GE Healthcare

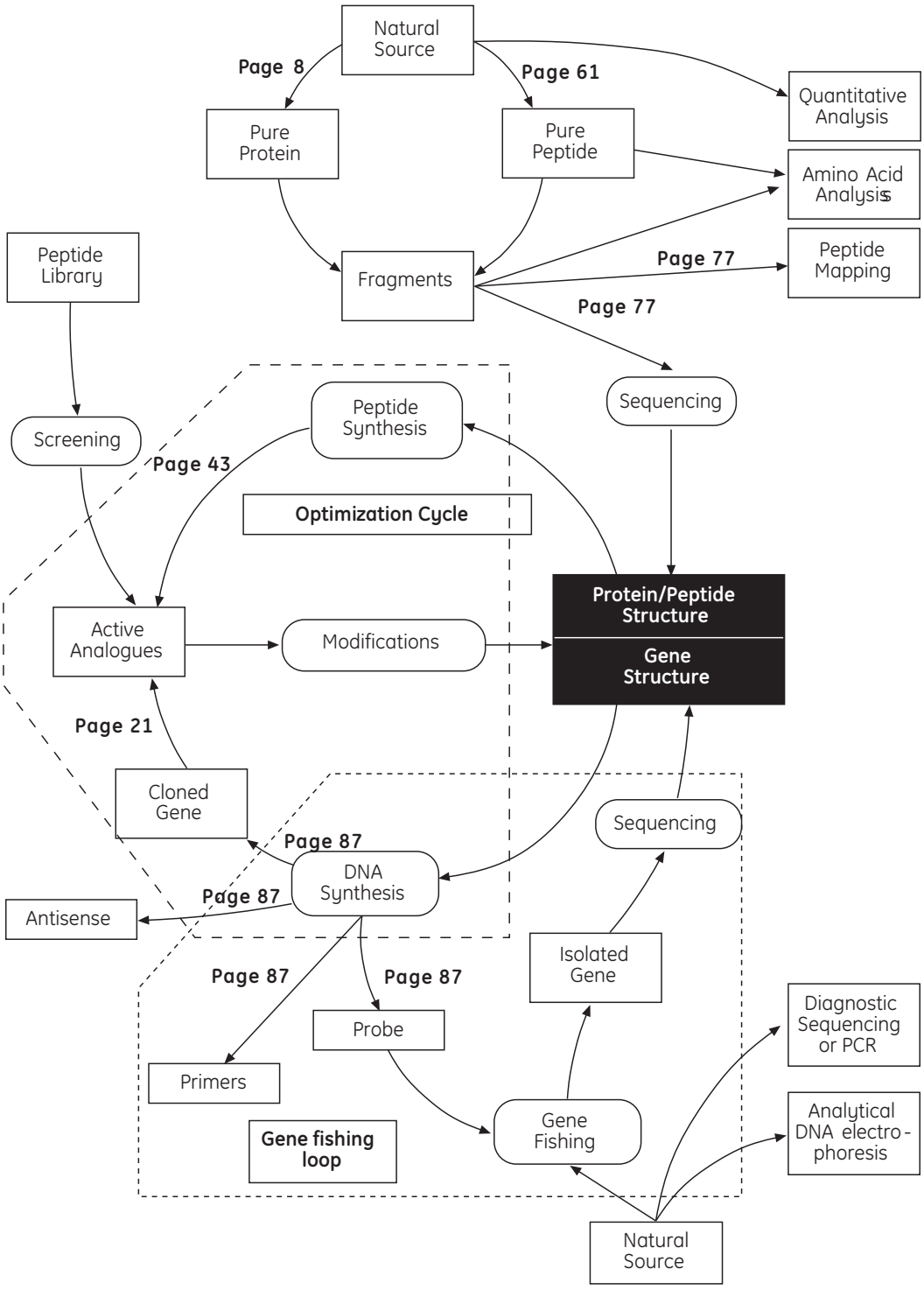
ÄKTAdesign Purification

Method handbook

ÄKTA™

Protocol Finder

The Map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Contents

1 Introduction to this handbook and UNICORN

1.1 About this handbook

This handbook is intended to help you make full use of your ÄKTAexplorer™ or ÄKTApurifier™ chromatography system. Rather than being a text book in separation science, the handbook is instead a collection of useful step-by-step protocols to aid your everyday purification work. Detailed instructions and recommendations are presented in a straightforward format, which should be easy to follow without needing a high level of expertise in programming or in chromatography.

Protocols specially adapted for the chromatography software UNICORN™ help you to plan, optimize and run your purification experiments.

All purification protocols are based on pre-constructed templates available in UNICORN. In combination with the UNICORN Column list, these templates make method creation very simple.

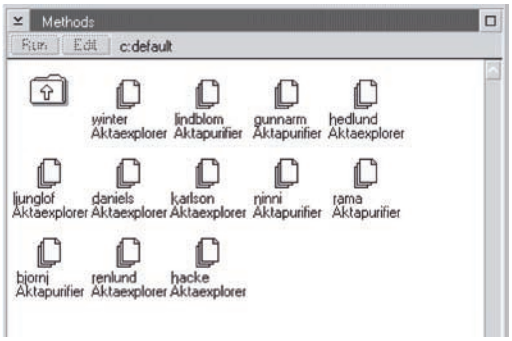
The handbook covers purification of proteins, peptides and oligonucleotides. However, before developing a suitable purification protocol, a development platform must be established, including:

- Required quality and quantity of the purified target molecule.
- Available information on the physical properties of the target molecule.
- Stability range for the starting material as well as the target molecule.
- Properties of the starting material.

The development of a suitable purification protocol starts with running Standard Purification Protocols. These protocols take advantage of the high overall resolution obtained by combining techniques with independent selectivities. This will provide sufficient purity in a large number of cases. When satisfactory results are not obtained with the Standard Purification Protocols, different optimizing measures are suggested.

If you are not familiar with how to operate UNICORN, read Making your first run before using the protocols of this handbook.

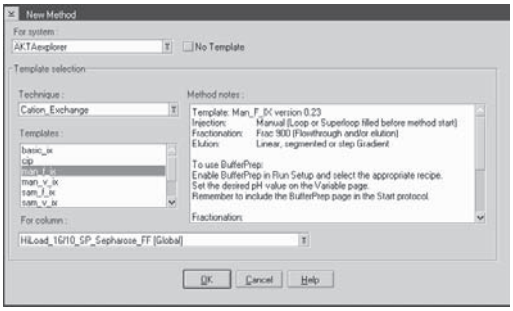
A full description of how to operate UNICORN is found in the UNICORN User Manual.



A brief theoretical background to the techniques used in this handbook is given in Appendix II on page 133 and further help is found in Appendix I on page 125.

1.2 About UNICORN templates for method editing

1.2.1 Pre-constructed method templates

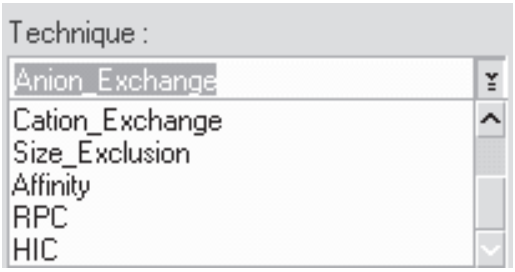


UNICORN contains a collection of pre-constructed method templates for different chromatographic situations. These templates are built up of blocks that are different parts used to build up a method, e.g. Column equilibration, Sample introduction, Fractionation, Elution, etc. Each method template contains a complete set of instruction variables within the blocks and, when an appropriate column is chosen from the Column list of UNICORN, column-specific default values (pressure limit, flow rate, etc.) are entered automatically. The default values represent the average chromatographic experiment and are used in many of the Standard Purification Protocols in this handbook. Creating a method can be achieved in a few simple steps:

1. Select a chromatographic technique
2. Select a suitable template.
3. Select an appropriate column from the Column list.
4. If necessary, change the variable values.
5. Save the method under a new name.

The pre-constructed templates are quite complex, so we do not recommend you to make any changes other than those allowed in the Run Set-up Variables page. However, you can create your own templates as described in the UNICORN User Manual.

1.2.2 Method templates available in UNICORN



UNICORN provides templates for the following chromatographic techniques:

- Anion exchange chromatography (AIEX)
- Cation exchange chromatography (CIEX)
- Hydrophobic interaction chromatography (HIC)
- Reversed phase chromatography (RPC)
- Size exclusion chromatography (SEC)
- Affinity chromatography (AC)

Besides a basic template for each chromatographic technique, templates are provided to support the use of different alternatives for sample injection and fraction collection. The latter also provide a higher degree of flexibility regarding gradient profiles, etc. These templates are named by abbreviations according to the following principle: xxx_y_zz, where xxx represents type of sample injection, y represents the type of fraction collection and zz represents the technique. The table below lists the available options and the corresponding abbreviations.

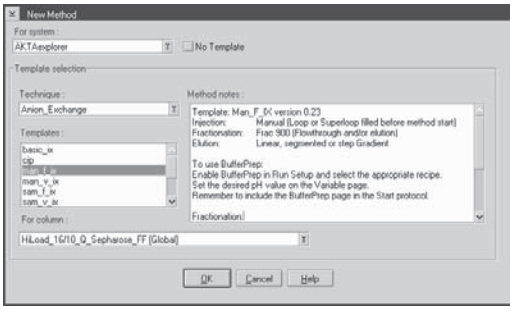
A number of special templates are also available. Their names, functions etc. are given in the table below.

1.3 Abbreviations in UNICORN method templates

1.3.1 Abbreviations for General Chromatography Templates

Type of Sample Injection			Type of Fraction Collection			Chrom. Technique	
Sample volume	Option	Abbreviation (xxx)	Fraction volume	Option	Abbreviation (y)	Option	ax, cx or ix[1]
0.1–2 ml	Fixed loop filled by sample pump	sam[1]	200 µl–70 ml	Fraction collector	f	Anion exchange or Cation exchange	gr
>2 ml	Via system pump	sys	2 ml–1 l	Outlet valve	v[1]	Reversed phase	gr
Fixed loop: 0.1–2 ml. Superloop: 1–150 ml	Manual	man	200 µl–70 ml	Peak fractionation	p	Hydrophobic interaction	gf
	Auto Sampler	aut				Size exclusion	ac
						Affinity Abbreviation (zz)	

[1] ÄKTAexplorer 100 only



1.3.2 Abbreviations for special feature templates

When selecting a template, **Method notes** will appear, summarising the characteristic of the template chosen.

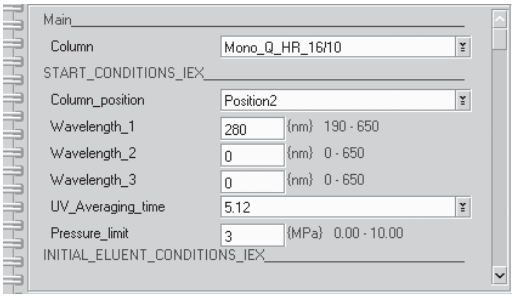
Abbreviation	Function	Available for	Type of injection	Type of fraction collection
varph_ax[1]	Optimizes running pH	Anion exchange	Fixed loop filled by sample pump	Fraction collector
varph_cx[1]	Optimizes running pH	Cation exchange	Fixed loop filled by sample pump	Fraction collector
cip	Cleans column automatically	All techniques	–	–
trioligo[1]	Standard method for the purification of synthetic oligo nucleotides	Anion exchange on SOURCE Q	Manual	Fraction collector

[1] ÄKTAexplorer 100 only

1.4 Method creation

The examples below serve to illustrate how the pre-constructed templates are accessed and used.

1.4.1 Example 1



Type of method to be created:

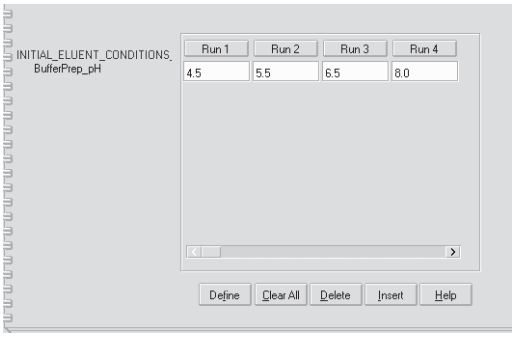
Anion exchange chromatography with the following characteristics:

- Sample injection via system pump.
- Elution by a linear gradient.
- Fraction collecting by multiple port outlet valve.

Actions:

1. Select **New: Method** in the menu bar of the Main Menu.
2. Select system to be used (here ÄKTAexplorer 100).
3. Select **Anion_Exchange** as the technique.
4. Select the template **sys_v_ix** (sys for sample application via System pump; v for fraction collection via multi-port outlet valve) from the **Template list**[1].
5. Check the characteristics of the template selected in **Method notes**.
6. Select an anion exchange column from the **columns list**[2].
7. Click on **OK**. The template method will now open.
8. Check or change the values of the method variables in the **Run Setup** Variables page.
9. Save the completed method under a new name.

1.4.2 Example 2



Type of method to be created:

Optimizing running pH for a purification on a cation exchanger.

Actions:

1. Select **New: Method** in the menu bar of the Main Menu.
2. Select type of system used (here ÄKTAexplorer 100).
3. Select **Cation_Exchange** as the technique.
4. Select the template **varph_cx** from the **Templates list**[1].
5. Check the characteristics of the template selected in **Method note**s.
6. Select a column from the **column list**[2].
7. Click on **OK**. The template method will now open.
8. Select the **BufferPrep ON button** and select the recipe 3.0 to 7.5 pH **CIEX** in the **Run Setup** BufferPrep page.
9. Check or change the values of the method variables in the **Run Setup** Variables page.
10. Enter a pH value for each consecutive run in the **Run Setup** Scouting page.
11. Save the completed method under a new name.

1 This template list shows only templates belonging to the technique chosen.

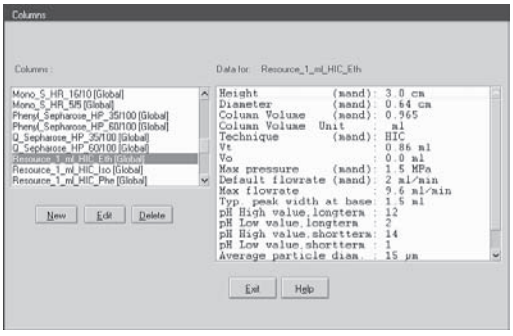
2 This Column list shows only columns belonging to the technique chosen.

1.5 About UNICORN column list

To make planning and running purification experiments with ÄKTAexplorer and ÄKTApurifier simple, a list of pre-packed
GE Healthcare columns is available in UNICORN.

The Column list is an integral part of the method creation procedure used in UNICORN. When a column in the list is selected, corresponding values for column-specific parameters like flow rate and pressure limit are automatically copied into the method. Other column-related information, such as typical values for peak volume, etc., will also appear. Operator-packed columns with corresponding column-specific parameter values may also be added to the list.

Full details about the UNICORN Column List are found in the UNICORN User Manual.



1.6 About automatic buffer preparation

ÄKTAexplorer and ÄKTApurifier are equipped with an automatic on-line buffer preparation unit called BufferPrep. BufferPrep prepares a selected eluent with defined pH and salt concentration from four stock solutions. It is intended primarily for unattended pH optimization experiments in ion exchange chromatography. However, for sample volumes up to 150 ml and flow rates up to 100 ml/min, it may also be used in general ion exchange chromatography experiments, and is thus very convenient when developing a purification protocol.

