monoclonal antibodies (MAbs) is one of the most exciting achievements in the biopharmaceutical industry, resulting in annual production requirements of, in some cases, several tonnes – joining insulin and plasma proteins in sheer scale of bulk production. MAbs are currently the largest category of biotech drugs on the market and are still rapidly growing with an annual growth rate of just below 10%. The clinical pipeline is strong with hundreds of projects in different clinical phases. Several MAb biosimilars are also appearing on the market. Furthermore, MAbs are being used in new ways to expand the breadth of their therapeutic application. For example, antibody-drug conjugates are being used to improve the potency and reduce the side effects of traditional chemotherapy drugs. To meet this demand, cell culture capacity increased rapidly between 1995 and 2010, with some reactor volumes approaching 25 000 l. In addition, expression levels, currently in the range of 3 to 5 g/l have increased approximately 10 fold over the same period, and are likely to increase further as improvements in upstream cell culture continue. This will put additional demand on the development of downstream purification tools such as high-throughput chromatography media and process solutions.

Key concerns in large-scale purification (downstream processing) differ from those typical at laboratory scale; the emphasis in large-scale purification is developing robust and cost-effective protocols and decreasing the number of unit operations in order to improve overall process economy. Current trends in antibody production show that affinity chromatography using Protein A chromatography media (e.g., MabSelect chromatography media family) is the most cost-effective approach for capture of antibodies. MabSelect SuRe is one of the most well-established protein A chromatography media globally and is currently used in numerous FDA-approved monoclonal antibody processes.

Platform technologies in MAb purification

Platform technologies in MAb purification refers to a standard set of unit operations, conditions, and methods applied to the purification of molecules of a given class, in order to facilitate rapid and economical process development and scale-up. For MAb purification at large scale, the platform recommended by GE consists of a protein A-based capture step followed by one or two additional chromatography steps. The protein A step is a rapid, robust unit operation yielding highly pure MAbs (typically > 99%) with high recovery. Additional chromatography unit operations such as cation exchange chromatography (CIEX), anion exchange chromatography (AIEX), and hydrophobic interaction chromatography (HIC) can be considered for the intermediate and polishing steps. A suitable purification process can be designed based on properties of the individual MAb, such as pI, hydrophobicity, stability, glycosylation pattern, impurity profile, and tendency to form aggregates. However, the goal of a purification platform is to establish a generic process which works for a wide variety of MAbs, thereby eliminating the need for molecule-specific process development which is expensive and can slow time-to-market.

Affinity chromatography

The principles of affinity chromatography using protein A are discussed in Chapter 3.

Ion exchange chromatography

In general, a MAb has a higher pI than most host cell proteins (HCP). This gives a good opportunity to use ion exchange chromatography (IEX) for purification. A CIEX step can be designed for the MAb to bind to the chromatography medium (often described as bind-elute mode) while most impurities like DNA, endotoxins, and HCP flow through the column or are washed away with a washing buffer. Alternatively, an AIEX step can be designed for use under nonbinding conditions, allowing the MAb to pass in the flowthrough (often described as flowthrough mode), while impurities such as DNA, endotoxins, leached protein A ligand, and HCP remain bound to the medium. When used as a polishing step, the AIEX alternatives provide an advantage in terms of capacity since only impurities are adsorbed. In addition, AIEX is a generally good technique for removal of viruses.

Hydrophobic interaction chromatography

Many antibodies form dimers or aggregates, in particular at high expression levels. The aggregates are more hydrophobic and will bind more strongly to HIC media compared with the corresponding monomer. Therefore, HIC is an efficient tool for aggregate and dimer removal in flowthrough mode. Aggregates bind to the medium while the antibody passes straight through. HIC is also useful for removing HCP and endotoxins.

Multimodal chromatography media

New types of chromatography media that have a ligand with additional interaction mechanisms in combination with ion charges, commonly called multimodal chromatography media, are now also available. Capto adhere is one example of a multimodal medium that effectively removes DNA, viruses, endotoxins, leached protein A ligand, and HCP. Capto adhere is a multimodal strong anion exchanger, and is designed for operation in flowthrough mode for the MAb.

Multimodal chromatography media can also be operated in bind-elute mode for cases where a flowthrough step does not remove impurities such as antibody fragments. Capto adhere ImpRes is based on the same ligand as Capto adhere, but with a smaller particle size and hence higher resolution. Capto adhere ImpRes is well-suited to operation under binding (bind-elute mode) conditions.

Process design

In designing a platform technology for purification of MAbs, one must consider the properties of the antibodies to be purified, the process constraints presented by the facility, and the performance of the chromatography media available. Figure 7.1 provides an overview of several recommended platform designs using BioProcess chromatography media.

Usually, three chromatography unit operations are used, with Protein A as the first capture step (Fig 7.1, right-hand side). For an antibody with low aggregate level, the process often has two steps after affinity chromatography: CIEX or a multimodal medium in bind-elute mode and AIEX or a multimodal medium in flowthrough mode. For an antibody that has formed significant amounts of aggregates, both polishing steps may be performed in bind-elute mode to further increase aggregate removal. Alternatively, a well-optimized chromatography step with Capto adhere or Capto adhere ImpRes can often eliminate an entire step of the process, allowing the development of a two-step strategy (Fig 7.1, left-hand side). A two-step strategy decreases production costs significantly by reducing process time, buffer consumption, chromatography media costs, while at the same time increases product yield.

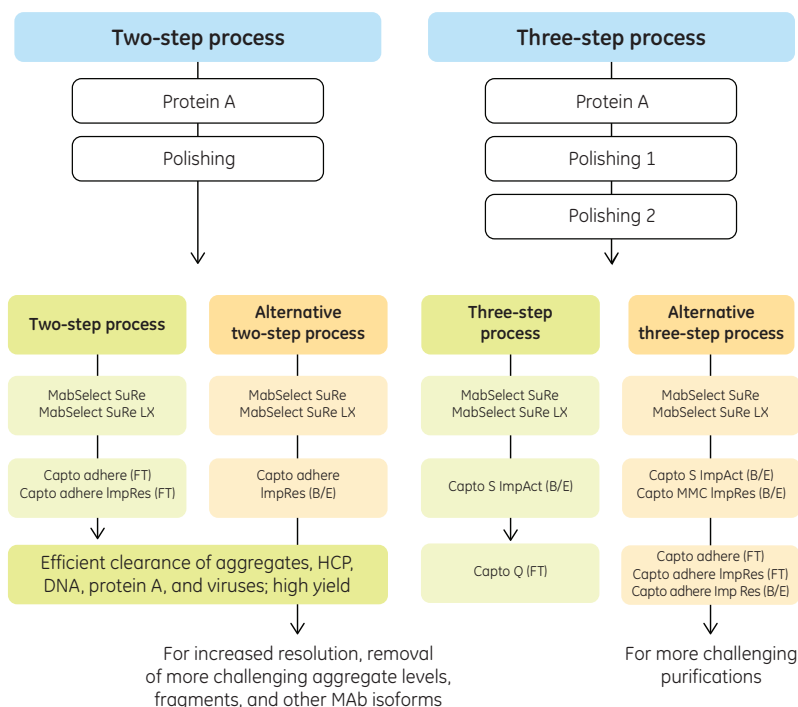


Fig 7.1. Overview of different combinations of chromatography unit operations in a MAb purification process.

High productivity chromatography media for MAb purification

GE has been the leading supplier of chromatography media for downstream processing of a broad range of biomolecules since the late 1950's. The latest additions to the portfolio include the MabSelect SuRe range of products, for MAb capture and the Capto family, which has been specifically designed to meet the increasing demand for high capacity and high throughput.

Table 7.1 briefly describes each of the products recommended for efficient and cost-effective large-scale purification of MAbs.

