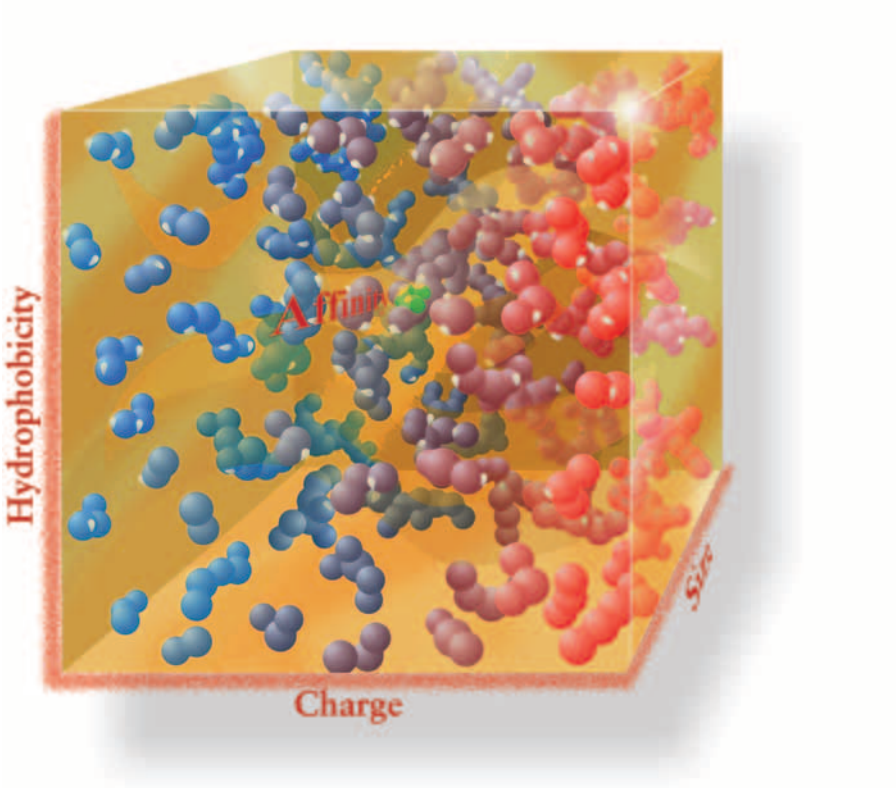
Protein and Peptide Purification



Technique selection guide

18-1128-63
Edition AD

Amersham Biosciences

Ion Exchange Chromatography (IEX)

High Resolution
High Capacity

IEX separates proteins with differences in charge. The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. Proteins bind as they are loaded onto a column. Conditions are then altered so that bound substances are eluted differentially. This elution is usually performed by increases in salt concentration or changes in pH. Most commonly, samples are eluted with salt (NaCl), using a gradient elution, as shown Figure 1. Target proteins are concentrated during binding and collected in a purified, concentrated form.

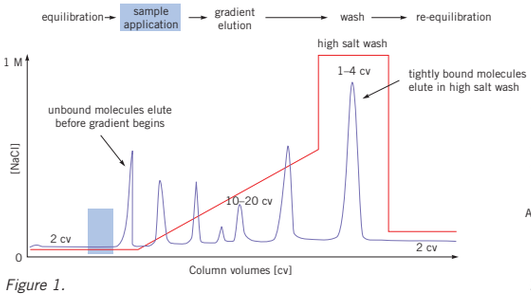


Figure 1.

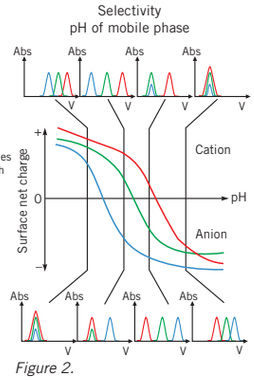


Figure 2.

The net surface charge of proteins varies according to the surrounding pH. IEX can be repeated at different pH values to separate several proteins which have distinctly different charge properties, as shown in Figure 2.

Choice of ion exchanger

Begin with a strong exchanger, to allow work over a broad pH range during method development.

Strong ion exchangers

Q (anion exchange), SP (cation exchange): fully charged over a broad pH range (pH 2–12).