The following recipes are available in UNICORN:

Broad range buffers

Anion exchange	pH range	Cation exchange	pH range
AIEX mixture	5 to 9.5	CIEX mixture	3 to 7.5

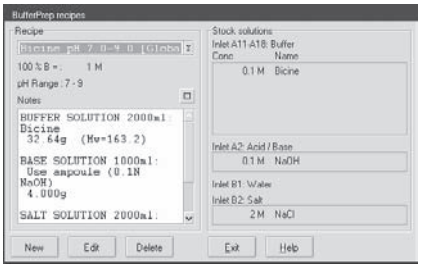
Narrow range buffers

Anion exchange	pH range	Cation exchange	pH range
0.1 M 1_methyl-piperazine	5.0–5.7	0.03 M Phosphate	2.2–3.3
0.1 M Bis-Tris	5.8–7.7	0.1 M Formate	2.5–4.5
0.1 M Piperazine	6.0–6.7	0.03 M Citrate	2.5–6.2
0.1 M Tris	7.5–8.5	0.1 M Acetate	3.8–5.7
0.1 M 1_methyl-piperazine	8.4–10.3	0.1 M Mes	5.5–6.7
0.1 M Piperazine	9.2–10.5	0.03 M Phosphate	6.2–7.6
		0.1 M Hepes	6.6–8.2
		0.1 M Bicine	7.0–9.0

Recipes may also be prepared according to your own needs (refer to ÄKTAexplorer or ÄKTApurifier User Manuals).

BufferPrep does not depend on pH measurements for the calculation of the correct mixing ratios, but uses an algorithm that takes into account both the pKa values of the buffer substances used and the salt content. For proper function, however, it is essential that the stock solutions are prepared with great accuracy.

Full details about how to operate BufferPrep are found in the ÄKTAexplorer User Manual or ÄKTApurifier User Manual.



1.7 About adviser in UNICORN

UNICORN provides on-line advice concerning:

- Method editing suited to your specific purification needs.
- All media and columns available for use with ÄKTAdesign™ systems.
- Detailed descriptions of the system components of ÄKTAdesign systems.
- Troubleshooting.
- Buffer preparation, both manual and automatic.

The method adviser provides detailed information about what template and column to choose, what variables to fill in and how the results are evaluated in different standard purification and optimization protocols. The method adviser works as a complement to this Method Handbook.

Adviser is accessible in UNICORN by clicking on the adviser button in the tool bar of the Main menu and Method editor.

2 The purification of biomolecules

2.1 Purpose of purification

Generally speaking, the reasons for purifying biomolecules may be twofold:

1) In a commercial situation, the final goal is to deliver material which fulfils certain criteria.

2) In a research project, the final goal is to gain information about the biochemical mechanisms and structures of the biomolecules involved.

Situation 1) very often aims at economically optimized purification and much effort is thus spent on optimizing the purification protocol. Screening media and scouting running conditions to optimize each constituent purification step are integral and justified parts of developing the protocol.

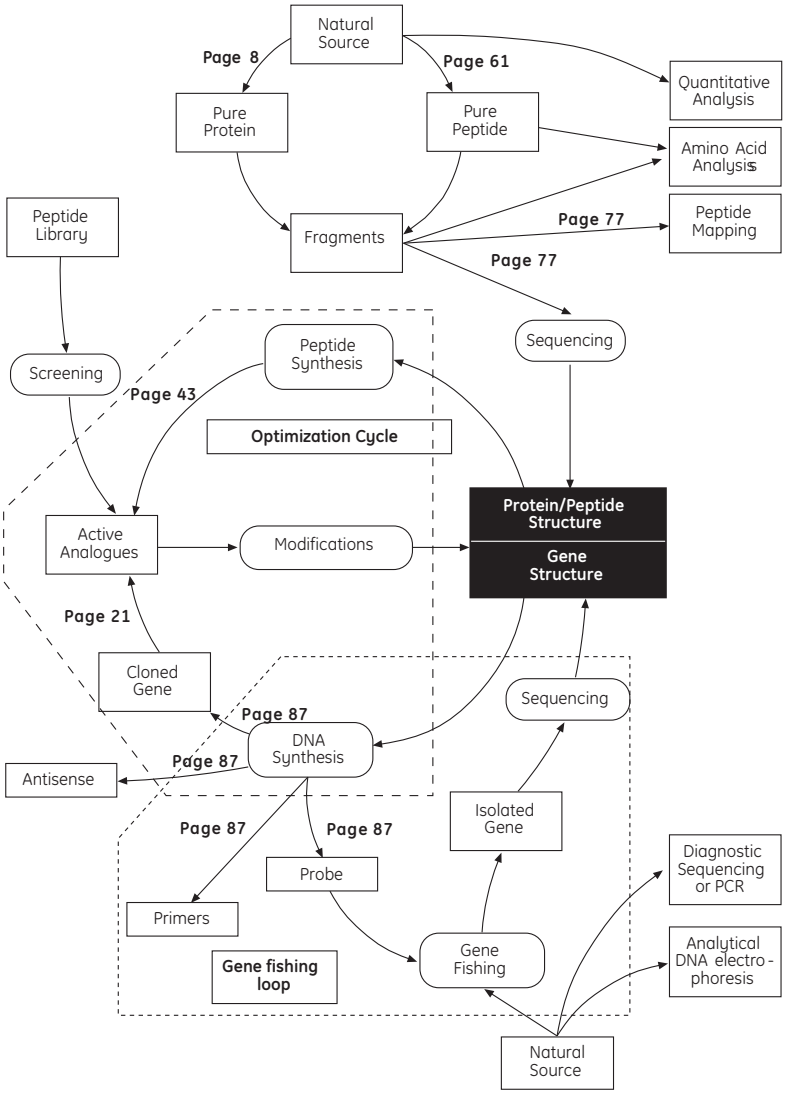
Situation 2), on the other hand, typically aims at obtaining the target molecule pure enough as soon as possible.

Here the purification in itself is not a final goal, but rather a necessary phase on the way to the final research goal. Standard purification protocols to render the target molecule pure enough will thus be the alternative of choice. Developing purification protocols "from scratch" will be turned to only if other alternatives are not at hand.

Obtaining information on the structure-activity relationship is often the ultimate goal in life science research and the main activities involved are shown in the scheme below.

Routes marked with Roman figures involve chromatography of proteins, peptides or nucleic acids.

2.2 The map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Analytical DNA electro-phoresis
Gene fishing loop
Natural Source

2.3 How to develop purification protocols

2.3.1 Introduction

Purification serves to deliver enough quantities of the target substance, pure enough and in a biological state to suit its further use. The requirements on the end product may vary considerably as may the type and amount of starting material, circumstances that will strongly influence the development of the purification. A structured way of developing a purification protocol is therefore the quickest and safest way to acceptable results.

Purity required is nearly always synonymous with *freed from interfering substances* and is thus determined by the intended further use of the substance to be purified. For instance, a certain enzyme may be judged pure enough for activity studies at a relatively moderate mass purity, provided that the end product is free of substances interfering with the activity of the enzyme and that the biological activity is retained.

Pure enough for structure analysis of the enzyme, on the other hand, means a very high mass purity, since interfering substances now include any protein or peptide, while biological activity will be of no relevance at all for the analysis.

Recovery or yield measures the relation between the amount of starting material applied and the amount of purified substance obtained after purification. It will determine the amount of starting material required to reach the separation goal. This, together with the richness and complexity of the starting material, heavily influences the strategy when planning the purification.

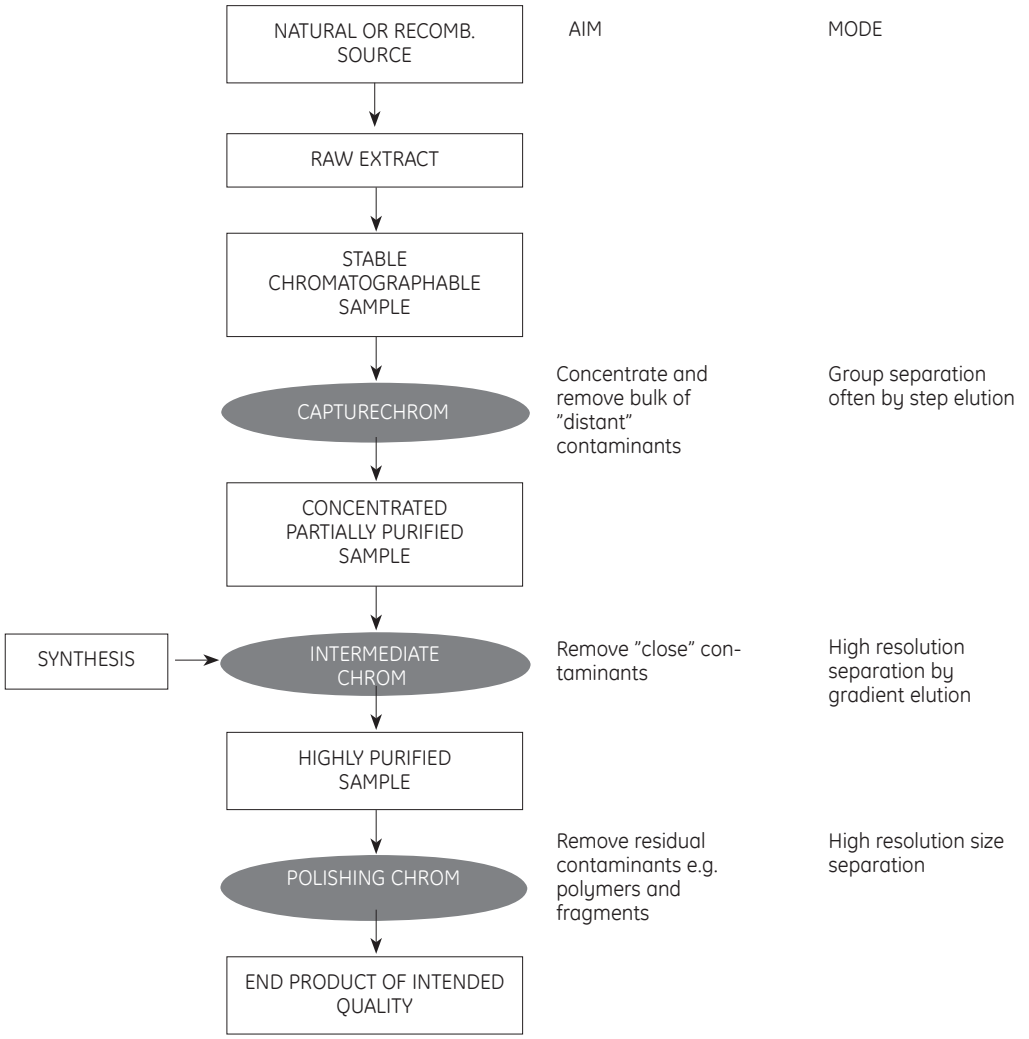
Sometimes the amount of starting material is very limited and full optimization of the purification protocol cannot be performed. In such cases, one has to rely upon a safe standard protocol requiring a minimum of adjustment and optimization steps. Such a protocol may not be optimal with respect to experimental time, recovery, economy, etc. but will be as close to the purification goal as the circumstances allow. However, when the available amount of starting material allows a more complete development of the protocol, this process should be driven to the point where the protocol is effective enough. The amount of work needed to reach the separation goal will depend mainly on the available information on sample and target molecule properties. It will therefore always pay off to gather such information from the literature or from databases.

2.3.2 General construction of purification protocols

The source of the substance to be purified strongly influences the construction of the purification protocol and can generally be divided into the following groups:

- Natural and recombinant sources.
- Synthetic sources.
- Fragments of a certain (pure) biomolecule.

A purification protocol can be described in general terms by the following scheme:



The different stages in the scheme address different types of purification problems, as explained below:

A stable chromatographable sample is a necessary pre-requisite when employing chromatographic techniques. Particulate matter and nucleic acids have to be removed or columns will be clogged or blocked. Proteases have to be rendered inactive or removed or the over-all yield will suffer.

The capture step aims at concentrating the sample and removing the bulk of the contaminants. Emphasis is here put primarily on speed and load capacity. Media with high load capacities and good flow properties are used often under step elution conditions.

The intermediate step(s) aims at separating the components of the concentrated, partially purified sample. Since the components remaining at this stage have rather closely related chromatographic properties, emphasis has to be put on maximum resolution. This is accomplished by using gradient elution on media of smaller bead sizes to provide high efficiencies and by combining techniques of independent selectivities. Optimizing resolution means that flow rates and load capacities will be restricted. On the other hand, both the volume and amount of material are much reduced at this stage of the purification.

The polishing step(s) serves to eliminate contaminants such as polymer etc,. and is also often used to condition the final product, e.g. to remove salts, when the final product is to be lyophilised.

The number of steps necessary to obtain the intended quality of the end product depends primarily on the nature of the starting material and the choice and optimization of the different techniques used. The complexity of natural and recombinant sources calls for a reduction of both sample volume and amount before the onset of high resolution techniques or the purification will be both unnecessarily expensive and time-consuming.

When the starting material consists of synthetic peptides or oligonucleotides, the main problem is to eliminate wrongly synthesised components. Normally these have chromatographic properties rather close to the target substance, while the number and amount of contaminants is rather limited. For this kind of sample, high resolution techniques have to be employed from the very start.

No chromatographic technique will provide 100 % yields of active material and the overall yield will therefore depend on the number of steps included in the purification protocol.

	Over-all recovery after n steps				
Recovery per step (%)	**n = 1**	**n = 2**	**n = 3**	**n = 4**	**n = 5**
99	99.0	98.0	97.0	96.1	95.1
95	95.0	90.3	85.7	81.5	77.4
90	90.0	81.0	72.9	65.6	59.0
80	80.0	64.0	51.2	41.0	32.8
70	70.0	49.0	34.3	24.0	16.8

To minimise the number of steps, method development should aim at optimizing each step for the intended purpose and arrange them in an order that minimises inter-step treatments.

A typical purification protocol for protein purification starts with IEC. The medium employed is a Fast Flow or HR type and packed in short and wide columns to allow high flow rates. HIC is then typically used for the first intermediate step. The column is now smaller and longer to allow elution with high-resolving shallow gradients. Since selectivity in HIC is rather independent of running pH, and descending salt gradients are used, conditioning the sample for this step is done simply by adding ammonium sulphate to match the buffer A concentration. If HIC had been used before IEC, the ionic strength would have to be lowered to fit that of buffer A for the IEC step either by dilution, dialysis or buffer exchange on a gel filtration column. This would have cost extra time and possibly some loss of active material.

Polishing is then performed on a gel filtration column and the purification is complete.

The standard purification protocol for proteins is built up this way. The conditions are, however, chosen to cover a broad range of target proteins.

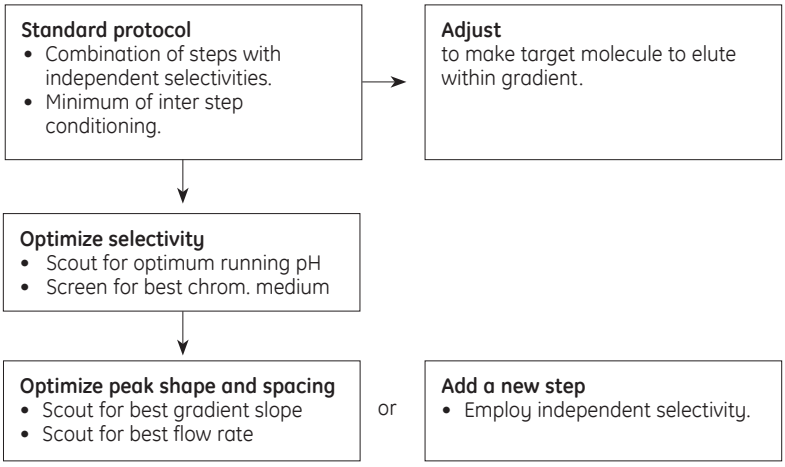
If the purity is insufficient, however, optimization of the different steps is performed or an extra intermediate purification step is added. Very often this extra step is another IEC step, but this time under completely different conditions.

2.3.3 Basic principles for the development of a purification protocol

The scheme below gives the main elements involved in developing a purification protocol.

By performing the optimization measures in the order of their effect on the final result, the “pure enough level” is reached rather quickly and without much trouble. In practice, however, the standard protocol alone often provides enough purity.

In some situations, the amount of sample allows only for a minimum of optimization. Here the best way to reach the final goal is to start with the standard protocol and when necessary add new steps with independent selectivities rather than employing scouting measures.