Table 7.1. Overview of recommended chromatography media for downstream purification of monoclonal antibodies

Purification step	Chromatography method	Product features
Capture		
MabSelect SuRe LX	Affinity (protein A)	High binding capacity. Increased alkali stability facilitates cleaning-in-place; high throughput and purity.
MabSelect SuRe	Affinity (protein A)	Increased alkali stability facilitates cleaning-in-place; high throughput and purity.
Intermediate purification and polishing		
Capto Q	Anion exchange	High capacity and throughput.
Capto S ImpAct	Cation exchange	High capacity, resolution, and throughput.
Capto adhere	Multimodal anion exchange	Enables a two-step process; removes host cell proteins, leached protein A ligand, and dimers/aggregates.
Capto adhere ImpRes	Multimodal anion exchange	Enables two-step process and well suited to be operated both in bind-elute and flowthrough mode.
Capto MMC ImpRes	Multimodal cation exchange	Designed for MAb purification, with a different selectivity and window of operation compared to traditional cation exchangers.
Capto Phenyl ImpRes	Hydrophobic interaction	High capacity and selectivity; removes dimers/aggregates.

Prepacked, disposable solutions speed up the downstream process

In addition to a wide range of industrial-scale columns, such as AxiChrom™ columns, and bulk chromatography media for purification of MABs, GE offers large-scale, disposable ReadyToProcess™ columns. These columns are prepacked, prequalified, and presanitized process chromatography columns available with a range of BioProcess chromatography media—including MabSelect SuRe and Capto product families. ReadyToProcess columns are available in different sizes (Fig 7.2), are ready-to-use, and the design makes them easy to connect to chromatography systems and to dispose of after completed production.

ReadyToProcess columns are designed for purification of biopharmaceuticals (e.g., proteins and antibodies, vaccines, plasmids, and viruses) for clinical phase I and II studies. Depending on the scale of operations, the columns can also be used for manufacturing, as well preclinical studies. As ReadyToProcess columns make column packing, column qualification, and sanitization redundant in the purification process, significant time savings can be achieved in the downstream process.



Fig 7.2. ReadyToProcess columns are easily connected to the system and can be disposed after completed production.

Custom Designed Media and columns

Custom Designed Media (CDM) can be produced for specific industrial process separations when suitable 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.gelifesciences.com/cdm for more information.

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.gelifesciences.com/custom-column-packing for more information.

Appendix 1

Products for antibody purification

Characteristics of protein G and protein A chromatography media

Tables A1.1 to A1.2 summarize key characteristics of bulk protein G and protein A Sepharose chromatography media.

Table A1.1. Characteristics of Protein G Sepharose chromatography media

Characteristics	Protein G Sepharose 4 Fast Flow	Protein G High Performance
Ligand	Recombinant protein G lacking albumin-binding region	Recombinant protein G lacking albumin-binding region
Ligand coupling method	Cyanogen bromide activation	N-hydroxysuccinimide activation
Matrix	Highly cross-linked agarose, 4%	Highly cross-linked agarose, 6%
Binding capacity	> 20 mg human IgG/ml medium	> 25 mg human IgG/ml medium
Average particle size	90 µm	34 µm
Ligand density	~ 2 mg protein G/ml medium	~ 2 mg protein G/ml medium
Recommended flow rate	50 to 300 cm/h	N/A
Chemical stability	Stable in all commonly used aqueous buffers	Stable in all commonly used aqueous buffers
pH stability ¹		
Long term	3 to 9	3 to 9
Short term	2 to 10	2 to 9
Storage	20% ethanol	20% ethanol
Storage temperature	2°C to 8°C	2°C to 8°C

¹ pH below 3.0 is sometimes required to elute strongly bound IgG species. Note that protein ligands can hydrolyze at very low pH.

Table A1.2. Characteristics of Protein A Sepharose chromatography media

Characteristics	nProtein A Sepharose 4 Fast Flow	rProtein A Sepharose 4 Fast Flow	Protein A High Performance
Ligand	Native protein A	Recombinant protein A (<i>E. coli</i>)	Native protein A
Ligand coupling method	Cyanogen bromide activation	Epoxy activation, thioether coupling	N-hydroxysuccinimide activation
Matrix	Highly cross-linked agarose, 4%	Highly cross-linked agarose, 6%	Highly cross-linked agarose, 6%
Binding capacity	> 30 mg human IgG/ml medium	> 50 mg human IgG/ml medium	> 20 mg human IgG/ml medium
Average particle size	90 µm	90 µm	34 µm
Ligand density	~ 6 mg native protein A/ml medium	~ 6 mg recombinant protein A/ml medium	~ 3 mg protein A/ml medium
Recommended flow rate	50 to 300 cm/h	50 to 300 cm/h	N/A
Chemical stability	Stable in all commonly used aqueous buffers	Stable in all commonly used aqueous buffers	Stable in all commonly used aqueous buffers
pH stability ¹			
Long term	3 to 9	3 to 10	3 to 9
Short term	2 to 10	1 to 11	2 to 9
Storage	20% ethanol	20% ethanol	20% ethanol
Storage temperature	2°C to 8°C	2°C to 8°C	2°C to 8°C

¹ pH below 3.0 is sometimes required to elute strongly bound IgG species. Note that protein ligands can hydrolyze at very low pH.

Characteristics of MabSelect chromatography media

Table A1.3 to A1.4 summarizes key characteristics of bulk MabSelect chromatography media.

Table A1.3. Characteristics of MabSelect and MabSelect Xtra

Characteristics	MabSelect	MabSelect Xtra
Ligand	Recombinant protein A (<i>E. coli</i>)	Recombinant protein A (<i>E. coli</i>)
Ligand coupling method	Epoxy activation	Epoxy activation
Matrix	Rigid, highly cross-linked agarose	Rigid, highly cross-linked agarose
Binding capacity	> 30 mg human IgG/ml medium	> 40 mg human IgG/ml medium
Average particle size	85 µm	75 µm
Recommended flow rate	100 to 500 cm/h	100 to 300 cm/h
Chemical stability	Stable in all aqueous buffers commonly used in protein A chromatography	Stable in all aqueous buffers commonly used in protein A chromatography
pH stability		
Long term	3 to 10	3 to 10
Short term	2 to 12	2 to 12
Storage	20% ethanol	20% ethanol
Storage temperature	2°C to 8°C	2°C to 8°C

Table A1.4. Characteristics of MabSelect SuRe and MabSelect SuRe LX

Characteristics	MabSelect SuRe	MabSelect SuRe LX
Ligand	Alkali-stabilized protein A-derived (<i>E. coli</i>)	Alkali-stabilized protein A-derived (<i>E. coli</i>)
Ligand coupling method	Epoxy activation	Epoxy activation
Matrix	Rigid, highly cross-linked agarose	Rigid, highly cross-linked agarose
Binding capacity	> 35 mg human IgG/ml medium	> 60 mg human IgG/ml medium
Average particle size	85 µm	85 µm
Recommended flow rate	100 to 500 cm/h	< 500 cm/h
Chemical stability	Stable in all aqueous buffers commonly used in protein A chromatography	Stable in all aqueous buffers commonly used in protein A chromatography
pH stability		
Long term	3 to 12	3 to 12
Short term	2 to 14	2 to 14
Storage	20% ethanol	20% ethanol
Storage temperature	2°C to 8°C	2°C to 8°C

Thiophilic adsorption AC media

HiTrap IgY Purification HP and HiTrap IgM Purification HP are packed with a thiophilic adsorption medium, 2-mercaptopyridine coupled to Sepharose High Performance. Table A1.5 summarizes the characteristics of 2-mercaptopyridine chromatography media used for purification of IgY and IgM, respectively.