Weak ion exchangers

DEAE and ANX (anion exchange) and CM (cation exchange): fully charged over a narrower pH range (pH 2–9 and pH 6–10, respectively), but give alternative selectivities for separations.

Sample volume and capacity

For optimal separations with gradient elution, use approximately one fifth of the total binding capacity. IEX is a binding technique, independent of sample volume.

Media and Column Selection

Refer to Ion Exchange Selection Guide Code no: 18-1127-31. Use HiTrap IEX Selection Kit for media scouting and method optimisation.

Sample Preparation

Samples should be at the same pH and ionic strength as the starting buffer, and free from particulate matter.

Buffer Preparation

If charge characteristics are unknown try these conditions first:

Anion Exchange

Start buffer (A): 20 mM Tris-HCl, pH 7.4

Elution buffer (B): 20 mM Tris-HCl + 1 M NaCl, pH 7.4

Gradient: 0–100% elution buffer in 10–20 column volumes

Cation Exchange

Start buffer (A): 20 mM $Na_2HPO_4 \times 2H_2O$, pH 6.8

Elution buffer (B): 20 mM $Na_2HPO_4 \times 2H_2O$ + 1 M NaCl, pH 6.8

Gradient: 0–100%B in 10–20 column volumes

Optimisation Parameters

1. Select optimal ion exchanger.
2. Select for optimum pH.
3. Select steepest gradient to give acceptable resolution at selected pH.
4. Select highest flow rate which maintains resolution and minimises separation time.
5. For large scale purifications and capture steps transfer to a step elution to reduce separation times and buffer consumption.

Hydrophobic Interaction Chromatography (HIC)

Good Resolution
Good Capacity

HIC separates proteins with differences in hydrophobicity. The separation is based on the reversible interaction between a protein and the hydrophobic surface of a chromatographic medium. This interaction is enhanced by high ionic strength buffer which makes HIC an ideal 'next step' for purification of proteins which have been precipitated with ammonium sulphate or eluted in high salt during IEX chromatography. Samples in high ionic strength solution (e.g. 1.5 M NH_2SO_4) 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. Most commonly, samples are eluted with a decreasing gradient of ammonium sulphate, as shown in Figure 3. Target proteins, which are concentrated during binding and collected in a purified, concentrated form. Other elution procedures are available.

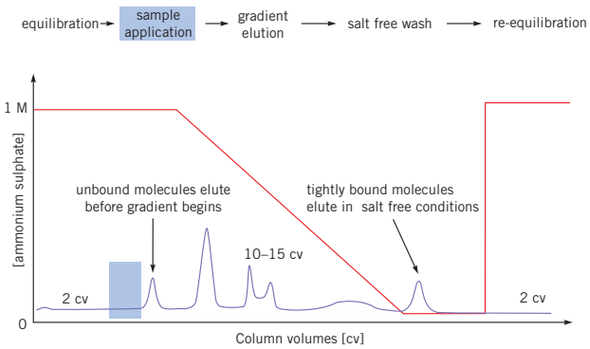


Figure 3. Typical gradient elution.

Choice of hydrophobic ligand

Select from a range of ligands. Typically the strength of binding of a ligand to a protein increases in the order: ether, isopropyl, butyl, octyl, phenyl.

Highly hydrophobic proteins bind tightly to highly hydrophobic ligands. Screen several hydrophobic media. Begin with a medium of low hydrophobicity if the sample has very hydrophobic components. Select the medium which gives the best resolution and loading capacity at a low salt concentration.

Sample volume and capacity

For optimal separations during gradient elution, use approximately one fifth of the total binding capacity of the column. HIC is a binding technique, independent of sample volume.

Media and Column Selection

With HIC the chromatographic medium as well as the hydrophobic ligand affect selectivity. Parameters such as sample solubility, scale of purification and availability of the correct ligand at the required scale should be considered. Use HiTrap HIC Selection Kit or RESOURCE™ HIC Test Kit for media scouting and method optimisation.

Sample Preparation

Samples should be at the same pH as the starting buffer, in high ionic strength solution and free from particulate matter.