3 Planning protein purification

3.1 Introduction

The purification of proteins from natural and recombinant sources generally requires multi-step purification protocols, unless an affinity chromatography method specific for the target protein is available. The effectiveness of such protocols, measured in terms of end product quality, yield, recovery of biological activity and experimental time, is heavily dependent on their proper construction. In the majority of cases, however, satisfactory results are readily arrived at just by following a set of simple rules.

This section will help you through the different steps involved in developing a purification protocol adapted to your specific needs.

3.1.1 The purification protocol development scheme

A detailed scheme for the development of protein purification protocols is presented on page 25. This helps you to decide which path to take in different situations and refers you to a set of step-by-step protocols used at the different stages outlined in the scheme.

Development work involves four basic phases:

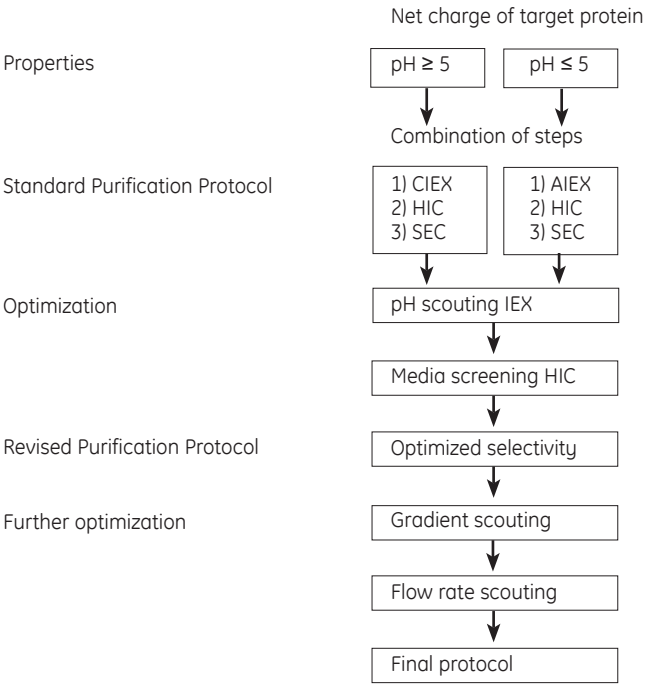
1. **Forming a platform for the development**
   The platform should include:
   - Intended quantity and quality of the end product.
   - Available information on the physical properties of the target protein.
   - Available information on the type of starting material and content of the target protein.
   - Information on the range of conditions compatible with sample stability (the sample stability window, SSW).
2. **Running initial purification experiments.**
   The Standard Purification Protocol for proteins from recombinant and natural sources is used as a starting point for the development. In many cases, the Standard Purification Protocol will provide sufficient purity, while in others it may need to be further optimized.
3. **Optimizing the purification protocol.**
   Guided by the outcome of the standard protocol experiment, the purification steps are optimized and/or recombined until satisfactory results are obtained.

4. Adapting the scale of the final purification protocol.

By following simple rules, the purification protocol can be adapted to perform almost identically at any chosen scale.

Note: An already existing purification protocol may also be optimized. The development scheme is then analogous to that given above.

Purification strategy



If the target protein is incompatible with HIC, this technique is replaced by IEX complementary to that of step one. This require an extra conditioning step.

If the final purity is unsatisfactory despite the optimization measures above, another step is added.

3.1.2 General purification protocol development scheme

		Protocol on page	Further reading
1.	**Purification protocol platform**		
1.1	Establish the intended final quality of the target protein.	27	
1.2.	List available information on: • Target protein properties. • Starting material properties.	27	133
1.3	Perform a Sample Stability Test to establish the sample stability window (SSW). • If the sample stability is incompatible with HIC, proceed to # 3.2. • If the sample stability is incompatible with pH 4.5, proceed to # 3.1.		125
2.	**Initial purification experiment**		
2.1	Run the Standard Purification Protocol within the SSW.		
	• If the results obtained are OK, proceed to # 7.	28	
	• If the target protein does not elute within the gradient of step one or if the final purity is too low, proceed to # 3.1.	33	136
	• If the target protein does not elute within the gradient of step two, proceed to # 4.	33	142
3.	**Optimization of the IEX running pH**		
3.1	Perform pH scouting for CIEX and AIEX within the SSW to establish the running pH providing the best resolution. • With AIEX, increase the running pH in steps of one pH unit from the target protein pI to the upper pH limit of the SSW. • With CIEX, decrease the running pH in steps of one pH unit from the target protein pI to the lower pH limit of the SSW. • Analyse gradient eluted fractions only in runs lacking the target protein in the flow-through fraction. • Select the running pH providing the highest purity of the target protein. • Replace step one of the Standard Purification Protocol with the IEX method selected above and run the revised Standard Purification Protocol. • If the results obtained are OK, proceed to # 7. • If the final purity is too, low proceed to # 4.	35	136
3.2	Perform pH scouting for CIEX and AIEX within the SSW as described in # 3.1 above and revise the Standard Purification Protocol as follows: • Replace step one with the pH optimized IEX method leaving max background protein unbound. • Replace step two with the pH optimized IEX complementary to that of step one. • Run the revised Standard Purification Protocol. • If the results obtained are OK, proceed to # 7. • If the final purity is too low, proceed to # 5.1.	35	136

4.	**Screening for optimal HIC medium**	28	134
4.1	Perform HIC media (ligand) screening within the SSW. • Analyse gradient eluted fractions only in the runs lacking target protein in the flow-through fraction. • Select the HIC medium providing the highest purity of the target protein. • If the target protein does not elute, remains unbound or if the recovery is substantially reduced, replace HIC with the IEX method from # 3.1 complementary to that of step one. • Revise the Standard Purification Protocol and rerun it. • If the results obtained are OK, proceed to # 7. • If the final purity is too low, proceed to # 5.		
5.	**Gradient optimization**	29	
5.1	Scout the IEX and/or the HIC steps for the minimal gradient length proving satisfactory resolution. • Revise the Standard Purification Protocol and re-run it. • If the results obtained are OK, proceed to # 7. • If the final purity is too low, proceed to # 6.		
6.	**Addition of step(s)**	30	136
6.1	Add a RPC step if compatible with the sample stability.		
6.2	Add a complementary IEX (# 3.1) step or fine adjust the running pH for the already present IEX step(s).	29	128
7.	**Scaling up of the final purification prototcol**	92	
7.1	The following procedure will not alter the final purity provided media used, column lenghts, linear flow rates and gradient parameters are kept constant. • For each step of the protocol, increase the column volume in proportion to the intended increase in sample load in step one. • With pre-packed columns volume should be chosen to be larger or equal to the theoretical value.		

3.1.3 Planning platform protocol

End product quality

Quality aspect	Level	Determined by
Activity retained		
Total protein content		
Purity		
Content of defined	Nucleic acids: contaminants	Nucleic acids:
	Endotoxins:	Endotoxins:
	Others:	Others:

Target protein description

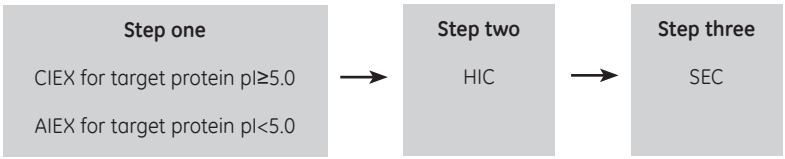
Property	Value	Determined by
Molecular mass		
Isoelectric point		
	pH range:	
Stability	$(NH_4)_2SO_4$ range:	

Starting material description

Property	Value	Determined by
Total protein content		
Total protein content		
Location of target protein		

3.2 Standard purification protocol for proteins

*The Standard Purification Protocol for protein*s is based on the combination of IEX, HIC and SEC, techniques with selectivities that are highly independent of each other. The order of steps is chosen to fully utilise the high loading capacity of IEX at the beginning of the purification and to avoid any inter-step treatments other than adding salt to the sample prior to the HIC step.



Running CIEX as the first step will effectively remove most of any DNA and endotoxins present in the starting material. Crude extracts from *E. coli* often precipitate at pH < 4.5 thus AIEX is recommended for target proteins having a pI lower than 5.0.

The Standard Purification Protocol in most cases provides a final purity in the range of 90–95 %.

Step one, ion exchange chromatography

Sample preparation

- Check that the sample is stable at running pH (see below) and in the range 0.1–1.0 M NaCl.
- Adjust sample conditions to match those of eluent A.
- Filter or spin the sample to remove any residual particulate matter.

Method

For target protein pI ≥5.0 or when the pI is unknown, run CIEX at pH 4.5.

For target protein pI <5.0, run AIEX at pH 8.0.

- Select a column to match the amount of sample.
- Use the default values for gradient and flow rate provided by the template.

Eluents

Use BufferPrep recipes 3.0–7.5 CIEX or 5.0–9.5 AIEX respectively.

Evaluation

- If the target protein elutes as a reasonably symmetrical peak within the gradient and the recovery of active material is satisfactory, proceed to STEP TWO.
- If the target protein elutes in the flow-through fraction, change the type of ion exchanger or perform pH scouting (page 35).
- If the target protein elutes during regeneration only, consult Trouble-shooting on page 101 or perform pH scouting (page 35).
- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101 or perform pH scouting (page 35).

Step two, hydrophobic interaction chromatography

Sample preparation

- Make up to 1.2 M with ammonium sulphate or to the maximum concentration compatible with sample stability.
- Filter or spin the sample to remove any residual particulate matter.

Method

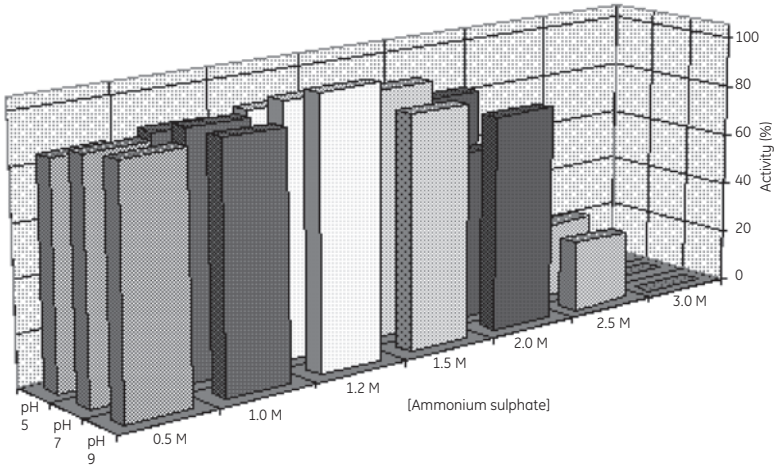
- Select a HIC column to match the amount of sample.
- Use the default values for gradient and flow rate provided by the template.

Eluents

- Prepare the following eluents:
  - Eluent A: 50 mM phosphate pH 7.0 containing the maximum concentration of ammonium sulphate compatible with sample stability.
  - Eluent B: 50 mM phosphate pH 7.0.
- Degas the eluents and filter through a 0.45 µm filter.

Evaluation of this step

- If the target protein elutes as a reasonably synmmetrical peak within the gradient and the recovery of active material is satisfactory, proceed to STEP THREE.
- If the target protein elutes in the flow-through fraction, perform HIC media screening (page 36).
- If the target protein does not elute or if the recovery is substantially reduced, replace HIC with the IEX method complementary to that of STEP ONE.
- If peak symmetry is unsatisfactory, consult Trouble-shooting on page 101.



Stability test of alkaline phosphatase in a sample from E. coli.

The diagram shows the enzyme activity as a function of ammonium sulphate concentration at different pH values. The enzyme tolerates up to 1.5 M ammonium sulphate in the pH interval tested.

Step three, size exclusion chromatography

Sample preparation

- Filter or spin the sample to remove any residual particulate matter.
- Adjust the sample concentration to ≤ 5 %.

Method

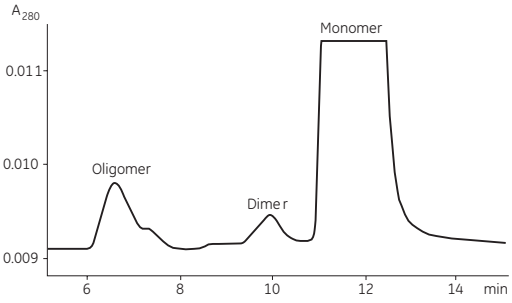
- Select Superdex™ 200 for target proteins with Mr > 50 000 or Superdex 75 for target proteins with Mr < 50 000.
- Select column dimensions to match the sample volume.
- Use the default flow rate provided by the template.

Eluents

- Prepare 150 mM NaCl in 50 mM phosphate, pH 7.0.
- Degas the eluent and filter through a 0.45 µm filter.

Evaluation

- If recovery or peak symmetry suffers, consult Trouble-shooting (page 101).
- If resolution is unsatisfactory, perform scouting for optimal flow rate (page 40).



Conditions

Sample: Preparation from an experimental batch of rhGH.

Sample volume: 50 µL.

Column: Superdex 75 HR 10/30.

Detection: UV at 280 nm.

Eluent: 0.1 M Na_2SO_4 in 0.05 M phosphate, pH7.3.

Flow rate: 1 ml/min.

3.3 *Adjustment and optimization*

3.3.1 Adjustment of the standard purification protocol

To work properly, the Standard Purification Protocol requires:

1) The sample to be compatible with the running conditions of the different steps.

2) The target protein to elute within the gradients of the different steps.

Incompatibility between running conditions and sample seldom occurs in IEX at the pH values recommended. However, such problems sometimes exclude the use of HIC.

Incorrect elution in IEX is most likely to happen when the pI of the target protein is unknown. In HIC, this may happen when the target protein does not fully adsorb under conditions compatible with sample stability. If necessary, adjust the Standard Purification Protocol according to the table below.

Problem	Actions	
	step one (IEX)	step two (HIC)
Incorrect elution or conditions not within sample stability window	1. Change to the alternative ion exchanger and pH given in step one.	1. Screen for a HIC medium allowing binding at compatible . conditions (see page 36)
	2. Scout for optimal running pH within the sample stability window (see page 35).	2. Replace HIC with IEX complementary to that used in step one.

3.3.2 Optimizing the final purity

When the Standard Protocol does not provide satisfactory purity, optimization of the individual steps must be carried out. However, optimization should advance only to the stage where the purification goals are fulfilled. This optimization protocol therefore deals with optimization parameters in the order of decreasing influence on the final purity (see table below) and stops when satisfactory results are reached. Measures influencing selectivity such as the combination of techniques, pH in IEX and choice of ligand in HIC, have the strongest impact on the final purity and should be dealt with first.

Adding an extra step will certainly affect the overall selectivity. However, chromatography techniques other than those already used in the Standard Protocol are all more or less limited in their application to protein purification and are often associated with low recoveries. For this reason, the addition of a RPC or a complementary IEX step comes only in fifth place in the table below.

It is often difficult to judge the overall effect of any single optimization effort by just looking at individual steps, since it is the contaminants that are not removed by "later steps" that need to be better resolved from the target protein. The success of optimization measures should therefore be judged by their effect on the whole protocol, i.e. the final result.

Optimization measure	Effects	Comments
A. Scout for optimal running pH in IEX	Influences elution order and spacing of peaks	Choosing the HIC medium is often a trade-off between binding strength and sample stability
B. Screen for optimal HIC medium	Influences salt conc. needed for binding, elution order and spacing of peaks	Reducing the gradient slope will broaden peaks
C. Scout for optimal gradient slope in IEX	Influences mainly spacing of peaks	Reducing the gradient slope will broaden peaks
C. Scout for optimal gradient slope in HIC	Influences mainly spacing of peaks	Useful examples are RPC when applicable or IEX complementary to step one. May reduce final recovery
D. Add a new step	Increases the overall selectivity	
E. Scout for optimal flow rates	Decreases peak width	Only limited effect on final purity

A. Optimizing running pH in IEX

Sample preparation

- For CIEX, reduce ionic strength and adjust sample pH to equal the most acidic run planned.
- For AIEX, reduce ionic strength and adjust sample pH to equal the most alkaline run planned.