Table A1.5. Characteristics of HiTrap IgY Purification HP and HiTrap IgM Purification HP

Characteristics	HiTrap IgY Purification HP	HiTrap IgM Purification HP
Ligand	2-mercaptopyridine	2-mercaptopyridine
Ligand density	~ 3 mg/ml medium	~ 2 mg/ml medium
Matrix	Highly cross-linked agarose, 6%	Highly cross-linked agarose, 6%
Medium	2-mercaptopyridine Sepharose High Performance	2-mercaptopyridine Sepharose High Performance
Binding capacity	100 mg pure IgY or ¼ egg yolk/5 ml column	5 mg human IgM/ml medium
Average particle size	34 µm	34 µm
Recommended flow rate	5 ml/min	1 ml/min
Maximum flow rate	20 ml/min	4 ml/min
pH stability ¹		
Long term	3 to 11	3 to 11
Short term	2 to 13	2 to 13
Column volume	5 ml	1 ml
Storage	20% ethanol	20% ethanol
Storage temperature	2°C to 8°C	2°C to 8°C

¹ pH below 3.0 is sometimes required to elute strongly bound antibody species. Note that protein ligands can hydrolyze at very low pH.

Characteristics of Capto L and Lambda FabSelect

The characteristics of Capto L and Lambda FabSelect chromatography media are shown in Table A1.6.

Table A1.6. Characteristics of Capto L and Lambda FabSelect

Characteristics	Capto L	Lambda FabSelect
Ligand	Recombinant protein L (<i>E. coli</i>), mammalian free	Recombinant protein (M _r 13 000), produced in <i>S. cerevisiae</i> , with affinity for the constant domain of the immunoglobulin lambda light chain
Matrix	Rigid, highly cross-linked agarose	Rigid, highly cross-linked agarose
Dynamic binding capacity	Approx 25 mg human Fab/ml medium ¹	Approximately 20 mg Fab/ml of medium ³
Average particle size	85 µm	75 µm
Recommended flow rate	<500 cm/h ²	Max 600 cm/h ⁴
Chemical stability	Stable in all commonly used aqueous buffers	Stable in all commonly used aqueous buffers
pH stability		
Long term	2 to 10	2 to 10
Short term	2 to 12	2 to 12
Storage	20% ethanol	20% ethanol
Storage temperature	2°C to 8°C	2°C to 8°C

¹ Dynamic binding capacities were measured at 10% breakthrough with a residence time of 4 min.

² At bed height 20 cm.

³ Dynamic binding capacity at 10% breakthrough measured in a Tricorn 5/50 column, 5 cm bed height, 4 min residence time (75 cm/h) for polyclonal Fab lambda reagent in PBS, pH 7.4.

⁴ Pressure/flow Specification: 300 kPa at 600 cm/h, 1 m diameter column, 20 cm bed height.

Products for antibody purification

Table A1.7 summarizes products for antibody purification.

Table A1.7. Summary of products for antibody purification with references to product data files

Product	Medium volume	Approx. protein binding capacity	High-throughput screening	Minipreps	Batch/gravity flow	Syringe compatible	Prepacked columns, AKTA compatible	Process development	Data file code number
HiTrap Protein G HP	1 ml 5 ml	25 mg/column 125 mg/column				• •	• •		11003558
Ab SpinTrap	100 µl	1 mg/column		•					28902030
Protein G HP SpinTrap	100 µl	1 mg/column		•					28906790
Protein G HP MultiTrap	50 µl	0.5 mg/well	•						28906790
MABTrap Kit ¹	1 ml	25 mg/column				•			18103414
Protein G Sepharose 4 Fast Flow	5 ml to 5 l	20 mg/ml	•	•	•				18101291
Protein G GraviTrap	1 ml	20 mg/column		•	•				28992104
Protein G Mag Sepharose Xtra ²	200 µl/500 µl	27 mg/ml	•	•					28976801
HiTrap Protein A HP	1 ml 5 ml	20 mg/column 100 mg/column				• •	• •		11003558
Protein A HP SpinTrap	100 µl	1 mg/column		•					28906789
Protein A HP MultiTrap	50 µl	0.5 mg/well	•						28906789
rProtein A Sepharose Fast Flow	5 ml to 60 l	50 mg/ml	•	•	•				18111394
HiTrap rProtein A FF	1 ml 5 ml	50 mg/column 250 mg/column				• •	• •		11003558
rProtein A GraviTrap	1 ml	50 mg/column		•	•				28992104
rProtein A/Protein G GraviTrap	1 ml	35 mg/column		•	•				28992104
nProtein A Sepharose 4 Fast Flow	5 ml to 10 l	30 mg/ml							18112519
Protein A Mag Sepharose Xtra ²	100 µl	27 mg/ml	•	•					28976801
MabSelect	From 25 ml	30 mg/ml						•	18114994
HiTrap MabSelect	1 ml 5 ml	30 mg/column 150 mg/column					• •	• •	11003490
HiScreen MabSelect	4.7 ml	140 mg/column					•	•	28930581
PreDicator MabSelect	6 µl 20 µl 50 µl	N/A ³ N/A ³ N/A ³	• • •					• • •	28925839
MabSelect SuRe	From 25 ml	30 mg/ml						•	11001165
HiTrap MabSelect SuRe	1 ml 5 ml	30 mg/column 150 mg/column					• •	• •	11003490
HiScreen MabSelect SuRe	4.7 ml	140 mg/column					•	•	28930581
PreDicator MabSelect SuRe	6 µl 20 µl 50 µl	N/A ³ N/A ³ N/A ³	• • •					• • •	28925839
MabSelect Xtra	From 25 ml	40 mg/ml						•	11001157
HiTrap MabSelect Xtra	1 ml 5 ml	40 mg/column 200 mg/column					• •	• •	11003490
HiScreen MabSelect Xtra	4.7 ml	188 mg/column					•	•	28930581
PreDicator MabSelect Xtra	6 µl 20 µl 50 µl	N/A ³ N/A ³ N/A ³	• • •					• • •	28925839
MabSelect SuRe LX	From 25 ml	60 mg/ml					•	•	28987062
HiScreen MabSelect SuRe LX	4.7 ml	282 mg/column					•	•	28930581
PreDicator MabSelect SuRe LX	6 µl 20 µl 50 µl	N/A ³ N/A ³ N/A ³	• • •					• • •	28925839

¹ Includes buffer stock solutions for approximately 20 purifications using a syringe.


² Paramagnetic beads.

³ The products are used for high-throughput screening of chromatographic conditions (i.e., capacity, selectivity, purity).

Appendix 2

Analytical assays during purification

Analytical assays are essential to follow the progress of purification. They are used to assess the effectiveness of each step in terms of yield, biological activity, 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 2x 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 A2.1).

Table A2.1. 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 below.

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.gelifesciences.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.gelifesciences.com/westernblotting. For further 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 GE.

Appendix 3

Immunoprecipitation techniques

Target proteins can be isolated and enriched from crude cell lysates by immunoprecipitation (also known as immunoaffinity or pull-down techniques). An antibody selected for its specificity is first affinity captured onto Protein A Sepharose or Protein G Sepharose chromatography media. In a second step, the immobilized antibody is used for capture and enrichment of the protein of interest (i.e., antigen). The target protein can be enriched several hundredfold, depending of the specificity of the antibody. In combination with other techniques, such as SDS-PAGE and immunoblotting, immunoprecipitation can be used to detect and quantitate antigens, determine relative molecular weights, monitor protein turnover and post-translational modifications, and check for enzyme activity.

By using the high specificity of protein A and protein G for the Fc regions of IgG molecules from a wide range of mammalian species, Protein A Sepharose and Protein G Sepharose chromatography media offer effective and rapid isolation and enrichment of such immune complexes.

Immunoprecipitation Starter Pack (Fig A3.1) from GE is a good starting point for immunoprecipitation work. The pack includes nProtein A Sepharose 4 Fast Flow (2 ml) and Protein G Sepharose 4 Fast Flow (2 ml) to enable work with a wide range of antibody species and selection of the optimal medium.



Fig A3.1. Immunoprecipitation Starter Pack for immunoprecipitation of a wide range of antibodies with different binding selectivities.

Protein G Mag Sepharose and Protein A Mag Sepharose (Fig A3.2) combine well-proven enrichment methods with the magnetic beads platform, which has excellent properties for small-scale sample preparation. Together with MagRack 6, a separation tool for six microcentrifuge tubes, up to six samples can be processed in parallel. Alternatively, Mag Rack Maxi can handle larger sample volumes of up to 50 ml.