Buffer Preparation

Try these conditions first if hydrophobic characteristics are unknown:

Start buffer (A): 50 mM $Na_2HPO_4 \times 2H_2O$ pH 7.0 + 1.0 M ammonium sulphate

Elution buffer (B): 50 mM $Na_2HPO_4 \times 2H_2O$ pH 7.0

Gradient: 0–100% B in 10–20 column volumes

Optimisation Parameters

1. Select medium from a HiTrap HIC Selection Kit or RESOURCE HIC Test Kit.
2. Select optimum gradient to give acceptable resolution. For unknown samples begin with 0–100%B (0%B=1 M ammonium sulphate).
3. Select highest flow rate which maintains resolution and minimises separation time.
4. For large scale purifications and capture steps transfer to a step elution.
5. Samples which adsorb strongly to a gel are more easily eluted by changing to a less hydrophobic medium.

Affinity Chromatography (AC)

High Resolution
High Capacity

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. AC can be used whenever a suitable ligand is available.

The target protein(s) is specifically and reversibly bound by a complementary binding substance (ligand). The sample is applied under conditions that favour specific binding. Unbound material is washed away, and the bound target protein is recovered by changing conditions to those favouring desorption. Desorption is performed specifically, using a competitive ligand, or non specifically, by changing the pH, ionic strength or polarity. Proteins, which are concentrated during binding, are collected in a purified, concentrated form. The key stages in a separation are shown in Figure 4.

AC may also used to remove specific contaminants e.g. Benzamidine Sepharose™ Fast Flow (high sub) removes serine proteases.

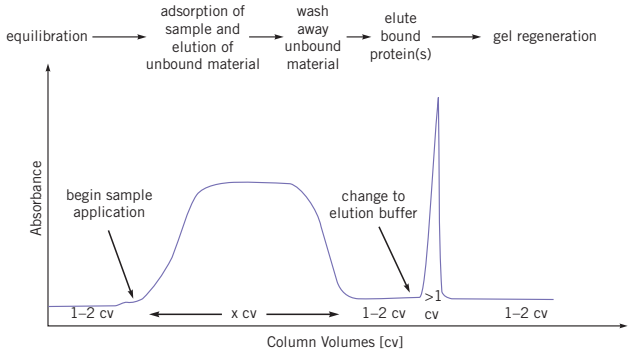


Figure 4. Typical affinity separation

	Application
Purification of immunoglobulins	IgG classes, fragments and subclasses including polyclonal rat IgG_3 strong affinity to monoclonal mouse IgG_1 and monoclonal rat IgG
Group Specific Media	nucleotide-requiring enzymes, coagulation factors, DNA binding proteins, α_2-macroglobulin, lipoprotein lipases, steroid receptors, hormones, interferon, protein syntheses factors
Media for coupling ligands	any $-NH_2$ containing ligand
Purification of recombinant fusion proteins	His fusion proteins, GST fusion protein

Table 1. Examples of affinity applications

Sample volume and capacity

Total binding capacity (target protein(s) bound per ml gel) is defined for commercially available affinity media. AC is a binding technique, independent of sample volume.

Media and Column Selection

Commercial availability of affinity matrices should be considered. Table 1 shows examples of applications for which ready to use affinity media are available. Specific affinity media are prepared by coupling a ligand to a selected gel matrix, following recommended coupling procedures. Further details on media are available in the Affinity Chromatography Product Profile Code No. 18-1121-86 and in the Convenient Protein Purification HiTrap Column Guide Code No. 18-1129-81. Use pre-packed HiTrap Affinity columns for method optimisation or small scale purification.

Sample Preparation

Samples must be free from particulate matter and contaminants which may bind non-specifically to the column e.g. lipids.

Buffer Preparation

Binding, elution and regeneration buffers are specific to each affinity medium. Follow instructions supplied.

Optimisation Parameters

1. Select correct specificity for target protein.
2. Follow manufacturer's recommendations for binding or elution conditions.
3. Select optimum flow rate to achieve efficient binding.
4. Select optimum flow rate for elution to maximise recovery.
5. Select maximum flow rate for column regeneration to minimise run times.

Gel Filtration (GF)

High Resolution (with Superdex)
Low Capacity (limited by sample volume)

Gel filtration separates proteins with differences in molecular size. The technique should be used when sample volumes have been minimised.

Since buffer composition does not directly affect resolution buffer conditions can be varied to suit the sample type or the requirements for the next purification, analysis or storage step.