Method

- For CIEX, decrease the running pH in steps of 0.5–1.0 pH units from the target protein pI to the lower limit of the sample stability window.
- For AIEX, increase the running pH in steps of 0.5–1.0 pH units from the target protein pI to the upper limit of the sample stability window.
- Use the default values for gradient and flow rate provided by the template.

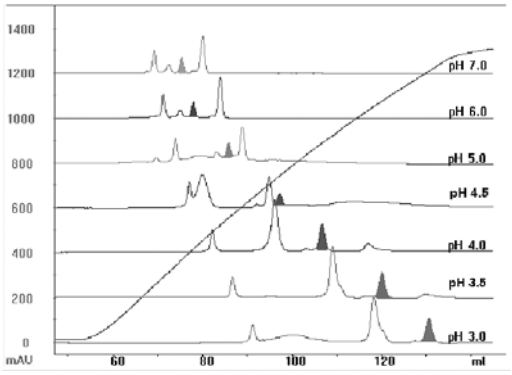
Eluents

Use BufferPrep recipes 3.0–7.5 CIEX or 5.0–9.5 AIEX respectively.

Evaluation

Select the running pH that provides the highest purity.

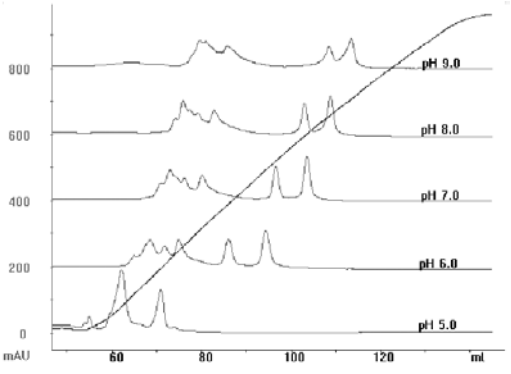
CIEX, pH range 3.0–7.5.



Conditions

Column:	Mono S™ HR 10/10.
Column volume:	8 ml.
Detection:	UV at 280 nm.
Buffer:	BufferPrep, CIEX pH range pH 3–7.5.
Gradient:	0 → 0.5 M NaCl.
Gradient vol:	10 column volumes.
Flow rate:	4.0 ml/min.
Sample volume:	500 µl.

AIEX, pH range 5.0–9.0.



Conditions

Column:	Mono Q™ HR 10/10.
Column volume:	8 ml.
Detection:	UV at 280 nm.
Buffer:	BufferPrep, AIEX pH range 5–9.5.
Gradient:	0 → 1.0 M NaCl.
Gradient vol:	10 column volumes.
Flow rate:	4.0 ml/min.
Sample volume:	500 µl.

B. Media screening for HIC

Sample preparation

- Adjust the content of ammonium sulphate to match that of eluent A.
- Adjust the sample pH to approximately 7.0.

Method

- Use the default values for gradient and flow rate provided by the template.

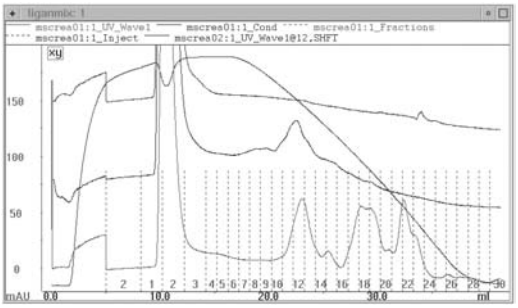
Eluents

- Prepare the following eluents:
  Eluent A: 50mM phosphate pH 7.0 containing the maximum concentration of ammonium sulphate compatible with sample stability.

  Eluent B: 50 mM phosphate pH 7.0.
- Filter the eluents through a 0.45 µm filter and degas them.

Evaluation

Select the HIC medium providing the highest purity and an acceptable recovery of the target protein.



Conditions

Sample:	Partically purified alkaline phosphatase.
Columns:	HIC test kit consisting of 1. RESOURCE™ 15ETH. 2. RESOURCE 15ISO. 3. RESOURCE 15PHE.
Column volume:	1 ml
Buffer A:	2.0 M ammonium sulphate in 100 mM phosphate, pH 7.0
Buffer B:	100 mM phosphate, pH 7.0
Gradient:	1.2 → 0 M Ammonium sulphate
Gradient vol:	20 column volumes.
Flow rate:	1.0 ml / min.
Sample volume:	1.0 ml.

Screening of HIC media for the purification of alkaline phosphatase.

C. Gradient optimization

Sample preparation

- Adjust sample conditions to match those of Eluent A.

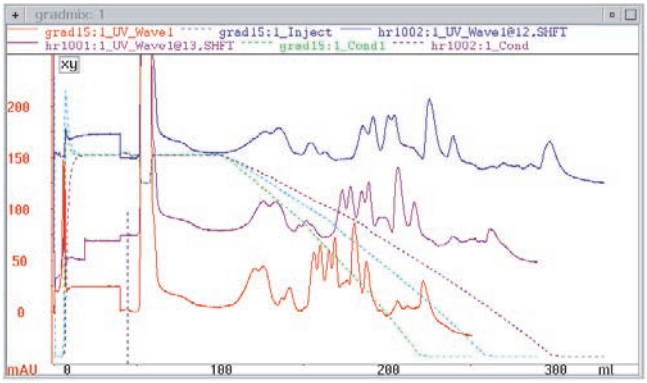
Method

- Use the medium and conditions arrived at above and the default flow rate provided by the template.

Evaluation

Select the steepest gradient providing satisfactory purity of the target protein.

Gradient volume scouting



Conditions

Sample:	Partically purified alkaline phosphatase.
Medium:	SOURCE™ 15PHE.
Column:	HR 10/10.
Column volume:	8 ml.
Buffer A:	2.0 M ammonium sulphate in 100 mM phosphate, pH 7.0
Buffer B:	100 mM phosphate, pH 7.0
Gradient:	1.2 → 0 M ammonium sulphate.
Gradient volume:	1. 15 column volumes. 2. 20 column volumes. 3. 25 column volumes.
Flow rates:	2.0 ml / min.
Sample volume:	7 ml.

D. Adding a new step

I. IEX

CIEX and AIEX may provide rather different selectivities even though the same sample property is utilised. Even a change in running pH may provide a useful change in selectivity. The wide applicability makes IEX one of the primary options.

The new step may either be inserted before or after STEP TWO of the Standard Protocol.

Choice of medium

Choose an ion exchanger complementary to the IEX step already present in the Standard Protocol.

Sample preparation

Adjust the sample pH and ionic strength to match that of eluent A.

Method

Use the default values for gradient and flow rate provided by the template.

Eluents

Within the BufferPrep broad range recipes, select a running pH at the opposite side of the target protein pI compared with that of STEP ONE.
The deviation from pI should be at least one pH unit.

II. RPC

RPC constitutes an excellent additional step when applicable. However, many proteins are either damaged by RPC conditions or irreversibly adsorbed to the column. This is especially true for large proteins or proteins with labile tertiary structures. However, the applicability of RPC broadens when the target protein does not have to be recovered in its native form.

Since the practical loading capacities of RPC media are generally rather limited, insert the new RPC step after STEP TWO of the Standard Protocol.

Choice of medium

The use of a polymer-based RPC medium may be advantageous over a silica-based one, since irreversibly adsorbed protein contaminants can be removed by alkali without harming the medium.

Sample preparation

Adjust the sample pH to match that of Eluent A.

Method

Use the default values for gradient and flow rate provided by the template.

Eluents

Proteins in general are not stable at very low pH values.
Thus run this step at pH in the interval 3 to 9.

E. Flow rate optimization

Sample preparation and eluents

See the original method.

Method

Minimum peak widths are obtained at very low flow rates with proteins.

Thus start with the default flow rate provided by the template and decrease it in rather large steps.

Select the maximum flow rate providing satisfactory resolution.

4 Planning peptide purification

4.1 Introduction

Peptides are substantially more stable than proteins and thus tolerant to a much broader range of conditions, such as high concentrations of organic solvents, extremely acidic conditions like TFA or 70 % formic acid and even boiling. Unlike most proteins, peptides are not normally inactivated by reversed phase chromatography. RPC provides very high resolution and has therefore become the primary purification technique for peptides. As with proteins, peptide extracts originating from natural sources are very complex and require multi-step purification protocols.

By contrast, peptide synthesis can often be carried out with great precision, leaving just a few by-products such as synthetic failures and modified peptides as the major contaminants. However, these side products are often structurally closely related to the target peptide and high resolution techniques are required to remove them.

Peptide mapping, i.e. the analysis of protein fragments produced by controlled proteolysis, is normally performed as a one-step procedure in which the pattern of the separated fragments provides the required information. However, with the introduction of MALDI TOF for molecular weight determination of the fragments, or when the primary structure of individual fragments is to be determined, precise fraction collection becomes essential.

The Standard Purification Protocol for peptide fragments starts with optimizing a one-step procedure based on RPC. Should these optimization efforts fail to separate all fragments needed, acidic RPC is combined with neutral RPC in a two-step procedure. In certain cases, adding an alternative technique is advantageous

4.1.1 The purification protocol development scheme

Detailed schemes for the development of peptide purification protocols are presented in the separate sections for:

- Synthetic Peptides (page 43)
- Peptides from Natural Sources (page 59)
- Protein Fragments (page 75)

These help you decide which path to take in different situations and refer you to a set of step-by-step protocols used at the different stages outlined in the scheme.

The development work involves four basic phases:

1. **Forming a platform for the development**
   The platform should include:
   - Intended quality of the end product.
   - Available information on the physical properties of the target peptide.
   - Available information on the type of starting material and content of the target peptide.
   - Information on the range of conditions compatible with sample solubility.
2. **Running initial purification experiments.**
   The Standard Purification Protocol is used as a starting point for the development. In many cases, the Standard Purification Protocol will provide sufficient purity, while in others it may need to be further optimized.
3. **Optimizing the purification protocol.**
   Guided by the outcome of the standard protocol experiment, the purification steps are optimized and/or recombined until satisfactory results are obtained.
4. **Adapting the scale of the final purification protocol.**
   By following simple rules, the purification protocol can be adapted to perform almost identically at any chosen scale.

Notes and comments

Testing the solubility of the target peptide is essential since this gives information on which techniques to use and what conditions to apply.

Physical properties of peptides with known structure can be calculated to an extent meaningful to purification. Since peptides expose all or most of the constituent amino acid side chains, these will directly influence the chromatographic behaviour. It may thus be of considerable benefit to calculate hydrophobicities, titration curves and number of charges as a function of pH when planning peptide purification.

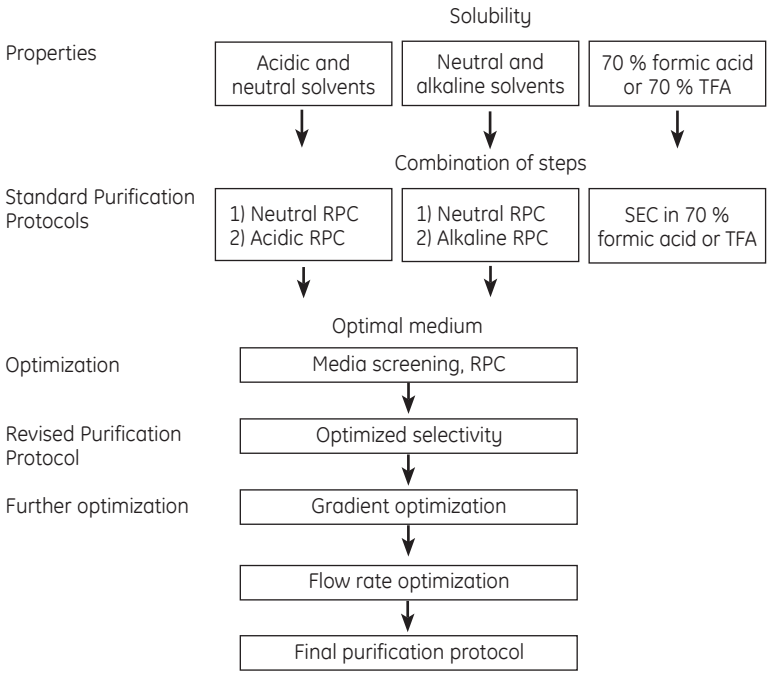
An already existing purification protocol may also be optimized. The development scheme is analogous to that given above.

5 Planning synthetic peptide purification

5.1 Introduction

Peptide synthesis can often be carried out with great precision, leaving just a few by-products such as synthetic failures and modified peptides as the major contaminants. However, these side products are often structurally closely related to the target peptide and high resolution techniques are required to remove them. (See also chapter 4).

5.1.1 Purification strategy



5.1.2 General purification protocol development scheme

1.	Purification planning platform.	Protocol on page	Further reading
1.1	Establish intended final quality of target peptide.		
1.2.	List available information on: • Target peptide properties. • Starting material properties.	46	
1.3	Perform a Sample Solubility Test. • If soluble both in acidic and in neutral solvents proceed to # 2.1. • If soluble only in neutral and basic solvents proceed to # 2.2. • If soluble only in alkaline solvents proceed to # 2.3.		125
2.	**Initial purification experiments.**		
2.1	Run the Standard Purification Protocol I. • If the final purity obtained is OK, proceed to # 5. • If the final purity is too low, proceed to # 3.1.	48	144
2.2	Run Standard Purification Protocol II. • If purity obtained is OK, proceed to # 5. • If purity obtained is too low, proceed to # 2.3.	50	144
2.3	Run C8, C18 and RESOURCE RPC according to the Media Screening Protocol, neutral eluent system. • If the purity obtained is OK, proceed to # 5. • If the purity obtained is too low, proceed to # 4.	56	144

3.	Screening for optimal RPC medium	Protocol on page	Further reading
3.1	Run C8, C18 and RESOURCE RPC according to the Media Screening Protocol, acid eluent system. • Select the medium giving the highest purity. • If the purity obtained is OK, proceed to # 5. • If the purity obtained is too low, proceed to # 3.2 for peptides soluble at acid and neutral pH or to # 4.1 for peptides soluble at acidic pH only.	56	144
3.2	Run C8, C18 and SOURCE RPC according to the Media Screening Protocol, neutral eluent system. • Select the medium providing the highest purity. • If the purity obtained is OK, proceed to # 5. • If the purity obtained is too low, proceed to # 3.3.	56	144
3.3	Run the Standard purification protocol with the medium selected in #3.1 and 3.2 • If the purity obtained is OK, proceed to # 5. • If the purity obtained is too low , proceed to # 4.1.		144
4.	**Gradient optimization**	58	144
4.1	Run the Gradient Optimization Protocol for RPC with the selected media and eluent systems. Select the gradient providing satisfactory purity at minimum gradient lenght.		
5.	**Scaling up of the final purification prototcol**	99	
5.1	The following procedure will not alter final purity provided media used, column lenghts, linear flow rates and gradient parameters are kept constant. For each step of the protocol, increase the column volume in proportion to the intended increase in sample load in step one. With pre-packed columns volume should be chosen to be larger or equal to the theoretical value.		

5.1.3 Planning platform protocol

End product quality

Quality aspect	Level	Purity Check method
Total peptide content		
Purity		

Target peptide description

Calculated properties	Value	Calculation method
Molecular mass		
Hydrophobicity		
Isoelectric point		
Net charge at pH 2.0:		
Net charge at pH 7.0:		
Net charge at pH 9.0:		

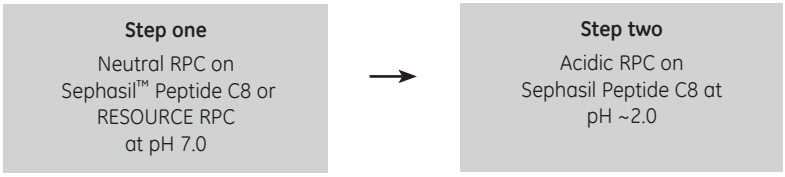
Target peptide solubility

Solvent	Solubility		
	0 % ACN	10 % ACN	20 % ACN
0.1 % TFA pH ~2			
20 mM Phosphate pH 7.0			
20 mM Tris-HCl pH 9.0			

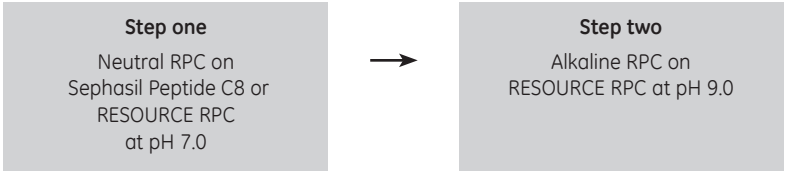
5.2 Standard purification protocol for synthetic peptides

The Standard Purification Protocol for synthetic peptides combines two RPC steps under conditions providing quite different selectivities and will, in most cases, be able to render a sufficiently pure target peptide.