Fig A3.2. Protein A or Protein G Mag Sepharose (left image) offer simple enrichment of target protein in small or large sample volumes (low microliter to high milliliter scale) by use of MagRack 6 or MagRack Maxi (center and right images).

Procedures for immunoprecipitation must often be optimized empirically to obtain satisfactory results. For example, the choice of cell lysis conditions is critical with regard to cell type and how the antigen is to be used. Whereas cells without cell walls (e.g., animal cells) are easily disrupted by treatment with mild detergent, other cells might need some type of mechanical shearing, such as sonication or Dounce homogenization.

☞ Refer to Table 3.1 in Chapter 3 to see which medium is likely to be suitable for the antibody source and subtype, or test using Immunoprecipitation Starter Pack.

Cell lysis conditions

Cell lysis must be harsh enough to release the target antigen, but mild enough to maintain its immunoreactivity. Some commonly used lysis buffers are listed in Table A3.1.

NP-40 (IGEPAL™ CA-630) and RIPA buffer release most soluble cytoplasmic or nuclear proteins without releasing chromosomal DNA and are a good choice for initial experiments.

☞ Parameters that affect the extraction of an antigen include salt concentration (0 to 1 M), nonionic detergents (0.1% to 2%), ionic detergents (0.01% to 0.5%), and pH (6.0 to 9.0).

Table A3.1. Common lysis buffers

Buffers and solutions	Contents	Ability to disrupt cells
Lysis buffers		
Low salt	1% IGEPAL CA-630, 50 mM Tris, pH 8.0, 1 mM phenylmethylsulfonyl fluoride (PMSF)	+
NP-40 (IGEPAL CA-630)	150 mM sodium chloride, 1% IGEPAL CA-630, 50 mM Tris, pH 8.0, 1 mM PMSF	++
RIPA	150 mM sodium chloride, 1% IGEPAL CA-630, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM PMSF	+++
High salt	500 mM sodium chloride, 1% IGEPAL CA-630, 50 mM Tris, pH 8.0, 1 mM PMSF	++++
Other buffers and solutions		
PBS	1 mM potassium hydrogen phosphate, 10 mM disodium hydrogen phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4	
Wash buffer	50 mM Tris, pH 8.0	
Sample buffer (reducing)	1% SDS, 100 mM dithiothreitol (DTT), 50 mM Tris, pH 7.5	

Choice of antibody

Polyclonal serum contains antibodies against multiple epitopes of an antigen. These antibodies help to stabilize the antigen-antibody-medium complexes, but can also create problems with high background during analysis.

Monoclonal antibodies (MAbs) are more specific, which reduces background, but can lead to the formation of less stable immune complexes due to lower affinity. This can be overcome by using pools of different MAbs.

Protein enrichment



Fig A3.3. Protein A HP SpinTrap spin columns and Protein A HP MultiTrap 96-well plates for the enrichment of proteins from a variety of biological samples.

GE protein A and G products (Protein A HP SpinTrap, Protein G HP SpinTrap, Protein A HP MultiTrap, and Protein G HP MultiTrap, as well as Protein G and Protein A Mag Sepharose) are designed for small-scale protein enrichment, for example for use upstream of gel electrophoresis, liquid chromatography, and mass spectrometry (Fig A3.3). There are two alternative protocols for protein enrichment using these products: the cross-link protocol and classic protocol.

In the cross-link protocol, antigen-capturing antibodies are covalently bound to the Protein A or Protein G Sepharose chromatography media by a cross-linking agent. The antigen is enriched from the sample, purified through washings, and eluted from the column whereas the antibody remains bound to the matrix.



Use the cross-link protocol if the desired protein/antigen has similar molecular weight as the heavy or light chain of the antibody, which causes problems with co-migration in SDS-PAGE analysis or if the antibody interferes with downstream analysis.

In the classic protocol, antigen-capturing antibodies are immobilized by binding to protein A or protein G coupled to Protein A or Protein G Sepharose chromatography medium, respectively. The bound antibody is then used for capture of the antigen of interest. The classic protocol requires that the capturing antibody used binds to protein A or protein G. The antigen of interest is enriched from the sample, purified through washings and eluted from the column or the magnetic bead media together with the antibody.

The optimal parameters for protein enrichment are dependent on the specific antibody-antigen combination. Optimization can be required for each specific antibody-antigen combination to obtain the desired results. Examples of parameters that might require optimization are sample pretreatment, amount of protein to be enriched, incubation time, choice of buffers, and number of washes.

For complete protocols for immunoprecipitation using Immunoprecipitation Starter Pack or protein enrichment using Protein A and Protein G HP SpinTrap, MultiTrap, or Mag Sepharose, please refer to the instructions for each respective product or download at www.gelifesciences.com.

Appendix 4

General instructions for affinity purification using HiTrap columns

Alternative 1. Manual purification with a syringe



Fig A4.1. Using HiTrap columns with a syringe. (A) Prepare buffers and sample. Remove the column's top cap and twist off the end. (B) Equilibrate the column, load the sample and begin collecting fractions. (C) Wash and elute, continuing to collect fractions.

1. Fill the syringe with binding buffer. Remove the stopper and connect the column to the syringe (use the connector supplied) "drop to drop" to avoid introducing air into the column.
2. Remove the snap-off end at the column outlet.
3. Equilibrate the column with 5 column volumes of binding buffer.
4. Apply the pretreated sample using a syringe fitted to the Luer connector on the column. For optimal results, use a flow rate of 0.2 to 1 ml/min (1 ml column) and 0.5 to 5 ml/min (5 ml column) during sample application*.
5. Wash with 5 to 10 column volumes of binding buffer or until no material appears in the effluent. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for washing. Optional: collect the flowthrough (in 1 ml fractions for the 1 ml column and 2 ml fractions for the 5 ml column) and reserve until the procedure has been successfully completed. Retain a sample for analysis by SDS-PAGE to measure the efficiency of protein binding to the medium.
6. Elute with 5 to 10 column volumes of elution buffer. Maintain a flow rate of 0.2 to 1 ml/min (ml column) and 0.5 to 5 ml/min (5 ml column) for elution.
7. After elution, regenerate the column by washing it with 3 to 5 column volumes of binding buffer. The column is now ready for a new purification.

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



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

Alternative 2. Simple purification with ÄKTAprime plus

ÄKTAprime plus contains preprogrammed templates for purification of IgG, IgM, and IgY using the appropriate HiTrap columns.



Prepare at least 500 ml of each buffer.

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

(A)



(B)



(D)



(C)



Fig A4.2. Typical procedures using ÄKTAprime plus. (A) Prepare the buffers. (B) Connect the column. (C) Prepare the fraction collector. (D) Load the sample.

Appendix 5

Column packing and preparation

Prepacked columns from GE 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 GE (Fig A5.1).



Fig A5.1. 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 GE 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/adaptor 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 A5.2.

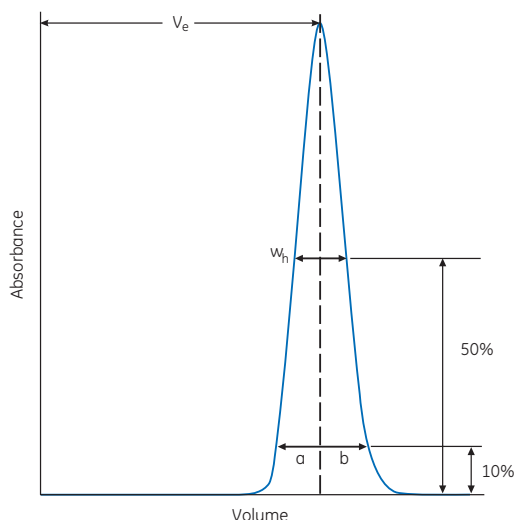


Fig A5.2. Measurements taken to calculate column efficiency.