The key stages in a separation are shown in Figure 1.

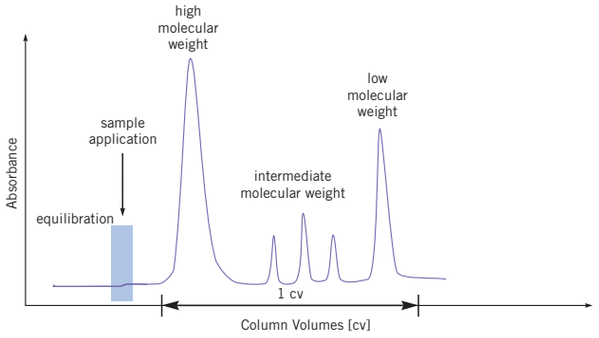


Figure 5. Typical gel filtration elution

Sample volume and capacity

To achieve highest resolution the sample volume must not exceed 5% of the total column volume. Gel filtration is independent of sample concentration.

Media and Column Selection

Refer to Gel Filtration Selection Guide Code: 18-1124-19. In gel filtration efficient column packing is essential. Use pre-packed columns to ensure reproducible results and highest performance.

Sample Preparation

Samples must be free from particulate matter. Viscous samples should be diluted. During separation, sample buffer is exchanged with buffer in the column.

Buffer Preparation

Select a buffer in which the purified product should be collected and which is compatible with protein stability and activity. Ionic strength can be up to 150 mM NaCl, to avoid non-specific ionic interactions with the matrix.

When working with a new sample try these conditions first:

Buffer: 0.5 M $Na_2HPO_4 \times 2H_2O$, pH 7.0 + 0.15 M NaCl or
select the buffer in which the sample should be eluted for the next step

Optimisation Parameters

1. Select a medium which has your target protein close to the middle of its separation range.
2. Select the highest flow rate which maintains resolution and minimises separation time. Lower flow rates improve resolution of high molecular weight components, faster flow rates may improve resolution of low molecular weight components.
3. Determine the maximum sample volume which can be loaded without reducing resolution (sample volume should be 0.5–5% of total column volume).
4. To further improve resolution increase column length by connecting two columns in series.

Group separations

For sample preparation and clarification use Sephadex G-25 for desalting, buffer exchange and removal of lipids and salts from proteins >5000.

Gel filtration is also ideal for sample preparation before or between purification steps. Sample volumes of up to 30% of the total column volume are loaded. In a single step, the sample is desalted, exchanged into a new buffer, and low molecular weight materials are removed.

Any sample volume can be processed rapidly and efficiently. The high sample volume load gives a low resolution separation but with minimal sample dilution.

Reversed Phase Chromatography (RPC)

High Resolution

RPC separates molecules of differing hydrophobicity based on the reversible interaction between the molecule and 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 matrices, the binding is usually very strong and requires the use of organic solvents and other additives (ion pairing agents) for elution. Elution is usually performed by increases in organic solvent concentration, most commonly acetonitrile. Molecules, which are concentrated during the binding process, are collected in a purified, concentrated form. The key stages in a separation are shown in Figure 6.

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

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

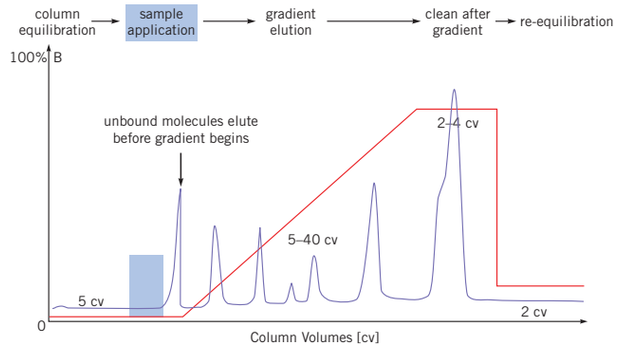


Figure 6. Typical RPC gradient elution

Choice of ligand hydrophobicity

Select a polymer or silica based matrix either C4, C8 or C18 n-alkyl hydrocarbon ligands according to the degree of hydrophobicity required. Highly hydrophobic molecules bind tightly to highly hydrophobic ligands, e.g. C18. Screen several RPC media. Begin with a medium of low hydrophobicity, e.g. C4 or C8, if the sample has very hydrophobic components (more likely with larger biomolecules). Select the medium which gives the best resolution and loading capacity.