Protocol I,
for peptides soluble in acidic and neutral solvents



Protocol II,
for peptides soluble in neutral and alkaline solvents



Optional additions to protocol I & II

The following alternative techniques may be added to the standard protocols when purity is unsatisfactory even after normal optimization has been carried out.

Addition alternative I: CIEX at pH 3.0 with 30 % ACN added to the eluent.

Addition alternative II: SEC in eluents containing ACN.

For details in the protocols, see the following pages!

5.2.1 Protocol I, for peptides soluble in neutral and acidic solvents

Sample preparation

- Check that the sample is stable at neutral and acidic conditions, and in 0 to 60 % ACN.
- Adjust sample conditions to match those of eluent A.

Column

- Select a Sephasil Peptide C8 for STEP ONE.
- Select a Sephasil Peptide C8 or a RESOURCE RPC column for STEP TWO.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

Prepare the following eluents:

Step one:

- Eluent A: 10 mM ammonium phosphate pH 7.0 in water.
- Eluent B: 10 mM ammonium phosphate in water containing 60 % acetonitrile (final conc.).

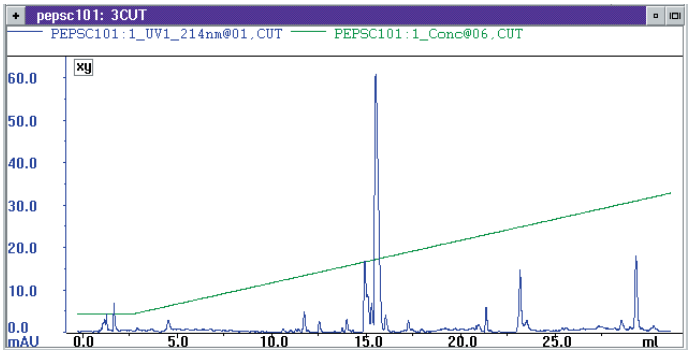
Step two:

- Eluent A: 0.06 % TFA in water.
- Eluent B: 0.05 % TFA in water containing 60 % acetonitrile (final conc.).

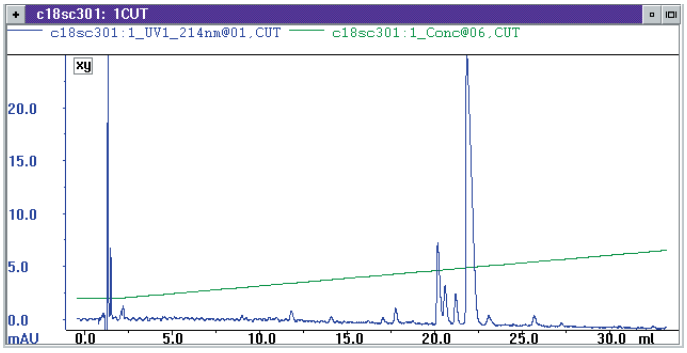
Evaluation

- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101.
- Check purity of collected fractions by:
  - Chromatography on µRPC or Mini S.
  - Capillary electrophoresis.
  - MS.
- If purity is unsatisfactory, proceed to Optimization on page 54 or add an alternative step (page 58).

Step one



Step two



Purification of a synthetic peptide with the structure:
EPPTAAREKQYLINSQQKSLLQPGEKLVSD.

Conditions

Column:	Sephasil Peptide C18 5 µm ST 4.6/100.
Detection:	UV at 214 nm.
Running pH:	Step one 7.0.
	Step two 2.5.
Gradient length:	20 CV.
Gradient concentration:	Step one 10–40 % B.
	Step two 25–35 % B.
Injected amount:	0.6 mg.

5.2.2 Protocol II, for peptides soluble in neutral and alkaline solvents

Sample preparation

- Check that the sample is stable at neutral and alkaline conditions and in 0 to 60 % ACN.
- Adjust sample conditions to match those of eluent A.

Column

- Select a Sephasil Peptide C8 or RESOURCE RPC column for STEP ONE.
- Select a RESOURCE RPC column for STEP TWO.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

Prepare the following eluents:

Step one:

- Eluent A: 10 mM ammonium phosphate pH 7.0 in water.
- Eluent B: 10 mM ammonium phosphate in water containing 60 % acetonitrile (final conc.).

Step two:

- Eluent A: 20 mM Tris-HCl pH 9.0 in water.
- Eluent B: 20 mM Tris-HCl pH 9.0 in water containing 60 % acetonitrile (final conc.).

Evaluation

- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101.
- Check purity of collected fractions by:
  - Chromatography on µRPC or Mini S
  - Capillary electrophoresis
  - MS
- If purity is unsatisfactory, proceed to Optimization on page 54 or add an alternative step (page 58).

5.2.3 Addition alternative I, cation exchange

Sample preparation

- Check that the sample is stable at acidic conditions and in 30 % ACN.
- Adjust sample conditions to match those of eluent A.

Column

- Select a Mono S or a RESOURCE S column.

Method

- Use the default values for gradient and flow rate provided by the template.

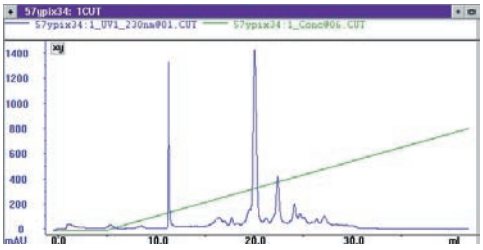
Eluents

Prepare the following eluents:

- Eluent A: 5 mM phosphate pH 3.0 in water containing 30 % acetonitrile (final conc.).
- Eluent B 5 mM phosphate pH 3.0 in water containing 30 % acetonitrile and 1.0 M NaCl (final conc.).

Evaluation

- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101.
- Check purity of collected fractions by:
  - Chromatography on µRPC.
  - Capillary electrophoresis.
  - MS.



Conditions

Sample:	200 µg of crude synthetic peptide.
Column:	Mono Q HR5/5.
Detection:	UV at 230 nm.
Eluent A:	30 % acetonitrile in 5 mM sodium phosphate, pH: 7.0.
Eluent B:	Eluent A containing 0.75 mol/L NaCl.
Gradient:	0–75 % B in 40 column volumes.
Flow rate:	1 ml/min.

Purification of an acid labile synthetic peptide by IEX.

Peptide sequence: ISPDGHEYIY(PO$_3$)VDPMQLPY

5.2.4 Addition alternative II, size exclusion chromatography

Sample preparation

- Check that the sample is stable at the conditions chosen.
- Adjust sample conditions to match those of eluent A.

Column

- Select a Superdex peptide column for target peptides with Mr ≤3 000.
- Select a Superdex 75 column for target peptides with Mr >3 000.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

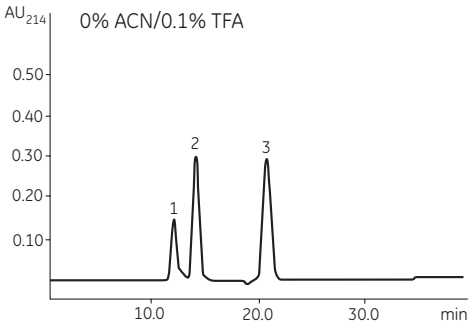
Depending on the solubility of the target peptide, prepare one of the alternatives below:

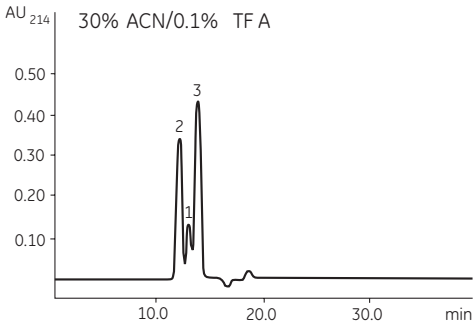
- 50 mM phosphate, pH 7.0 in water containing 30 % acetonitrile (final conc.).
- 0.1 % TFA in water containing 30 % acetonitrile (final conc.).
- 70 % formic acid in water (applicable to very hydrophobic peptides).
- 70 % acetonitrile in water (applicable to very hydrophobic peptides).

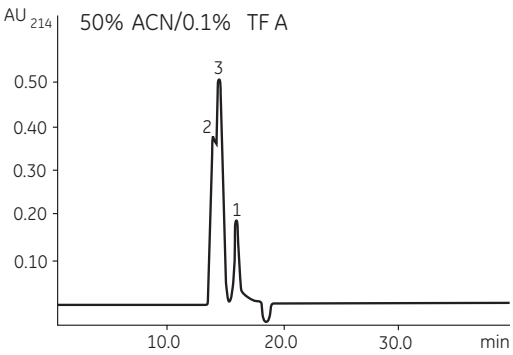
Evaluation

- If peak symmetry or recovery is unsatisfactory, consult Trouble Shootingon page 101.
- Check purity of collected fractions by:
  - Chromatography on µRPC.
  - Capillary electrophoresis.
  - MS.

Note: The resolution of peptides in SEC is affected by the ACN concentration. ACN concentrations higher or lower than 25–30 % will cause varying degrees excessive retardation.



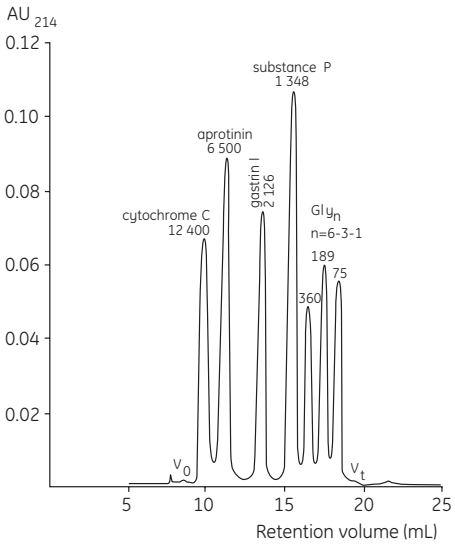




Conditions

Column:	Superdex Peptide HR 10/30.
Detection:	UV at 214 nm.
Eluent:	1. 0 % ACN in 0.1 % TFA. 2. 30 % ACN in 0.1 % TFA. 3. 50 % ACN in 0.1 % TFA.
Flow rate:	1.0 ml/min.

Effect of varying concentrations of ACN on elution behaviour of peptides in SEC.



Conditions

Sample:	200 mg/ml of each peptide.
Sample volume:	25 µL
Column:	Superdex Peptide HR 10/30.
Detection:	UV at 214 nm.
Eluent:	= 0.25 M NaCl in 0.02 M phoshpate, pH: 7.2.
Flow rate:	0.25 ml/min.

5.3 Adjustment and optimization

5.3.1 Adjustment of the standard purification protocol

To work properly, the Standard Purification Protocol requires:

1) The sample to be compatible with the running conditions of the different steps.

2) The target protein to elute within the gradients of the different steps.

Incompatibility between running conditions and sample seldom occurs in the acidic region. However, oxidation of SH-containing peptides may occur under alkaline conditions unless the eluent is thoroughly degassed.

Although rare, incorrect elution in RPC may occur if the peptide is either too hydrophilic or too hydrophobic.

If necessary, adjust the Standard Purification Protocol according to the table below.

Problem	**Actions step one and two, (RPC)**
Incorrect elution	1. Increase pH to reduce retention.
	2. Decrease pH to increase retention.
	3. Change ion pairing agent.
Oxidation	1. Degas eluent by helium sparging.
	2. Cool eluents.

5.3.2 Optimizing the final purity

When the Standard Protocol does not provide satisfactory purity, optimization of the individual steps must be carried out. However, optimization should advance only to the stage where the purification goals are fulfilled. This optimization protocol therefore deals with optimization parameters in the order of decreasing influence on the final purity (see table below) and stops when satisfactory results are reached.

It is often difficult to judge the overall effect of any single optimization effort by just looking at individual steps, since it is the contaminants that are not removed by "later steps" that need to be better resolved from the target peptide. The success of optimization measures should therefore be judged by their effect on the whole protocol, i.e. the final result.

5.3.3 Selectivity optimization

Measures influencing selectivity, such as the combination of techniques, pH and the choice medium in RPC, have the strongest impact on the final purity and should be dealt with first.

The effect of running pH, however, has already been taken into account in the standard protocols.

Adding an extra step will certainly affect the overall selectivity.

5.3.4 Further optimization

Decreasing the gradient slope will mainly increase the spacing of the peaks and sometimes also affect the order of elution.

Optimization measure	Effects	Comments
A. Screen for optimal RPC medium.	Influences elution order and spacing of peaks	Type of base matrix and the ligand fixation chemistry are more important than the choice of ligand type.
B. Scout for optimal gradient slope.	Influences mainly the spacing of peaks	Reducing the gradient slope will broaden peaks
C. Add a new step. (CIEX or SEC)	Changes overall selectivity.	May require the removal of ACN.
D. Scout for optimal flow rate	Decreases peak width	Only limited effect on final purity

A. Media Screening for RPC

Sample preparation

- Adjust the sample conditions to match eluent A.

Method

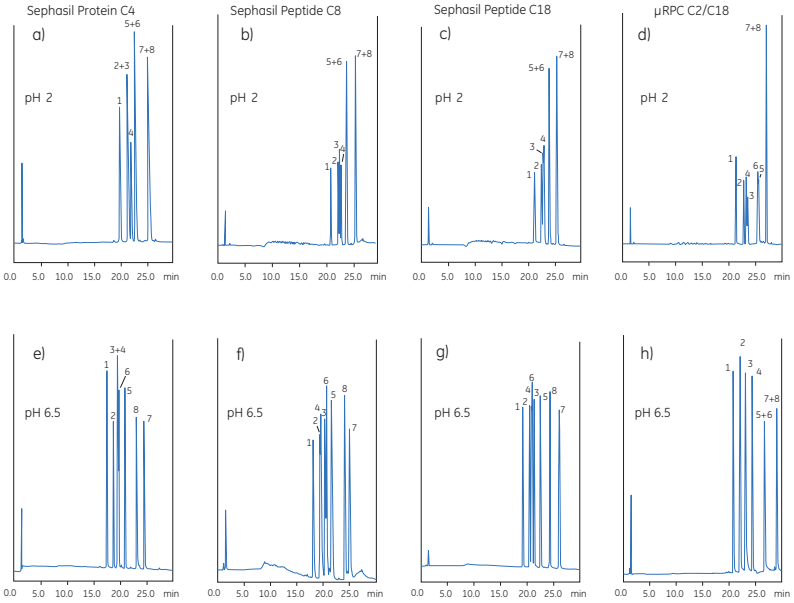
- Use the default values for gradient and flow rate provided by the template.

Eluents

- Prepare the originally used eluents.

Evaluation

- Select the RPC medium providing the highest purity and an acceptable recovery of the target peptide.
- If peak symmetry or recovery is unsatisfactory, consult Trouble shooting on page 101.



Selectivity comparision between different silica based media at two different pH. A mixture of closely related angiotensin (AT) peptides was used as sample.