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.gelifesciences.com/custom-column-packing for more information.

Column selection

Tricorn, XK, and HiScale columns are fully compatible with the high flow rates allowed by with modern media, and a broad range of column dimensions are available (see Table A5.1). In most cases the binding capacity of the medium and the amount of sample to be purified will determine the column size required. Also, Empty Disposable PD-10 Columns are available for single-use applications using gravity flow. For a complete listing of available columns, refer to www.gelifesciences.com.

Table A5.1. Column bed volumes and heights¹

	Column size		Bed volume (ml)	Bed height (cm)
	i.d. (mm)	Length		
Tricorn 5/20	5	20 mm	0.3 to 0.5	1.6 to 2.8
Tricorn 5/50	5	50 mm	0.9 to 1.1	4.6 to 5.8
Tricorn 10/20	10	20 mm	1.2 to 2.2	1.6 to 2.8
Tricorn 10/50	10	50 mm	3.6 to 4.5	4.6 to 5.8
Tricorn 10/100	10	100 mm	7.5 to 8.5	9.6 to 10.8
XK 16/20	16	20 cm	5.0 to 31.1	2.5 to 15.5
XK 16/40	16	40 cm	45.2 to 70.3	22.5 to 35.0
XK 26/20	26	18 cm	5.3 to 66.3	1.0 to 12.5
XK 26/40	26	40 cm	122.1 to 185.7	23.0 to 35.0
XK 50/20	50	18 cm	0 to 274.8	0 to 14.0
XK 50/30	50	30 cm	274.8 to 549.5	14.0 to 28.0
HiScale 16/20	16	200 mm	0 to 40.2	0 to 20.0
HiScale 16/40	16	400 mm	16.1 to 80.4	8.0 to 40.0
HiScale 26/20	26	200 mm	0 to 106.1	0 to 20.0
HiScale 26/40	26	400 mm	69.0 to 212.3	13.0 to 40.0
HiScale 50/20	50	200 mm	0 to 392.5	0 to 20.0
HiScale 50/40	50	400 mm	274.8 to 785.0	14.0 to 40.0
Empty Disposable PD-10 ²	15	7.4 cm	8.3	4.8 to 5.0

¹ Tricorn and XK, column specifications apply when one adapter is used. For HiScale, column specifications apply when two adapters are used.

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

Cleaning of Protein G and Protein A Sepharose media

After purification, the medium should be regenerated as follows:

1. After elution, wash with 2 to 3 column volumes of elution buffer.
2. Immediately re-equilibrate by washing with 2 to 3 column volumes of binding buffer.

Cleaning-in-place



When an increase in backpressure is seen, the medium should be cleaned. In some applications, substances like denatured proteins or lipids do not elute in the regeneration procedure.

To remove precipitated or denatured proteins:

1. Wash the medium with 2 column volumes of 6 M guanidine hydrochloride.
2. Immediately wash with at least 5 column volumes of binding buffer.

To remove strongly bound hydrophobic proteins, lipoproteins and lipids:

1. Wash with a nonionic detergent, for example, 0.1% Triton X-100 at 37°C for 1 min.
2. Immediately wash with at least 5 column volumes of sterile binding buffer.
- 2a. Alternatively, wash the column with 70% ethanol and let it stand for 12 h. After treatment, wash with at least 5 column volumes of binding buffer.



Reversed flow can improve the efficiency of the cleaning-in-place procedure. After cleaning, store in 20% ethanol.



Washing with 70% ethanol will increase backpressure. Use a lower flow rate when cleaning with 70% ethanol.

Cleaning of MabSelect media

All MabSelect media can be cleaned using the following procedures:

To remove precipitated or denatured substances:

1. Wash the medium with 2 column volumes of 50 mM sodium hydroxide in 500 mM sodium sulfate, or 50 mM sodium hydroxide in 1 M sodium chloride, or 100 mM sodium phosphate, or 6 M guanidine hydrochloride in 10 mM sodium hydroxide. Contact time: at least 10 min.
2. Immediately wash with at least 5 column volumes of sterile filtered binding buffer at pH 7.0 to 8.0.

MabSelect SuRe and MabSelect SuRe LX are alkali-tolerant, allowing the use of more concentrated solutions of sodium hydroxide:

1. Wash with 3 column volumes of binding buffer.
2. Wash with at least 2 column volumes of 100 mM to 500 mM sodium hydroxide. Contact time: 10 to 15 min.
3. Immediately wash with at least 5 column volumes of sterile and filtered binding buffer at pH 7.0 to 8.0.

To remove strongly bound hydrophobic proteins, lipoproteins, and lipids:

1. Wash with 2 column volumes of a nonionic detergent (e.g., 0.1% solution).
2. Immediately wash with at least 5 column volumes of sterile filtered binding buffer at pH 7.0 to 8.0.
- 2a. Alternatively, wash with 3 to 4 column volumes of 70% ethanol or 30% 2-propanol. Wash immediately with at least 5 column volumes of sterile filtered binding buffer at pH 7.0 to 8.0. Apply increasing gradients to avoid air bubble formation when using high concentrations of organic solvents.



Washing with 70% ethanol and 30% 2-propanol will increase backpressure. Use a lower flow rate when cleaning with 70% ethanol or 30% 2-propanol.

Appendix 6

Storage of biological samples



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

General recommendations

- Add stabilizing agents, when necessary. Stabilizing agents are often required for storage of purified proteins.
- Serum, culture supernatants, and 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 IgG₃ 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.

Appendix 7

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)

$$\begin{aligned}\text{Volumetric flow rate (ml/min)} &= \frac{\text{Flow velocity (cm/h)}}{60} \times \text{column cross sectional area (cm}^2\text{)} \\ &= \frac{Y}{60} \times \frac{\pi \times d^2}{4}\end{aligned}$$

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

$$\begin{aligned}\text{Volumetric flow rate} &= \frac{150 \times \pi \times 1.6 \times 1.6}{60 \times 4} \text{ ml/min} \\ &= 5.03 \text{ ml/min}\end{aligned}$$

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

$$\begin{aligned}\text{Flow velocity (cm/h)} &= \frac{\text{Volumetric flow rate (ml/min)} \times 60}{\text{column cross sectional area (cm}^2\text{)}} \\ &= Z \times 60 \times \frac{4}{\pi \times d^2}\end{aligned}$$

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 8

Conversion data: proteins, column pressures

Proteins

Mass (g/mol)	1 μ g	1 nmol	Protein	A ₂₈₀ for 1 mg/ml
10 000	100 pmol; 6×10^{13} molecules	10 μ g	IgG	1.35
50 000	20 pmol; 1.2×10^{13} molecules	50 μ g	IgM	1.20
100 000	10 pmol; 6.0×10^{12} molecules	100 μ g	IgA	1.30
150 000	6.7 pmol; 4.0×10^{12} molecules	150 μ g	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 9

Principles and standard conditions for different purification techniques

Affinity chromatography (AC)

AC separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand attached to a chromatographic matrix. The technique is well-suited for a capture or intermediate step and can be used whenever a suitable ligand is available for the protein(s) of interest. AC offers high selectivity, hence high resolution, and usually high capacity. Affinity chromatography is frequently used as the first step (capture step) of a two-step purification protocol, followed by a second chromatographic step (polishing step) to remove remaining impurities.

The target protein(s) is/are specifically and reversibly bound by a complementary binding substance (ligand). The sample is applied under conditions that favor specific binding to the ligand. Unbound material is washed away, and bound target protein is recovered by changing conditions to those favoring desorption. Desorption is performed specifically, using a competitive ligand, or nonspecifically, by changing the pH, ionic strength, or polarity. Samples are concentrated during binding, and the target protein is collected in purified and concentrated form. The key stages in an affinity chromatography separation are shown in Figure A9.1. AC is also used to remove specific contaminants; for example, Benzamidine Sepharose 4 Fast Flow can remove serine proteases.