Sample volume and capacity

RPC is a binding technique, independent of sample volume. Total capacity is strongly dependent upon experimental conditions and the properties of the gel and sample. For optimal conditions during gradient elution screen for a sample loading which does not reduce resolution.

Media and Column Selection

In RPC the chromatographic medium as well as the hydrophobic ligand affect selectivity. Screening of different RPC media is recommended. Reversed phase columns should be 'conditioned' by extended equilibration for first time use, after long term storage or when changing buffer systems.

Sample Preparation

Samples should be free from particulate matter and, when possible, dissolved in the start buffer. If sample is insoluble try 1) 10-30% acetic acid, 2) 70% formic acid, 3) 6 M guanidine-HCl, 4) 100% DMSO (dimethyl sulphoxide), 5) TFA (trifluoroacetic acid). Note that a very hydrophobic peptide dissolved in DMSO may precipitate or bind irreversibly to an RPC matrix. Test first with aliquots of sample.

Buffer Preparation

Try these conditions first when sample characteristics are unknown:

Start buffer (A): 0.065% TFA (trifluoroacetic acid) in water

Elution buffer (B): 0.05% TFA in acetonitrile

Gradient: 2–80% elution buffer in 20 column volumes

Optimisation Parameters

1. Select medium from screening results.
2. Select optimum gradient to give acceptable resolution. For unknown samples begin with 2–80%B.
3. Select highest flow rate which maintains resolution and minimises separation time.
4. For large scale purifications transfer to a step elution.
5. Samples which adsorb strongly to a gel are more easily eluted by changing to a less hydrophobic medium.

Protein and Peptide Purification

Decision point
Yes
No
Continue

Sample Preparation

Start here

Proteins and peptides, crude samples

Sample shows visible contamination

Change in buffer or ionic strength required

Filter or centrifuge

For desalting and buffer exchange use Sephadex™ media.

GF

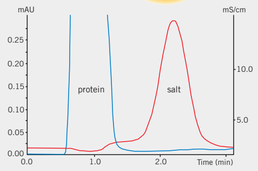
Further extraction or concentration required

Peptides or very hydrophobic proteins are not suitable for HIC separations. Go to GF

Ammonium sulphate or other precipitation

Screen for HIC medium (to avoid extra desalting step). Adjust $(NH_4)_2SO_4$ conc.

HIC medium selected



Sample Preparation
Buffer exchange
HiPrep™ 26/10 Desalting
Mouse plasma (10 ml)

Capture

Biospecific ligand available

AC

Purity OK or impurities differ in size from target molecule

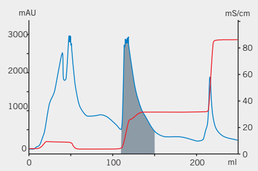
GF

or repeat affinity step after tag cleavage if only tag removal is required

IEX

Unknown isoelectric point (pI) or pI below pH 7, use AIEX. pI above 5 or high DNA contamination, use CIEX.

HIC



Capture
IEX
HiPrep 16/10 Q XL
DAOCS enzyme from clarified *E. coli* extract
Step elution

Intermediate Purification

IEX

Adjust ionic strength of sample before IEX.

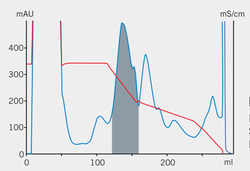
Purity OK

GF

HIC

Increase ionic strength of sample before HIC. Peptides or very hydrophobic proteins are not suitable for HIC separations. Go to GF

Adjust ionic strength of sample before IEX.



Intermediate Purification
HIC
SOURCE™ 15 ISO in HR 16/10
DAOCS enzyme

Polishing

GF

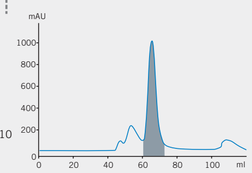
Purity OK

Target sensitive to organic solvents

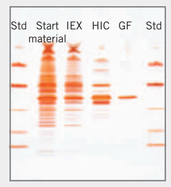
RPC

At this stage in a purification gel filtration can be used as the final step if impurities differ in size from the target molecule. However, *gel filtration is highly recommended as a final step in all purification strategies* to ensure removal of any low molecular weight contaminants.