Conditions

Sample:	1. Val4-Ile7-AT III (RVYVHPI) 2. Ile7-AT III (RVYIHPI) 3. Val4-AT III (RVYVHPF) 4. Sar1-Leu8-AT II (Sar-RVYIHPL) N-methylglycine 5. AT III (RVYIHPF) 6. AT II (DRVYIHPF) 7. des-Asp1-AT I (RVYIHPFHL) 8. AT I (DRVYIHPFHL)
Columns:	a) and e) Sephasil Protein C4 5 µm 4.6/100 b) and f) Sephasil Peptide C8 5 µm 4.6/100 c) and g) Sephasil Peptide C18 5 µm 4.6/100 d) and h) µRPC C2/C18 ST 4.6/100
Eluent A (pH 2):	0.065 % TFA in distilled water
Eluent B (pH 2):	0.05 % TFA, 75 % acetonitrile
Eluent A (pH6.5):	10 mM phosphate
Eluent B (pH6.5):	10 mM phosphate, 75 % acetonitrile
Flow rate:	1 ml/min
System:	ÄKTApurifier 10
Gradient:	5–95 % B in 20 column volumes

B. Gradient optimization

Sample preparation

- Adjust sample conditions to match those of Eluent A.

Method

- Use the medium and conditions arrived at above and the default flow rate provided by the template.

Evaluation

Select the steepest gradient providing satisfactory purity of the target protein.

C. Adding a new step

Choice of technique

I. IEX

CIEX may provide a rather different selectivity even though the pH parameter has been utilised in the standard protocol. Adding 30 % normally has a positive effect on peak symmetry.

Sample preparation

- Adjust sample conditions to match those of Eluent A. Especially reduce any content of ACN to 30 % or less.

Refer to Alternative Technique I on page 51.

II. SEC

SEC may be one of the few alternatives available for very hydrophobic peptides.

Though to a lesser degree, SEC also contributes to increased overall selectivity.

Varying the ACN content of the eluent may influence the order of elution of peptide samples and may be used to alter the selectivity even further.

Refer to Alternative Technique II on page 52.

D. Flow rate optimization

Sample preparation, and eluents

See the original method.

Method

Minimum peak widths are obtained at rather low flow rates with peptides.

Thus start with the default flow rate provided by the template and decrease it in rather large steps.

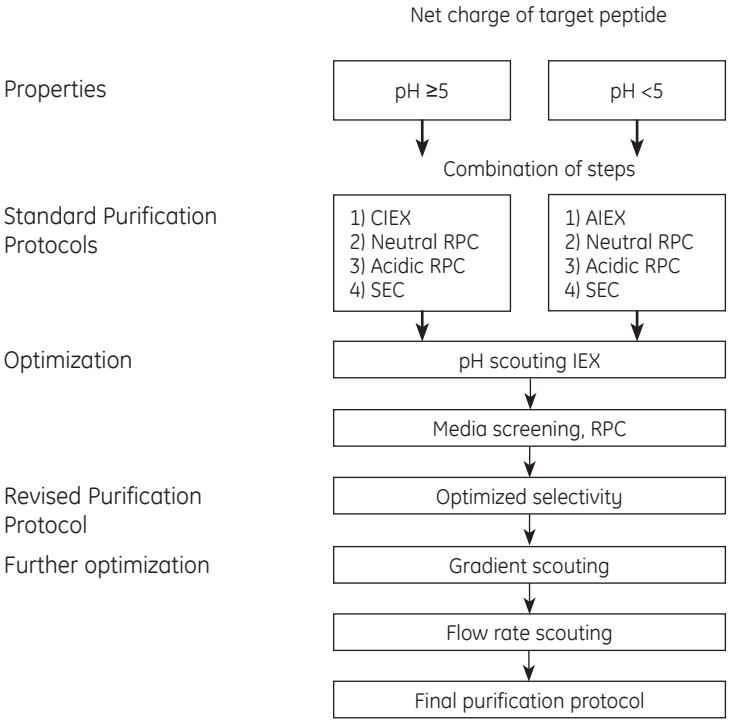
Select maximum flow rate providing satisfactory resolution.

6 Planning purification of peptides from natural sources

6.1 Introduction

Peptides in living matter normally occur in very low concentrations and large quantities of starting material may have to be dealt with to ensure that the purification results in enough material of the intended purity. The Standard Purification Protocol for peptides from natural sources combines IEX, RPC and SEC in a way that utilises their independent selectivities for maximum overall resolution. (See also chapter 4).

6.1.1 Purification strategy



6.1.2 Purification development scheme

		Protocol on page	Further reading
1.	**Purification protocol platform:**		
1.1	Establish the intended final quality of the target peptide.		
1.2.	List available information on:		
	• Target peptide properties	62	126
	• Starting material properties		
1.3	Perform a Sample Stability Test to establish the sample stability window (SSW).		125
2.	**Initial purification experiment:**		
2.1	Run the Standard Purification Protocol within the SSW.		136
	• If the results obtained are OK, proceed to # 7.	63	144
	• If the target peptide does not elute within the gradient of STEP ONE, or if the final purity is too low, proceed to # 3.1.		146
3.	**Optimization of IEX running pH:**		
3.1	Perform pH scouting for CIEX and AIEX within the SSW to establish the running pH providing the best resolution.	67	136
	• With AIEX, increase the running pH in steps of one pH unit from the target peptide pI to the upper pH limit of the SSW.		
	• With CIEX, decrease the running pH in steps of one pH unit from the target peptide pI to the lower pH limit of the SSW.		
	• Analyse the gradient eluted fractions only in runs lacking the target peptide the in flow-through fraction.		
	• Select the running pH providing highest purity of the target peptide.		
	• Replace STEP ONE of the Standard Purification Protocol with the IEX method selected above and run the revised Standard Purification Protocol.		
	If the results obtained are OK, proceed to # 7.		
	If the final purity is too low, proceed to # 4.		

		Protocol on page	Further reading
4.	**Screening for optimal RPC medium:**		
4.1	Perform RPC media (ligand) screening under acidic and neutral conditions. • Select the RPC medium providing the highest purity of the target peptide. • Revise the Standard Purification Protocol and re-run it. If the results obtained are OK, proceed to # 7. If the final purity is too low, proceed to # 5.	71	144
5.	**Gradient optimization:**		
5.1	Scout the IEX and/or the RPC steps for the minimal gradient length providing satisfactory resolution. • Revise the Standard Purification Protocol and re-run it. If the results obtained are OK, proceed to # 7. If the final purity is too low, proceed to # 6.	72	
6.	**Flow rate optimization:**		
6.1	Scout for maximal flow rate providing satisfactory resolution. • Start with STEP THREE, then STEP TWO and finally STEP ONE. • Stop when satisfactory resolution is obtained.	73	
7.	**Scaling up the final purification protocol:**		
7.1	The following procedure will not alter final purity provided media used, column lengths, linear flow rates and gradient parameters are kept constant. • For each step of the protocol, increase the column volume in proportion to the intended increase in sample load in step one. • With pre-packed columns, the volume should be chosen to be larger or equal to the theoretical value.	99	

6.1.3 Planning platform protocol

End product quality

Quality aspect Level	Purity Check method
Total peptide content	
Purity	

Target peptide description

Calculated properties	Value	Calculation method
Molecular mass		
Hydrophobicity		
Isoelectric point		
Net charge at pH 2.0:		
Net charge at pH 7.0:		
Net charge at pH 9.0:		

Target peptide solubility

Solvent	Solubility 0 % ACN	30 % ACN	60 % ACN
0.1 % TFA pH ~2			
20 mM Phosphate pH 7.0			
20 mM Tris-HCl pH 9.0			

6.2 Standard purification protocol for natural peptides

The order of steps is chosen to minimise the number of inter-step conditionings of the sample.

However, Alternative II in STEP FOUR requires the ACN content of the sample be reduced to 30 % or less. RESOURCE RPC in STEP TWO is chosen to allow the removement of any protein remaining irreversibly adsorbed to the column by alkali.

Step one, ion exchange chromatography

Sample preparation

- Check that the sample is stable at running pH and in the range 0.1–1.0 M NaCl.
- Adjust sample conditions to match those of eluent A.

Column

For target peptide pI ≥5.0 or when pI is unknown, run CIEX at pH 4.5.

For target peptide pI <5.0, run AIEX at pH 8.0.

- Select a column to match the amount of sample.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

Use BufferPrep recipes 3.0–7.5 CIEX or 5.0–9.5 AIEX respectively.

Evaluation

- If the target peptide elutes as a reasonably symmetrical peak within the gradient and the recovery of active material is satisfactory, proceed to STEP TWO.
- If the target peptide elutes in the flow-through fraction, change the type of ion exchanger or perform pH scouting (page 71).
- If the target peptide elutes during regeneration only, consult Trouble-shooting on page 101 or perform pH scouting (page 71).
- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101 or perform pH scouting (page 71).
- Check purity of collected fractions by:
  - Chromatography on µRPC or Mini S.
  - Capillary electrophoresis.
  - MS.

Step two, neutral RPC

Sample preparation

- Check that the sample is stable at neutral conditions and in 0 to 60 % ACN.
- Adjust sample conditions to match those of eluent A.

Column

- Select a RESOURCE RPC column for STEP TWO.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

Prepare the following eluents:

- Eluent A: 10 mM ammonium phosphate pH 7.0 in water.
- Eluent B: 10 mM ammonium phosphate in water containing 60 % acetonitrile (final conc.).

Evaluation

- If the target peptide elutes as a reasonably symmetrical peak within the gradient and the recovery is satisfactory, proceed to STEP TWO.
- If peak symmetry or recovery is unsatisfactory, consult Trouble Shooting on page 101.
- Check purity of collected fractions by:
  - Chromatography on µRPC or Mini S.
  - Capillary electrophoresis.
  - MS.
- If purity is unsatisfactory, proceed to Optimization on page 69.

Step three, acidic RPC

Sample preparation

- Check that the sample is stable at acidic conditions and in 0 to 60 % ACN.
- Adjust sample conditions to match those of eluent A.

Column

- Select a Sephasil C8 column.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

Prepare the following eluents:

- Eluent A: 0.06 % TFA in water.
- Eluent B: 0.05 % TFA in water containing 60 % acetonitrile (final conc.).

Evaluation

- If the target peptide elutes as a reasonably symmetrical peak within the gradient and the recovery of active material is satisfactory, proceed to STEP FOUR.
- If peak symmetry or recovery is unsatisfactory, consult Trouble Shooting on page 101.
- Check purity of collected fractions by:
  - Chromatography on µRPC or Mini S.
  - Capillary electrophoresis.
  - MS.
- If purity is unsatisfactory, proceed to Optimization on page 69.

Step four, alt I SEC

Sample preparation

- Check that the sample is stable at the conditions chosen.
- Adjust sample conditions to match those of eluent A.

Column

- Select a Superdex peptide column for target peptide Mr ≤3 000.
- Select a Superdex 75 column for target peptide Mr >3 000.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

Depending on the solubility of the target peptide, prepare one of the alternatives below:

- 50 mM phosphate, pH 7.0 in water containing 30 % acetonitrile (final conc.).
- 0.1 % TFA in water containing 30 % acetonitrile (final conc.).

Evaluation

- If peak symmetry or recovery is unsatisfactory, consult Trouble Shooting on page 101.
- Check purity of collected fractions by:
  - Chromatography on µRPC.
  - Capillary electrophoresis.
  - MS.

Note: The resolution of peptides in SEC is affected by the ACN concentration.

ACN concentrations higher or lower than 25–30 % will cause varying degrees of excessive retardation.

Step four, alt II complementary IEX + SEC

Sample preparation

- Check that the sample is stable at running pH and in the range 0.1–1.0 M NaCl.
- Reduce the sample content of ACN to 30 % or less by evaporation or freeze drying. Adjust sample conditions to match those of eluent A.

Column

- If STEP ONE utilised CIEX, run AIEX at pH 8.0.
- If STEP ONE utilised AIEX, run CIEX at pH 4.5.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

Use BufferPrep recipes 3.0–7.5 CIEX or 5.0–9.5 AIEX respectively.

Evaluation

- If the target peptide elutes as a reasonably symmetrical peak within the gradient and the recovery of active material is satisfactory, proceed to STEP FOUR, Alt I.
- If the target peptide elutes in the flow-through fraction, change the type of ion exchanger or perform pH scouting (page 71).
- If the target peptide elutes during regeneration only, consult Trouble-Shooting on page 101 or perform pH scouting (page 71).
- If peak symmetry or recovery is unsatisfactory, consult Trouble-Shooting on page 101 or perform pH scouting (page 71).

6.3 Adjustment and optimization

6.3.1 Adjustment of the standard purification protocol

To work properly, the Standard Purification Protocol requires:

1) The sample to be compatible with the running conditions of the different steps.

2) The target peptide to elute within the gradients of the different steps.

Incompatibility between running conditions and sample seldom occurs under the conditions of steps one to three. Incorrect elution in IEX is most likely to happen when the pI of the target peptide is unknown. Although rare, incorrect elution in RPC may occur if the peptide is either too hydrophilic or too hydrophobic.

If necessary, adjust the Standard Purification Protocol according to the table below.

Problem	Actions STEP ONE	TWO and THREE, (RPC)
Incorrect elution	1. Change to the alternative ion exchanger and pH given in step one. 2. Scout for optimal running pH within the sample stability window. (see page 71)	1. Increase pH to reduce retention. 2. Decrease pH to increase retention. 3. Change ion pairing agent.
Oxidation	1. Degas eluent by helium sparging. 2. Cool eluents.	1. Degas eluent by helium sparging. 2. Cool eluents.

6.3.2 Optimizing the final purity

When the Standard Protocol does not provide satisfactory purity, optimization of the individual steps must be carried out. However, optimization should advance only to the stage where the purification goals are fulfilled. The optimization protocol therefore deals with optimization parameters in the order of decreasing influence on the final purity (see table below) and stops when satisfactory results are reached.

It is often difficult to judge the overall effect of any single optimization effort by just looking at individual steps, since it is the contaminants that are not removed by ”later steps” that need to be better resolved from the target peptide. The success of optimization measures should therefore be judged by their effect on the whole protocol, i.e. the final result.

6.3.3 Selectivity optimization

Measures influencing selectivity, such as the combination of techniques, pH and the choice medium in RPC, have the strongest impact on the final purity and should be dealt with first.

The effect of running pH, however, has already been taken into account in the standard protocols.

Adding an extra step will certainly affect the overall selectivity.

6.3.4 Further optimization

Decreasing the gradient slope will mainly increase the spacing of the peaks and sometimes also affect the order of elution.

Optimization measure	Effects	Comments
A. Scout for optimal running pH in IEX	Influences elution order and spacing of peaks	
B. Screen for optimal RPC medium	Influences elution order and spacing of peaks	Type of base matrix and the fixation chemistry are more important than the choice of ligand type
C. Scout for optimal gradient slope in IEX	Influences mainly spacing of peaks	Reducing the gradient slope will broaden peaks
C. Scout for optimal gradient slope in RPC	Influences mainly spacing of peaks	Reducing the gradient slope will broaden peaks
D. Add a new step	Increases the overall selectivity	IEX complementary to step one May reduce final recovery
E. Scout for optimal flow rates	Decreases peak width	Only limited effect on final purity

A. Optimizing running pH in IEX

Sample preparation

- For CIEX, adjust the sample pH to equal the most acidic run planned.
- For AIEX, adjust the sample pH to equal the most alkaline run planned.

Method

- For CIEX, decrease the running pH in steps of 0.5–1.0 pH units from the target peptide pI to the lower limit of the sample stability window.
- For AIEX, increase the running pH in steps of 0.5–1.0 pH units from the target peptide pI to the upper limit of the sample stability window.
- Use the default values for gradient and flow rate provided by the template.

Eluents

Use BufferPrep recipes 3.0–7.5 CIEX or 5.0–9.5 AIEX respectively.

Evaluation

Select the running pH that provides the highest purity.

B. Media Screening for RPC

Sample preparation

- Adjust the sample conditions to match eluent A.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

- Prepare the originally used eluents.

Evaluation

- Select the RPC medium providing the highest purity and an acceptable recovery of the target peptide.
- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101.

C. Gradient optimization

Sample preparation

- Adjust sample conditions to match those of Eluent A.

Method

- Use the medium and conditions arrived at above and the default flow rate provided by the template.

Select the steepest gradient providing satisfactory purity of the target protein.