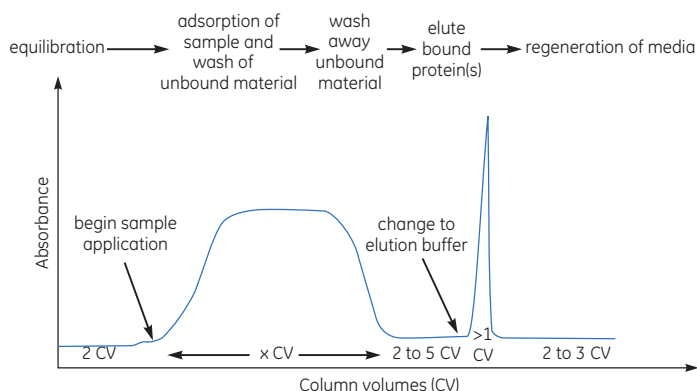


Fig A9.1. Typical affinity purification.

Further information

Strategies for Protein Purification Handbook, 28983331.

Affinity Chromatography Handbook, Principles and Methods, 18102229.

Chapter 3 in this handbook for the purification of antibodies.

Ion exchange chromatography (IEX)

IEX separates proteins with differences in surface charge to give a very high resolution separation with high sample loading capacity. The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatography medium.

Proteins bind as they are loaded onto a column. Conditions are then altered so that bound substances are eluted differentially. Elution is usually performed by increasing salt concentration or changing pH. Changes are made stepwise or with a continuous gradient. Most commonly, samples are eluted with salt (NaCl), using a gradient elution (Fig A9.2). Target proteins are concentrated during binding and collected in a purified, concentrated form.

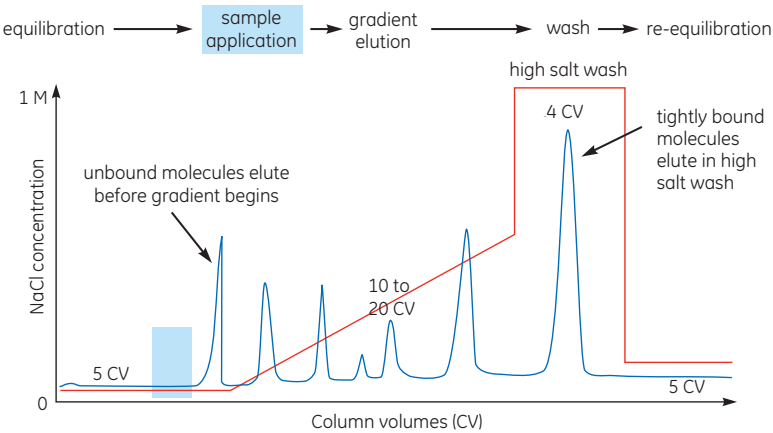


Fig A9.2. Typical IEX gradient elution.

The net surface charge of proteins varies according to the surrounding pH. Typically, when above its isoelectric point (pI) a protein will bind to an anion exchanger; when below its pI a protein will bind to a cation exchanger. However, it should be noted that binding depends on charge and that surface charges can thus be sufficient for binding even on the other side of the pI. Typically IEX is used to bind the target molecule, but it can also be used to bind impurities if required. IEX can be repeated at different pH values to separate several proteins that have distinctly different charge properties, as shown in Figure A9.3.

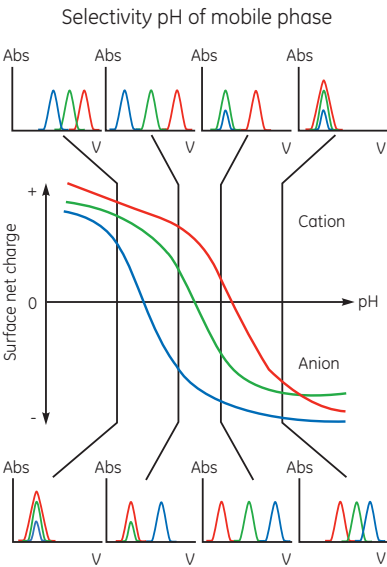


Fig A9.3. Effect of pH on protein elution patterns.

Method development (in priority order)

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



To reduce separation times and buffer consumption, transfer to a step elution after method optimization as shown in Figure A9.4. It is often possible to increase sample loading when using step elution.

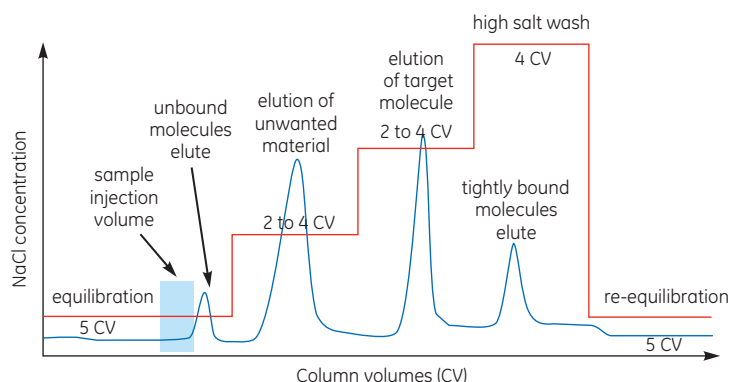


Fig A9.4. Step elution.

Further information

Protein Purification Handbook to Strategies for Protein Purification Handbook, 28983331.

Ion Exchange Chromatography and Chromatofocusing Handbook, Principles and Methods, 11000421.

Hydrophobic interaction chromatography (HIC)

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

Elution is usually performed by decreases in salt concentration (Fig A9.5). Changes are made stepwise or with a continuous decreasing salt gradient. Most commonly, samples are eluted with a decreasing gradient of ammonium sulfate. Target proteins are concentrated during binding and collected in a purified and concentrated form. Other elution procedures include reducing eluent polarity (ethylene glycol gradient up to 50%), adding chaotropic species (urea, guanidine hydrochloride) or detergents, changing pH or temperature.

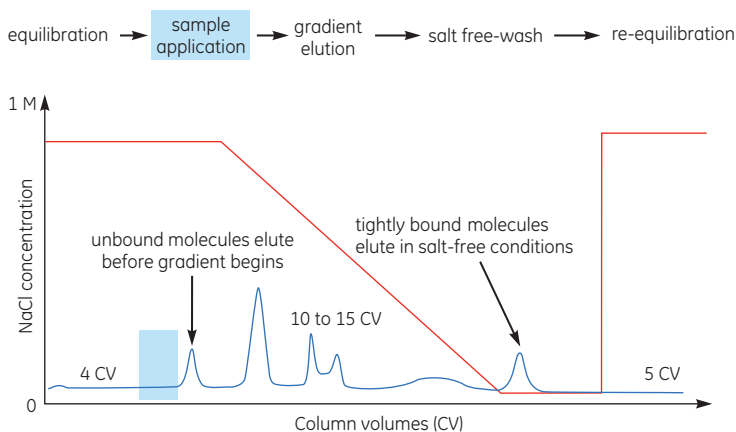


Fig A9.5. Typical HIC gradient elution.

Method development (in priority order)

1. The hydrophobic behavior of a protein is difficult to predict, and binding conditions must be studied carefully. Use HiTrap HIC Selection Kit select the medium that gives optimal binding and elution over the required range of salt concentration. For proteins with unknown hydrophobic properties begin with 0% to 100% B (0% B, e.g., 1 M ammonium sulfate). Knowledge of the solubility of protein in the binding buffer is important because high concentrations of, for example, ammonium sulfate can precipitate proteins.
2. Select a gradient that gives acceptable resolution.
3. Select the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.
4. If samples adsorb strongly to a medium, separation conditions such as pH, temperature, chaotropic ions, or organic solvents can have caused conformational changes and should be altered. Conformational changes are specific to each protein. Use screening procedures to investigate the effects of these agents. Alternatively, change to a less hydrophobic medium.



To reduce separation times and buffer consumption, transfer to a step elution after method optimization, as shown in Figure A9.6. It is often possible to increase sample loading when using step elution.