Evaluate results of each step. Evaluate purification strategy. Use alternative IEX selectivity to improve resolution or recovery. Use alternative ligand.



Polishing
GF
HiLoad™ 16/60 Superdex™ 75 prep grade
DAOCS enzyme



Analysis of fractions from DAOCS purification
SDS PAGE, silver stain

Peptide Purification

Start here

Synthetic peptides
product from cleavage, de-protection or ether precipitation
Natural peptides
products from, for example, cleaned tissue extracts

Large peptide (~50 amino acids)

AIEX

Isoelectric point below pH 7.0

CIEX

RPC

Purity OK

Media selection. Screen for optimum pH and suitable selectivity polymeric matrix pH 1–12 silica matrix pH < 7.

Hydrophobic peptide

Use polymeric matrix or C18 silica matrix. C8 or C4 if peptide is very hydrophobic.

Use different selectivity, mobile phase or pH on RPC for purity check or removal of minor contaminants if required. Analyse with MS.

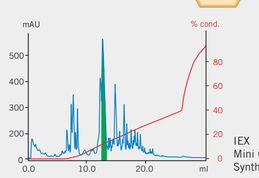
recycling of product improves purity but reduces yield

Use silica or polymeric matrix at low pH.

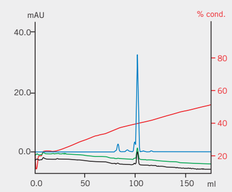
Very acidic (~25%)

Use polymeric matrix at high pH.

Try alternative matrices or conditions. Screen for optimum pH and suitable selectivity polymeric matrix pH 1–12 silica matrix pH < 7.



IEX
Mini Q™ 4.6/50 PE
Synthetic peptide



RPC
RESOURCE RPC
Synthetic peptide

Micropurification and Analysis

Start here

Protein and peptide fragments
e.g. from protein digests

Reduction in number of fragments per analysis needed
High molecular weight target fragments
> M_r 3 000–5 000

RPC

Resolution OK

silica matrix media screening elution optimisation

Reduction in number of fragments per analysis needed

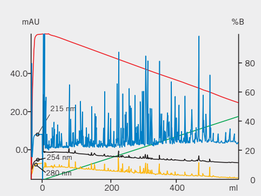
Change to RPC with alternative selectivity, mobile phase, pH or media.

Use different selectivity, mobile phase or pH on RPC for purity check or removal of minor contaminants if required. Analyse with MS.

GF
Superdex Peptide

Re-analyse fractions collected from GF.

silica matrix media screening elution optimisation

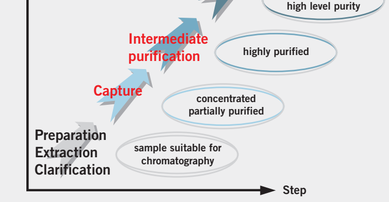


RPC
µRPC C2/C18 ST 4.6/100
Tryptic digest of a native M_r 165 000 protein

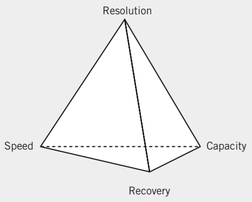
Purification Strategies

This selection guide offers general guidelines for protein and peptide purification strategies.

Selection of the final strategy will always depend upon specific sample characteristics, the condition of the starting material and the required level of purity.



For every technique used there is a balance between:



Note: In this model capacity refers to amount of target molecule bound per unit volume of gel. Capacity can also refer to sample size, both absolute volume and sample concentration, that can be handled in a single step.

Guidelines for Purification

1. Define objectives
   purity, activity, quantity required for final product

   Examples of approximate purity level requirements:
   Extremely high >99% *therapeutic use or in vivo studies*
   High 95–99% *x-ray crystallography and most physico-chemical characterisation methods*
   Moderate <95% *Antigen for antibody production*
2. Develop analytical assays
   fast detection of protein activity and recovery to work efficiently
3. Define sample characteristics
   to simplify technique selection and optimisation
4. Minimise sample handling at every stage
   avoid lengthy procedures which risk losing activity or reducing recovery
5. Minimise the use of additives
   additives may need to be removed in an extra purification step or may interfere with assays
6. Remove damaging contaminants early
   for example, proteases
7. Use a different technique at each step
   take advantage of sample characteristics which can be used for separation (size, charge, hydrophobicity, ligand specificity)
8. Minimise number of chromatographic steps
   extra steps reduce yield and increase time, combine steps logically

KEEP IT SIMPLE!