D. Adding a new step

IEX may provide a rather different selectivity even though the pH parameter has been utilised in the standard protocol. Adding 30 % ACN normally has a positive effect on peak symmetry.

Sample preparation

- Adjust sample conditions to match those of Eluent A.

Especially reduce any content of ACN to 30 % or less.

Column

- If STEP ONE used CIEX, run AIEX at pH 8.0.
- If STEP ONE used AIEX, run CIEX at pH 4.5.

Eluents

- For CIEX, prepare:
  - Eluent A: 5 mM phosphate pH 3.0 in water containing 30 % acetonitrile (final conc.).
  - Eluent B: 5 mM phosphate pH 3.0 in water containing 30 % acetonitrile and 1.0 M NaCl (final conc.).
- For AIEX, prepare:
  - Eluent A: 20 mM TRIS-HCl pH 8.0 in water containing 30 % acetonitrile (final conc).
  - Eluent B: 20 mM TRIS-HCl pH 8.0 in water containing 30 % acetonitrile and 1 MNaCl (final conc).

E. Flow rate optimization

Sample preparation, column and eluents

See the original method

Method

Minimum peak widths are obtained at rather low flow rates with peptides.

Thus start with the default flow rate provided by the template and decrease it in rather large steps.

Select the maximum flow rate providing satisfactory resolution.

7 Planning purification of protein fragments

7.1 Introduction

Peptide Mapping, i.e. the analysis of protein fragments such as those produced by controlled proteolysis, is normally performed as a one-step procedure in which the pattern of the separated fragments provides the required information.

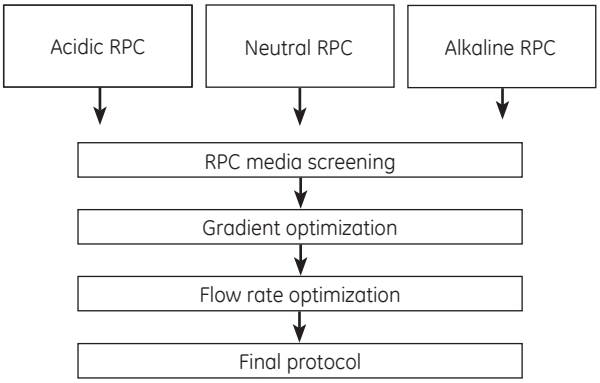
However, with the introduction of MALDI TOF for molecular weight determination of the fragments, or when the primary structure of individual fragments is to be determined, precise fraction collection then becomes essential.

The Standard Purification Protocol for protein fragments starts with optimizing a one-step procedure based on RPC.

Should these optimization efforts fail to separate all fragments needed, acidic RPC is combined with neutral RPC in a two-step procedure.

In certain cases, adding an alternative technique is advantageous. Protocols for IEX and SEC are, therefore, also included in this section.

7.1.1 Purification strategy



Further resolution can be obtained by re-running unresolved peaks on RPC using a complementary solvent system or on CIEX.

7.1.2 Purification development scheme

		Protocol on page	Further reading
1.	**Optimal eluent system:**		
1.1	• Run acidic and neutral eluent systems on Sephasil C8 and run the alkaline eluent system on RESOURCE RPC.	77	144
	• Select the eluent system providing the best resolution.	78	
	If the resolution is unsatisfactory, proceed to # 2.	79	
2.	**RPC media screening:**		
2.1	• Run acidic or neutral media screening for RPC.	82	144
	• Select the RPC medium providing the best resolution.		
	If the resolution is unsatisfactory, proceed to # 3.		
3.	**Gradient optimization:**		
3.1	• Run gradient scouting with the selected RPC medium.	82	144
	If the resolution is unsatisfactory, proceed to # 4.		
4.	**Flow rate optimization:**		
4.1	• Run gradient scouting at the selected gradient	82	146
	If the resolution is unsatisfactory, proceed to # 5.		
5.	**Combination of techniques:**		
5.1	Re-run unresolved peaks on RPC using a complementary eluent system (see #1.1!), on CIEX or on SEC.	83	

7.2 Purification protocol for protein fragments

Acidic RPC

Sample preparation

- Check that the sample is stable under acidic conditions and in 0 to 60 % ACN.
- Adjust sample conditions to match those of eluent A.

Column

- Select a Sephasil C8 column.

Method

- Use the default values for gradient and flow rate provided by the template.

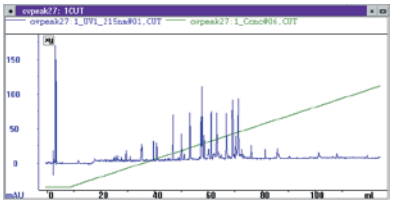
Eluents

Prepare the following eluents:

- Eluent A: 0.06 % TFA in water.
- Eluent B: 0.05 % TFA in water containing 60 % acetonitrile (final conc.).

Evaluation

- If the eluted peaks are symmetrical and the recovery is satisfactory, proceed to Neutral RPC.
- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101.
- Count the number of separated peaks.



Separation of pyridylethylated ovalbumin tryptic fragments.

Instrument:	ÄKTApurifier.
Conditions	
Sample:	Reduced and pyridylethylated ovalbumin digested with modified trypsin, 1 nmol.
Column:	Sephasil Peptide C18 5 µm ST 4.6/250.
Column volume:	4.16 ml.
Detection:	UV at 215 nm.
Eluent A:	0.06 % TFA.
Eluent B:	0.055 % TFA in 84 % acetonitrile.
Running pH:	2.5.
Gradient:	0–75 % B in 30 column volumes.
Flow rate:	1.0 ml/min.

Neutral RPC

Sample preparation

- Check that the sample is stable under neutral conditions and in 0 to 60 % ACN.
- Adjust sample conditions to match those of eluent A.

Column

- Select a Sephasil Peptide C8 or RESOURCE RPC column.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

Prepare the following eluents:

- Eluent A: 10 mM ammonium phosphate pH 7.0 in water.
- Eluent B: 10 mM ammonium phosphate in water, containing 60 % acetonitrile (final conc.).

Evaluation

- If the eluted peaks are symmetrical and the recovery is satisfactory, proceed to Alkaline RPC.
- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101.
- Count the number of separated peaks.

Alkaline RPC

Sample preparation

- Check that the sample is stable under alkaline conditions and in 0 to 60 % ACN.
- Adjust sample conditions to match those of eluent A.

Column

- Select a RESOURCE RPC column.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

Prepare the following eluents:

- Eluent A: 20 mM Tris-HCl pH 9.0 in water.
- Eluent B: 20 mM Tris-HCl pH 9.0 in water containing 60 % acetonitrile (final conc.).

Evaluation

- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101.

Select the method that provides the largest number of separated peaks.

If resolution is unsatisfactory, proceed to Optimization on page 80.

7.3 Adjustment and optimization

7.3.1 Adjustment of the standard purification protocol

To work properly, the Standard Purification Protocol requires:

1) The sample to be compatible with the running conditions of the different steps.
2) The target protein to elute within the gradients of the different steps.

Incompatibility between running conditions and sample seldom occurs in the acidic region. However, oxidation of SH-containing peptides may occur under alkaline conditions unless the eluent is thoroughly degassed.

Although rare, incorrect elution in RPC may occur if the peptide is either too hydrophilic or too hydrophobic.

If necessary, adjust the Standard Purification Protocol according to the table below.

Problem	**Actions STEP ONE and TWO, (RPC)**
Incorrect elution	1. Increase pH to reduce retention.
	2. Decrease pH to increase retention.
	3. Change ion pairing agent.
Oxidation	1. Degas eluent by helium sparging.
	2. Cool eluents.

7.3.2 Optimizing the final purity

When the Standard Protocol does not provide satisfactory purity, optimization of the individual steps must be carried out. However, optimization should advance only to the stage where the purification goals are fulfilled. The optimization protocol therefore deals with optimization parameters in the order of decreasing influence on the final purity (see table below!) and stops when satisfactory results are reached. It is often difficult to judge the overall effect of any single optimization effort by just looking at individual steps, since it is the contaminants that are not removed by "later steps" that need to be better resolved from the target peptide. The success of optimization measures should therefore be judged by their effect on the whole protocol, i.e. the final result.

7.3.3 Selectivity optimization

Measures influencing selectivity, such as the combination of techniques, pH and the choice medium in RPC, have the strongest impact on the final purity and should be dealt with first.

The effect of running pH, however, has already been taken into account in the standard protocols.

Adding an extra step will certainly affect the overall selectivity.

7.3.4 Further optimization

Decreasing the gradient slope will mainly increase the spacing of the peaks and sometimes also affect the order of elution.

Optimization measure	Effects	Comments
A. Screen for optimal RPC medium.	Influences elution order and spacing of peaks.	Type of base matrix and the ligand fixation chemistry are more important than the choice of ligand type.
B. Scout for optimal gradient slope.	Influences mainly the spacing of peaks.	Reducing the gradient slope will broaden peaks.
C. Add a new step. (CIEX or SEC)	Changes overall selectivity.	May require the removal of ACN.
D. Scout for optimal flow rate	Decreases peak width.	Only limited effect on final purity.

A. Media screening for RPC

Sample preparation

- Adjust the sample conditions to match eluent A.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

- Prepare the originally used eluents.

Evaluation

- Select the RPC medium providing the highest purity and an acceptable recovery of the target peptide.
- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101.

B. Gradient optimization

Sample preparation

- Adjust sample conditions to match those of Eluent A.

Method

- Use the medium and conditions arrived at above and the default flow rate provided by the template.

Evaluation

Select the steepest gradient providing satisfactory purity of the target protein.

C. Adding a new step

Choice of technique

I. IEX

CIEX may provide a rather different selectivity eventhough the pH parameter has been utilised in the standard protocol. Adding 30 % normally has a positive effect on peak symmetry.

Sample preparation

- Adjust sample conditions to match those of Eluent A.
  Especially reduce any content of ACN to be 30 % or less.

Refer to Alternative Technique I on page 43.

II. SEC

SEC may be one of the few alternatives available for very hydrophobic peptides.

Though to a lesser degree, SEC also contributes to increased overall selectivity.

Varying the ACN content of the eluent may influence the order of elution of peptide samples and may be used to alter the selectivity even further.

Refer to Alternative Technique II on page 44.

D. Flow rate optimization

Sample preparation, and eluents

See the original method.

Method

Minimum peak widths are obtained at rather low flow rates with peptides.

Start with the default flow rate provided by the template and decrease it in rahter large steps.

Select the maximum flow rate providing satisfactory resolution.

8 Planning synthetic oligonucleotide purification

8.1 Introduction

Solid phase synthesis of oligonucleotides requires a very high coupling efficiency or total yield and purity will suffer. Since the overall coupling efficiency can never be 100 % and side effects like depurination may occur, the final product will be contaminated by sequences aberrant in one aspect or another. Whether such failure sequences can be accepted or whether they have to be removed, depends on the intended further use of the target oligonucleotide. This section will help you develop purification protocols for labelled, unlabelled or phosphorothioate synthetic oligonucleotides.

8.1.1 The purification development scheme

Detailed schemes for the development of purification protocols for synthetic oligonucleotides are presented under the separate headings below. These help you to decide which path to take in different situations and refer you to a set of step-by-step protocols used at the different stages out-lined in the scheme.

The development work involves four basic phases:

1. **Forming a platform for the development.**
   The platform should include:
   - Intended quantity and quality of the end product
   - Available information on the physical properties of the target oligonucleotide
2. **Running initial purification experiments.**
   The Standard Purification Protocol for synthetic oligonucleotides is used as a starting point for the development.
3. **Optimizing the purification protocol.**
   Guided by the outcome of the standard protocol experiment, the purification steps are optimized and/or recombined until satisfactory results are obtained.
4. **Adapting the scale of the final purification protocol.**
   By following simple rules, the purification protocol can be adapted to perform almost identically at any chosen scale.

Notes and comments

On-line detection of oligonucleotides is accomplished by UV monitoring at 254 to 260 nm. However, UV monitoring is not capable of discriminating between the target oligonucleotide and contaminating failure sequences.

Estimation of purity obtained can only be carried out by off-line techniques after collecting fractions. Suitable techniques are analytical IEX (Mini Q), analytical RPC (*RPC) or capillary electrophoresis.

SEC can be used to remove low molecular weight contaminants such as cleaved protecting groups, salts and oligonucleotides up to ten bases in length and/or buffer exchange.

Complementary structures may cause inter- or intramolecular aggregation of the target oligonucleotide under non-denaturing conditions. At pH 12, however, complementary binding is minimal and oligonucleotides will separate as individual molecules.

8.2 Standard purification protocol for synthetic phosphorothioate oligonucleotides

Note: This protocol is only available for ÄKTAexplorer 100.

The standard purification protocol for synthetic phosphorothioate oligonucleotides combines removal of non-tritylated contaminants, on-column de-tritylation and further purification of de-tritylated material in a one-step experiment on SOURCE Q media. The separation of non-tritylated oligonucleotides from tritylated ones utilises the increased retardation of tritylated material caused by the weak hydrophobic character of this medium. The standard purification protocol includes the following steps:

- The raw product from synthesis is loaded onto the column without prior removal of trityl groups.
- A salt gradient is applied to elute non-tritylated material. As soon as the UV absorption in the eluate reaches a pre-set value, the gradient is stopped and returned to zero % B. This arrangement will elute all non-tritylated material, while leaving the tritylated material still adsorbed to the column.
- When the UV absorption has returned to baseline, 0.2 % TFA is pumped onto the column and left for 30 minutes to de-tritylate the still adsorbed material. At the now low salt concentration, de-tritylated material stays adsorbed to the column due to its charge properties, while cleaved trityl groups will stay adsorbed by hydrophobic forces.
- After washing out all TFA, a new salt gradient is applied to elute and separate de-tritylated material.
- The column is then regenerated with 2 M NaCl in 30 % iso-propanol.

8.2.1 Purification protocol development scheme

		Protocol on page
1.	**Phosphorothioate oligonucleotides:**	
1.1	Run the Standard Purification Protocol for synthetic phosphorothioate oligonucleotides. • If the final purity obtained is OK, proceed to # 4. • If the final purity is too low, add an RPC step according to # 1.2.	91
1.2	Run the RPC Purification Protocol: • If the final purity obtained is OK, proceed to # 4. • If the final purity is too low, proceed to # 2.1.	95
2.	**Run the gradient optimization protocol:**	
2.1	• Select the gradient resulting in satisfactory resolution at minimum gradient length. • If the final purity obtained is OK, proceed to # 4. • If the final purity is too low, proceed to # 3.1.	97
3.	**Optimization of flow rate:**	
3.1	Run the Flow Rate Optimization Protocol under conditions arrived at above. • Select the highest flow rate resulting in satisfactory resolution. • Proceed to # 4.	97
4.	**Adapting the scale of the final purification protocol:**	99
4.1	The following procedure will not alter the final purity provided that the media used, column lengths, linear flow rates and gradient parameters are kept constant. • For each step of the protocol, increase the column volume in proportion to the intended increase in sample load in step one. • With pre-packed columns, a volume should be chosen that is equal to or larger than the theoretical value.	

8.2.2 Planning platform protocol

End product quality

Quality aspect	Level	Determined by
Total oligonucleotide content		
Purity		

Target oligonucleotide description

Property	Value	Determined by
Number of bases		
Content of Adenine		
Content of complementary sequences		

8.2.3 Standard purification protocol, synthetic phosphorothioate oligonucleotides

Sample preparation

- Adjust sample conditions to match those of eluent A, e.g. by buffer exchange.

Column

- Select a RESOURCE Q column or pack a column with SOURCE Q medium by following the instructions in Adviser.

Method

Select Trioligo as the template (only available in ÄKTAexplorer 100).

Eluents

Prepare the following eluents:

- Eluent A: 10 mM NaOH.
- Eluent B: 2.0 M NaCl in 10 mM NaOH.
- Cleaving solution: 0.2 % TFA.
- Regeneration solution: 2 M NaCl in 30 % iso-propanol.