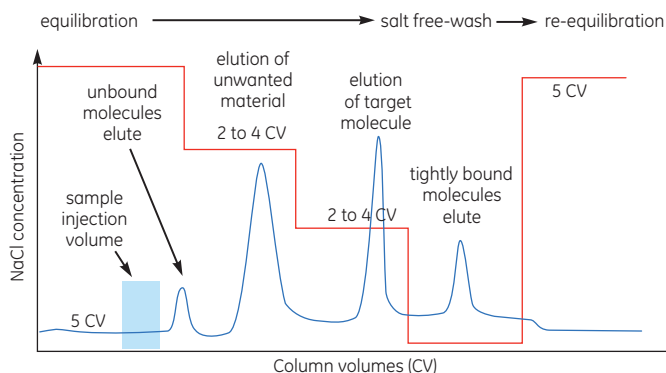


Fig A9.6. Step elution.

Further information

Strategies for Protein Purification Handbook, 28983331.

Hydrophobic Interaction Chromatography and Reversed Phase Handbook, Principles and Methods, 11001269.

Size exclusion chromatography (SEC)

SEC separates proteins with differences in molecular size. The technique is well-suited for the final polishing steps in purification when sample volumes have been reduced (sample volume significantly influences speed and resolution in SEC). Samples are eluted isocratically (single buffer, no gradient, Fig A9.7). Buffer conditions are varied to suit the sample type or the requirements for further purification, analysis, or storage, because buffer composition usually does not have major effects on resolution. Proteins are collected in purified form in the chosen buffer.

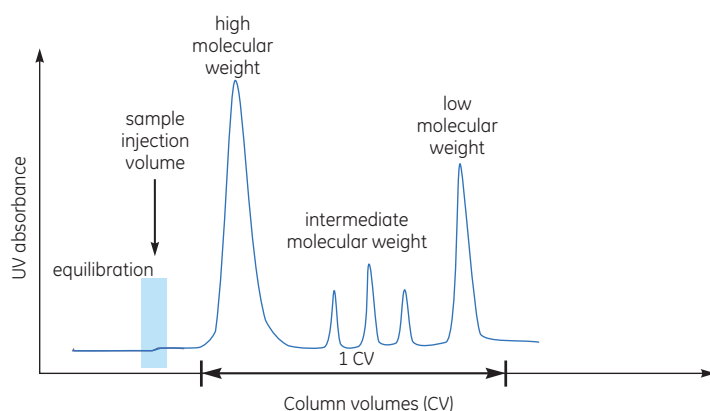


Fig A9.7. Typical SEC elution.

Further information

Strategies for Protein Purification Handbook, 28983331.

Size Exclusion Chromatography Handbook, Principles and Methods, 18102218.

Reversed phase chromatography (RPC)

RPC separates proteins and peptides with differing hydrophobicity based on their reversible interaction with the hydrophobic surface of a chromatographic medium. Samples bind as they are loaded onto a column. Conditions are then altered so that the bound substances are eluted differentially. Due to the nature of the reversed phase matrixes, binding is usually very strong. Binding may be modulated by the use of organic solvents and other additives (ion pairing agents). Elution is usually performed by increases in organic solvent concentration, most commonly acetonitrile. Samples that are concentrated during the binding and separation process are collected in a purified, concentrated form. The key stages in a separation are shown in Figure A9.8.

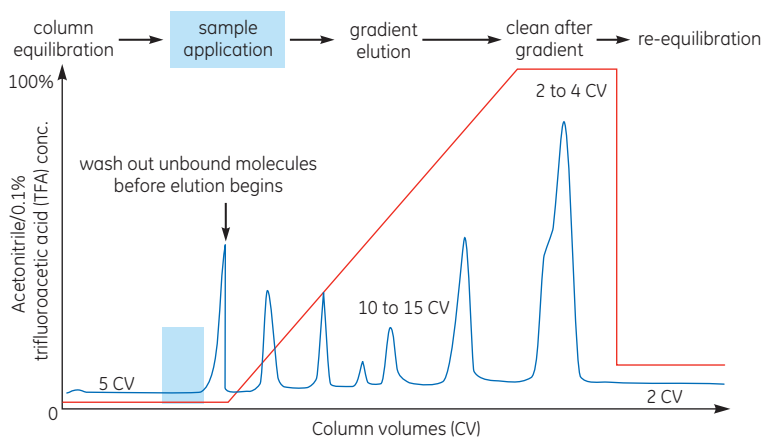


Fig A9.8. Typical RPC gradient elution.

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

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

Method development

1. Select medium from screening results.
2. Select optimal gradient to give acceptable resolution. For unknown samples begin with 0% to 100% elution buffer.
3. Select highest flow rate that maintains resolution and minimizes separation time.
4. For large-scale purification, transfer to a step elution.
5. Samples that adsorb strongly to a medium are more easily eluted by changing to a less hydrophobic medium.

Further information

Strategies for Protein Purification Handbook, 28983331.

Hydrophobic Interaction and Reversed Phase Chromatography Handbook, Principles and Methods, 11001269.

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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
Selection guides/brochures	
Affinity columns and media, Selection guide	18112186
Size exclusion chromatography columns and media, Selection guide	18112419
Ion exchange columns and media, Selection guide	18112731
Prepacked chromatography columns for ÄKTA systems, Selection guide	28931778
Pure simplicity for tagged proteins, Brochure	28935364
Sample preparation for analysis of proteins, peptides, and carbohydrates, Selection guide	18112862
Solutions for antibody purification, Selection guide	28935197
Years of experience in every column, Brochure	28909094

Ordering information

Product	Quantity	Code number
Affinity chromatography		
Prepacked columns and multiwell plates		
Protein G HP MultiTrap	4 × 96-well plates	28903135
Protein G HP SpinTrap	16 columns	28903134
Ab SpinTrap	50 columns	28408347
Protein G GraviTrap	10 × 1 ml	28985255
rProtein A GraviTrap	10 × 1 ml	28985254
rProtein A/Protein G GraviTrap	10 × 1 ml	28985256
HiTrap Protein G HP	1 × 1 ml	29048581
	2 × 1 ml	17040403
	5 × 1 ml	17040401
	1 × 5 ml	17040501
	5 × 5 ml	17040503
MABTrap Kit	HiTrap Protein G HP (1 × 1 ml), accessories, premade buffers for 10 purifications	17112801
Protein A HP MultiTrap	4 × 96-well plates	28903133
Protein A HP SpinTrap	16 columns	28903132
HiTrap Protein A HP	1 × 1 ml	29048576
	2 × 1 ml	17040203
	5 × 1 ml	17040201
	1 × 5 ml	17040301
	5 × 5 ml	17040303
HiTrap rProtein A FF	2 × 1 ml	17507902
	5 × 1 ml	17507901
	1 × 5 ml	17508001
	5 × 5 ml	17508002
HiTrap MabSelect	5 × 1 ml	28408253
	1 × 5 ml	28504255
	5 × 5 ml	28408256
HiScreen MabSelect	1 × 4.7 ml	28926973
HiTrap MabSelect SuRe	5 × 1 ml	11003493
	1 × 5 ml	11003494
	5 × 5 ml	11003495
HiScreen MabSelect SuRe	1 × 4.7 ml	28926977
HiScreen MabSelect SuRe LX	1 × 4.7 ml	17547415
HiTrap MabSelect Xtra	5 × 1 ml	28408258
	1 × 5 ml	28408260
	5 × 5 ml	28408261
HiScreen MabSelect Xtra	1 × 4.7 ml	28926976
HiTrap Protein L	5 × 1 ml	17547851
	1 × 1 ml	29048665
	1 × 5 ml	17547815
	5 × 5 ml	17547855