Glossary of Terms

Sample Characteristics include: Size, charge, hydrophobicity, affinity for a specific ligand, isoelectric point (pI), pH and temperature stability.

Sample Preparation: Clarification before first chromatographic separation step. May include extraction and/or concentration procedures.

Capture Initial purification of the target molecule from crude or clarified source material.
Goal: Rapid concentration, stabilisation and isolation.

Intermediate purification Removal of bulk contaminants.
Goal: Purification and concentration.

Polishing Removal of trace contaminants e.g. structural variants.
Goal: End product of required high level purity.

IEX Ion exchange chromatography (IEX)
Anion exchange chromatography (AIEX)
Cation exchange chromatography (CIEX)

AC Affinity chromatography (AC)

HIC Hydrophobic interaction chromatography (HIC)

GF Fractionation by gel filtration (GF)

RPC Reversed phase chromatography (RPC)

GF Sample clean-up by gel filtration (GF)

EBA *Expanded bed adsorption is a technique in which target proteins are purified from crude, particulate sample, combining sample preparation and capture in a single step. It is most suitable for large scale purification and therefore not shown in this guide.*

Technical information overleaf

Amersham Biosciences

Further Information

Selection Guides: to select the correct chromatographic medium for a separation step.

Handbooks: to learn more about the details of each chromatographic technique including applications and trouble shooting.

		Code No.
Purification	Protein Purification Handbook	18-1132-29
Affinity	Affinity Chromatography Product Profile	18-1121-86
	Affinity Chromatography: Principles and Methods	18-1022-29
	Antibody Purification Handbook	18-1037-46
Gel Filtration	Gel Filtration Selection Guide	18-1124-19
	Gel Filtration: Principles and Methods	18-1022-18
	Desalting and buffer exchange Selection Guide	18-1128-62
Hydrophobic Interaction & Reversed Phase	Hydrophobic Interaction Chromatography: Principles and Methods	18-1020-90
	Reversed Phase: Principles and Methods	18-1112-93
Ion Exchange	Ion Exchange Selection Guide	18-1127-31
	Ion Exchange Chromatography: Principles and Methods	18-1114-21
Applications	The Recombinant Protein Handbook	18-1105-02
	GST Gene Fusion System Handbook	18-1157-58
Interactive learning	Protein Purifier CD	18-1155-49
	Column Packing the Movie	18-1165-33

BioProcess Media – Made for bioprocessing

BioProcess Media

Secure Supply	• Large capacity production integrated with clear ordering and delivery routines-means availability in the right quantity, at the right place, at the right time. • Chromatography is our business; making BioProcess Media a safe investment for long-term production.
Validated Manufacture	• Validated methods for manufacturing & quality control within ISO9001 certified quality system. • A certificate of analysis is available for every lot and an MSDS for every product.
Regulatory support	• Regulatory Support Files detail performance, stability, extractable compounds and analytical methods. The essential information in these files is an invaluable starting point for process validation, as well as support for clinical and marketing applications submitted to regulatory authorities.
Capture to Polishing	• BioProcess Media are designed for each chromatographic stage in a process from Capture to Polishing. Take a systematic approach to method development by using BioProcess Media for every stage.
High Productivity	• High flow rates, capacities and recoveries available with BioProcess Media contribute to the overall economy of industrial processes.
Sanitization & CIP/Scalability	• All BioProcess Media can be cleaned and sanitized in place. • Packing methods are established for a wide range of scales. • Use the same BioProcess Media for development work, pilot studies and routine production for a direct scale up.
Custom Designed Media	• Provide large-scale users with media designed for specific applications through variations in ligand, coupling chemistry and base matrix. • Custom Designed Media (CDM) are fully tested and quality controlled • Some CDM's are made on an exclusive basis for specific customers; others are available on receipt of order.

For further information, visit us at:

www.chromatography.amershambiosciences.com

or

www.bioprocess.amershambiosciences.com

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Fax: 01 6941 9677

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