Product	Quantity	Code number
HiScreen Capto L	1 × 4.7 ml	17547814
Immunoprecipitation Starter Pack	2 × 2 ml	17600235
Protein A Sepharose 4 Fast Flow		
Protein G Sepharose 4 Fast Flow		
HiTrap IgY Purification HP	1 × 5 ml	17511101
HiTrap IgM Purification HP	5 × 1 ml	17511001
HiTrap NHSactivated HP	5 × 1 ml	17071601
	1 × 5 ml	17071701
HiTrap Blue HP	5 × 1 ml	17041201
	1 × 5 ml	17041301
Companion product		
Ab Buffer Kit	10× stock solutions of binding buffer, elution buffer, and neutralization buffer	28903059
Magnetic bead media		
Protein A Mag Sepharose	500 µl	28944006
	4 × 500 µl	28951378
Protein A Mag Sepharose Xtra	2 × 1 ml	28967056
	5 × 1 ml	28967062
Protein G Mag Sepharose	500 µl	28944008
	4 × 500 µl	28951379
Protein G Mag Sepharose Xtra	2 × 1 ml	28967066
	5 × 1 ml	28967070
Lab packs of bulk chromatography media¹		
Protein G Sepharose 4 Fast Flow	5 ml	17061801
	25 ml	17061802
nProtein A Sepharose 4 Fast Flow	5 ml	17528001
	25 ml	17528004
rProtein A Sepharose 4 Fast Flow	5 ml	17127901
	25 ml	17127902
Protein A Sepharose CL 4B	1.5 g	17078001
MabSelect	25 ml	17519901
MabSelect SuRe	25 ml	17543801
MabSelect SuRe LX	25 ml	17547401
MabSelect Xtra	25 ml	17526907
Capto L	5 ml	17547806
	25 ml	17547801
NHSactivated Sepharose Fast Flow	25 ml	17090601
CNBractivated Sepharose 4 Fast Flow	10 g	17098101
Chelating Sepharose Fast Flow	50 ml	17057501
Blue Sepharose 6 Fast Flow	50 ml	17094801
Benzamidine Sepharose 4 Fast Flow (high sub)	25 ml	17512310

¹ Larger quantities available on request. Please contact GE for more information.

Product	Quantity	Code number
Ion exchange chromatography		
Prepacked columns		
HiTrap IEX Selection Kit	7 × 1 ml	17600233
HiTrap Q XL 1 ml		
HiTrap SP XL 1 ml		
HiTrap ANX FF (high sub) 1 ml		
HiTrap DEAE FF 1 ml		
HiTrap CM FF 1 ml		
HiTrap Q FF 1 ml		
HiTrap SP FF 1 ml		
HiTrap Q HP	1 × 1 ml	29051325
	5 × 1 ml	17115301
	5 × 5 ml	17115401
HiTrap SP HP	1 × 1 ml	29051324
	5 × 1 ml	17115101
	5 × 5 ml	17115201
Lab packs of bulk chromatography media¹		
SP Sepharose Fast Flow	25 ml	17072910
Capto Q	25 ml	17531610
Capto S	25 ml	17544110
Capto S ImpAct	25 ml	17371701
Capto MMC	25 ml	17531710
Capto MMC ImpRes	25 ml	17371601
Capto adhere	25 ml	17544410
Capto adhere ImpRes	25 ml	17371501
Hydrophobic interaction chromatography		
Prepacked columns		
HiTrap HIC Selection Kit	7 × 1 ml	28411008
HiTrap Phenyl HP		
HiTrap Phenyl FF (low sub)		
HiTrap Phenyl FF (high sub)		
HiTrap Butyl HP		
HiTrap Butyl FF		
HiTrap ButylS FF		
HiTrap Octyl FF		
HiTrap Phenyl FF (high sub)	5 × 1 ml	17135501
	5 × 5 ml	17519301
HiTrap Phenyl FF (low sub)	5 × 1 ml	17135301
	5 × 5 ml	17519401
HiTrap Phenyl HP	5 × 1 ml	17135101
	5 × 5 ml	17519501
HiTrap Butyl FF	5 × 1 ml	17135701
	5 × 5 ml	17519701
HiTrap Octyl FF	5 × 1 ml	17135901
	5 × 5 ml	17519601

¹ Larger quantities available on request. Please contact GE for more information.

Product	Quantity	Code number
Lab packs of bulk chromatography media¹		
Phenyl Sepharose 6 Fast Flow (high sub)	25 ml	17097310
Phenyl Sepharose 6 Fast Flow (low sub)	25 ml	17096510
Phenyl Sepharose High Performance	75 ml	17108201

Size exclusion chromatography (desalting and buffer exchange)

Prepacked columns

HiTrap Desalting	5 × 5 ml	17140801
Disposable PD10 Desalting Columns	30 columns	17085101
PD SpinTrap G-25	50 columns	28918004
PD MultiTrap G-25	4 × 96well plates	28918006
PD MiniTrap G-25	50 columns	28918007
PD MidiTrap G-25	50 columns	28918008
HiPrep 26/10 Desalting	1 × 53 ml	17508701

Companion products

MiniSpin Adapter	10	28923243
MidiSpin Adapter	10	28923244
PD-10 Spin Adapter	10	28923245
LabMate PD10 Buffer Reservoir	10	18321603
Collection plate 500 µl (Vbottom)	5 × 96 well plates	28403943

Size exclusion chromatography (high resolution)

Prepacked columns

Superdex 200 Increase 3.2/300	1 × 2.4 ml column	28990946
Superdex 200 Increase 5/150 GL	1 × 3 ml column	28990945
Superdex 200 Increase 10/300 GL	1 × 24 ml column	28990944
Superdex Peptide 3.2/300	1 × 2.4 ml column	29036231
Superdex Peptide 10/300 GL	1 × 24 ml column	17517601
Superdex 75 3.2/300	1 × 2.4 ml column	29036230
Superdex 75 5/150 GL	1 × 3 ml column	28920504
Superdex 75 10/300 GL	1 × 24 ml column	17517401
HiLoad 16/600 Superdex 30 prep grade	1 × 120 ml column	28989331
HiLoad 26/600 Superdex 30 prep grade	1 × 320 ml column	28989332
HiLoad 16/600 Superdex 75 prep grade	1 × 120 ml column	28989333
HiLoad 26/600 Superdex 75 prep grade	1 × 320 ml column	28989334
HiLoad 16/600 Superdex 200 prep grade	1 × 120 ml column	28989335
HiLoad 26/600 Superdex 200 prep grade	1 × 320 ml column	28989336

Product	Quantity	Code number
Western blotting		
Amersham Hybond P 0.45 PVDF	25 sheets	10600069
Amersham Protran Premium 0.45 NC	10 sheets	10600048
Amersham Hybond LFP 0.2 PVDF	10 sheets	10600060
Amersham ECL Prime Western Blotting Detection Reagents	for 1000 cm ²	RPN2232
Amersham ECL Select Western Blotting Detection Reagent	for 1000 cm ²	RPN2235
Amersham ECL Western Blotting Detection Reagent	for 1000 cm ²	RPN2109
Amersham ECL Plex Western Blotting Combination Pack (Cy™3, Cy5, Amersham Protran Premium 0.45)	for 1000 cm ²	RPN998
Amersham ECL Plex Western Blotting Combination Pack (Cy3, Cy5, Amersham Hybond LFP)	for 1000 cm ²	RPN999
Amersham ECL start Western Blotting Detection Reagent	for 2000 cm ²	RPN3243
Empty columns		
Tricorn 5/20	1	28406408
Tricorn 5/50	1	28406409
Tricorn 10/20	1	28406413
Tricorn 10/50	1	28406414
Tricorn 10/100	1	28406415
XK 16/20	1	28988937
XK 16/40	1	28988938
XK 26/20	1	28988948
XK 26/40	1	28988949
XK 50/20	1	28988952
XK 50/30	1	28988953
Empty Disposable PD-10 Desalting columns	50	17043501
HiScale 16/20	1	28964441
HiScale 16/40	1	28964424
HiScale 26/20	1	28964514
HiScale 26/40	1	28964513
HiScale 50/20	1	28964445
HiScale 50/40	1	28964444

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