Evaluation

- Check purity of collected fractions by:
  - Chromatography on µRPC or Mini S.
  - Capillary electrophoresis.
  - MS.

8.3 Standard purification protocol for synthetic oligonucleotides

AIEX and RPC are used to remove failure sequences from the target oligonucleotide.

Non-tritylated oligonucleotides are eluted in order of increasing number of bases both in AIEX and in RPC.

Tritylated oligonucleotides are eluted much later and in order of decreasing number of bases in RPC. In principle, tritylation should not alter elution behaviour in AIEX. In practice, however, most AIEX media exhibit weak hydrophobic properties that may cause increased retardation of tritylated material.

Complementary structures may cause inter- or intramolecular aggregation of the target oligonucleotide under non-denaturing conditions. At pH 12, however, complementary binding is minimal and oligonucleotides will separate as individual molecules.

Short oligonucleotides containing 40 bases or less are separated preferably by AIEX for the following reasons:

- Separation can be carried out under denaturing conditions (pH 12) to avoid association of complementary sequences.
- Resolution after de-tritylation is better than with silica-based RPC.

Long oligonucleotides containing more than 40 bases may require a combination of RPC and AIEX to reach the intended purity. Despite the risk of unintentional de-tritylation, the RPC step is then performed before de-tritylation to increase resolution.

Labelled oligonucleotides are purified either by anion exchange or reversed phase chromatography depending on the type of label used. The table below indicates which technique is best suited for a number of different labels:

Label	Technique	
	RPC	AIEX
Biotin	X	X
CY3	X	
CY5	X	
FITC	X	

8.3.1 Purification protocol development scheme

		Protocol on page
1.	**Synthetic oligonucleotides:**	
1.1	Run the Anion Exchange Purification Protocol. • If the final purity obtained is OK, proceed to # 5. • If the final purity is too low, proceed to # 2.	94
2.	**Optimization of gradient length:**	
2.1	Run the Gradient Optimization Protocol for AIEX: • Select the gradient resulting in satisfactory resolution at minimum gradient length. • If the final purity obtained is OK, proceed to # 5. • If the final purity is too low, proceed to # 3.	97
3.	**Optimization of flow rate:**	
3.1	Run the Flow Rate Optimization Protocol for AIEX under the conditions arrived at above. • Select the highest flow rate resulting in satisfactory resolution. • If the final purity obtained is OK, proceed to # 5. • If the final purity is too low, proceed to # 4.	97
4.	**Addition of step:**	
4.1	Run the RPC Purification Protocol.	95
5.	**Adapting the scale of the final purification protocol:**	
5.1	The following procedure will not alter the final purity provided that the media used, column lengths, linear flow rates and gradient parameters are kept constant. • For each step of the protocol, increase the column volume in proportion to the intended increase in sample load in step one. • With pre-packed columns, a volume should be chosen that is equal to or larger than the theoretical value.	99

8.3.2 Planning platform protocol

End product quality

Quality aspect	Level	Determined by
Total oligonucleotide content		
Purity		

Target oligonucleotide description

Property	Value	Determined by
Number of bases		
Content of Adenine		
Content of complementary sequences		

8.3.3 Anion exchange purification protocol for synthetic oligonucleotides

Sample preparation

- Adjust sample conditions to match those of eluent A, e.g. by buffer exchange according to instructions in the Adviser.

Column

- Select a RESOURCE Q column or pack a column with SOURCE Q medium according to instructions in the Adviser.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

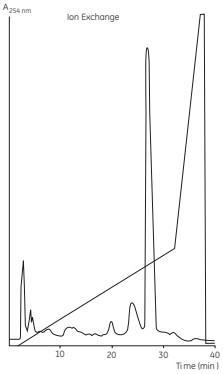
Prepare the following buffers:

– Eluent A: 10 mM NaOH.

– Eluent B: 2.0 M NaCl in 10 mM NaOH.

Evaluation

- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101.
- Check purity of collected fractions by:
  - Chromatography on µRPC or Mini S.
  - Capillary electrophoresis.
  - MS.
- If purity is unsatisfactory, proceed to Optimization on page 96.



Conditions

Sample:	20-mer without DMTr group
Column:	Mono Q HR 5/5
Flow:	1 ml/min
Buffer A:	10 mM NaOH, 0.5 M NaCl
Buffer B:	10 mM NaOH, 1.0 M NaCl

Purification of a 20-mer synthetic oligonucleotide by AIEX.

8.3.4 RPC purification protocol for synthetic oligonucleotides

Sample preparation

- Adjust sample conditions to match those of eluent A, e.g. by buffer exchange.

Column

- Select a RESOURCE RPC column or Sephasil C18 column.

Method

- Use the default values for gradient and flow rate provided by the template.

Eluents

Prepare the following buffers:

For RESOURCE RPC:

– Eluent A: 5 % acetonitrile containing 10 mM NaOH, pH 12.0.

– Eluent B: 40 % acetonitrile containing 10 mM NaOH, pH 12.0.

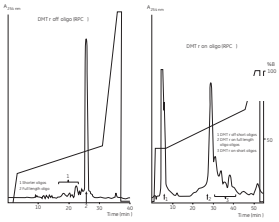
For Sephasil C18:

– Eluent A: 5 % acetonitrile containing 100 mM TEAA, pH 7.0.

– Eluent B: 40 % acetonitrile containing 100 mM TEAA, pH 7.0.

Evaluation

- If peak symmetry or recovery is unsatisfactory, consult Trouble-shooting on page 101.
- Check purity of collected fractions by:
  - Chromatography on µRPC or Mini S.
  - Capillary electrophoresis.
  - MS.
- If purity is unsatisfactory, proceed to Optimization on page 96.



Conditions

Sample: 20-mer synthetic oligonucleotide.
Column: PepRPC HR 5/5.
Detection: UV at 254 nm.
Eluent A: 100 mM TEAA in 5 % acetonitrile.
Eluent B: 100 mM TEAA in 30 % acetonitrile.
Flow rate: 1 ml/min.

1. Shorter oligos
2. Full length oligo
3. DMTr off short oligos
4. DMTr on full length oligo oligos
5. DMTr on short oligos

Purification of a 20-mer synthetic trityl-on oligonucleotide by RPC.

8.4 Optimizing the final purity

When the Standard Protocol does not provide satisfactory purity, optimization of the individual steps must be carried out. However, optimization should advance only to the stage where the purification goals are fulfilled. The optimization protocol therefore deals with optimization parameters in the order of decreasing influence on the final purity (see table below!) and stops when satisfactory results are reached.

It is often difficult to judge the overall effect of any single optimization effort by just looking at individual steps, since it is the contaminants that are not removed by "later steps" that need to be better resolved from the target molecule.

The success of optimization measures should therefore be judged by their effect on the whole protocol i.e. the final result.

8.4.1 Selectivity optimization

The combination of AIEX and RPC provides the strongest means of optimization.

8.4.2 Further optimization

Decreasing the gradient slope will mainly increase the spacing of the peaks and sometimes also affect the order of elution.

Optimizing the flow rate has the least effect on the final purity and should be performed only when the optimization measures above fail to provide satisfactory purity alone.

Optimization measure	Effects	Comments
– Combine AIEX with RPC.	Changes overall selectivity.	The two techniques provide quite different selectivities. Normally AIEX provides highest purity.
A. Scout for optimal gradient slope.	Influences mainly the spacing of peaks.	Reducing the gradient slope will broaden peaks.
B. Scout for optimal flow rate.	Decreases peak width.	Only limited effect on final purity.

A. Gradient optimization

Sample preparation, column and eluents

See the original method.

Method

- Use the default flow rate provided by the template.
- Increase length of gradient in steps of 5–10 column volumes.

Action

Select the steepest gradient (shortest gradient length) providing satisfactory purity.

B. Flow rate optimization

Sample preparation, column and eluents

See the original method.

Method

Minimum peak widths are obtained at rather low flow rates with oligonucleotides.

Thus start with the default flow rate provided by template and decrease it in rather large steps.

Select the maximum flow rate providing satisfactory resolution.

9 Changing the scale

Changing the scale of the final purification protocol is done by changing the column volumes in all steps in proportion to the intended sample load in the first step of the purification protocol. Resolution should remain constant provided that the media used, column lengths, linear flow rates and gradient parameters are also kept constant.

Actions

Calculate the scale factor as the intended sample amount over the original sample amount.

$$\text{Scale factor} = \frac{\text{Intended sample amount}}{\text{Original sample amount}}$$

Column

- Multiply the nominal load capacity of the original column by the scale factor.
- Select a column of the same type as the original one, but with a nominal load capacity equal to or larger than the figure calculated above.

Method

Select a template from the tables below.

ÄKTAexplorer 100

Sample volumes ≤ 150 ml (Sample injected by Superloop)		**Sample volumes > 150 ml (Sample loaded via system pump)**	
Fractions collected by fraction collector	Fractions collected by outlet valve	Fractions collected by fraction collector	Fractions collected by outlet valve
man_f_zz[1]	man_v_zz[1]	sys_f_gr[2]	sys_v_gr[2]

[1] zz represents the technique chosen.

[2] Since BufferPrep cannot be used with sys-templates, sys_f_ gr is used for all techniques in this mode.

ÄKTApurifier 10/10XT and ÄKTAexplorer 10/10XT

Sample volumes ≤ 150 ml (Sample injected by Superloop)	Sample volumes > 150 ml (Sample loaded via system pump)
Fractions collected by fraction collector	Fractions collected by fraction collector
man_f_zz[1]	sys_f_gr[2]

[1] *zz* represents the technique chosen.

[2] Since BufferPrep cannot be used with sys-templates, sys_f_ gr is used for all techniques in this mode.

Eluents

Prepare eluents equivalent to the original recipe, but multiply final volumes by the scale factor.

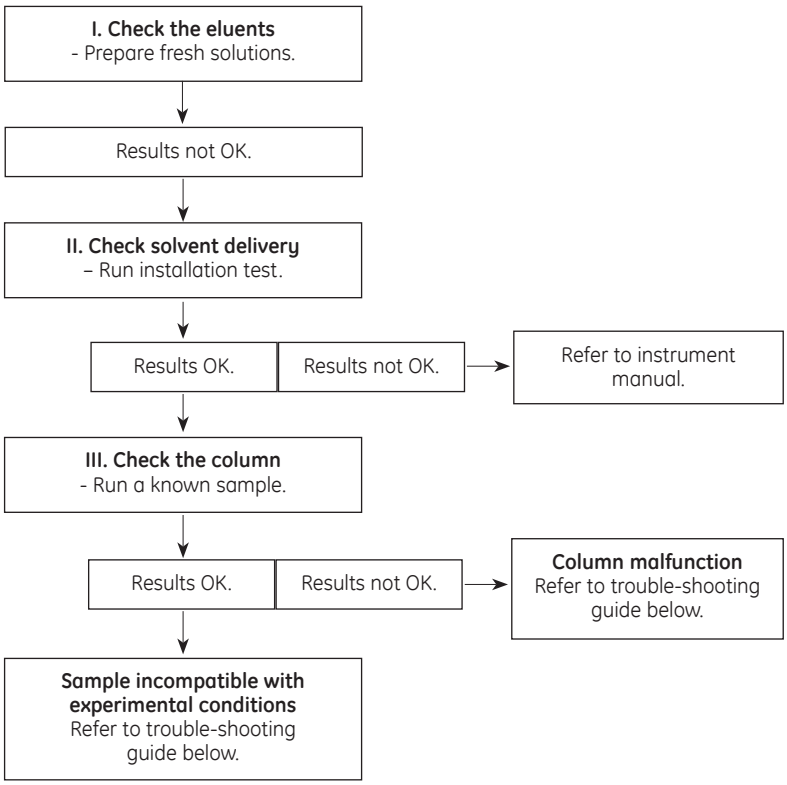
Note 1: Superloop can only be used together with low pressure columns, namely HiLoad™, HR, HiTrap™, HiPrep™ and RESOURCE. For high pressure columns, inject samples > 2.0 ml via the system pump.

Note 2: BufferPrep cannot be used when the system pump is used to load the sample.

10 Trouble-shooting

When a chromatogram shows unexpected deviations such as non-symmetrical or excessively broadened peaks, when recovery suffers, or when the system pressure deviates from normal, trouble-shooting has to be performed. The strategy outlined below starts with the perhaps most common and obvious source of malfunction; faulty eluents. Then make sure that the system performs normally to avoid the risk of misjudging column function tests. Finally, test sample behaviour.

General trouble-shooting strategy



The following table lists symptoms of malfunction. The actions recommended are intended as “first aids”.

Trouble-shooting guide

Symptom	Cause	Action
I. Pressure – Low	Air in the pump heads. Worn-out piston seals. Leaks.	Purge the pump with MeOH. Replace piston seals. (Refer to instrument manual)
– High	Clogged tubing or column.	Watch pressure while removing column and tubing in the "upstream" direction until the clogged part is identified.
– Irregular	Solvent delivery malfunction.	Re-run with fresh eluents Purge the pump with MeOHI. Replace piston seals. (Refer to instrument manual)
II. Aberrant peak shapes		Always check column first by running a known sample.
Triangular with sharp front	Overload.	Reduce sample load.
Excessively broad	Overload. Column bed irregularities. Air in the column.	Run column with 2 column volumes of MeOH to remove any trapped air. Perform recommended CIP.
Tailing	Column bed irregularities. Air in the column. Non-specific adsorption.	Run column with 2 column volumes of MeOH. Perform recommended CIP. Try additives like ACN or MeOH.
Fronting	Channels or air in the column bed.	Run column with 2 column volumes of MeOH to remove any trapped air.
Split peaks	Partially blocked frit. Column bed irregularities. Air in the column.	Run column "backwards" with 2 column volumes of MeOH to remove any particles in the frit or trapped air.
Irregular	Solvent delivery malfunction. Sample incompatible with experimental conditions.	Run Installation Test. Run sample stability test. Try additives like ACN or MeOH chaotropic salts. Change conditions, type of column or technique.
Ghost peaks (Peaks appearing even in blank runs)	Column regeneration incomplete. Quality of eluent chemicals and/or solvent too low. Aged eluents.	Increase eluting component concentration in eluent B. Use HPLC quality chemicals and solvents.
Unexpectedly low or missing peaks. Low recovery of activity.	Non-specific adsorption. Sample incompatible with experimental conditions.	Run sample stability test. Try additives like ACN or MeOH chaotropic salts. Try to block non-specific binding sites by injecting e.g. albumin. Change conditions, type of column or technique.
Base line instability – Irregular	Air in the detector cell. Detector problem	Check constrictor function. Run Installation Test.
– Drifting	Detector problem. Bleeding of precipitated or non-specifically adsorbed material. Faulty or aged eluents.	Run Installation Test. Perform specified CIP procedure. Re-run with fresh eluents.

11 Column maintenance

Chromatographic media are to be considered consumable with restricted life times. However, their life time is very dependent on proper column maintenance.

To prevent columns from deteriorating:

1. Always filter the sample before injection.
2. Ensure that the sample is stable in both eluents.
3. Always regenerate the column after a run.
4. When the column is not in use store it in the recommended storage solution.
5. Upon any sign of malfunction, clean the column by running the specified CIP procedure.

Further information on cleaning in place procedures is found in Adviser in UNICORN.

12 Applications

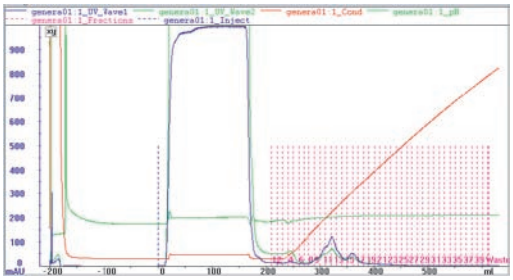
12.1. Purification of proteins

Example 1

The Fab fragment of a monoclonal antibody against the gp 120 envelope of the HIV-1 virus was expressed in *E. coli* and purified according to the Standard Purification Protocol on page 28. This protein has a molecular mass of 50 kD, an isoelectric point of ~11 and is located in the periplasmic space of the host.

Step one

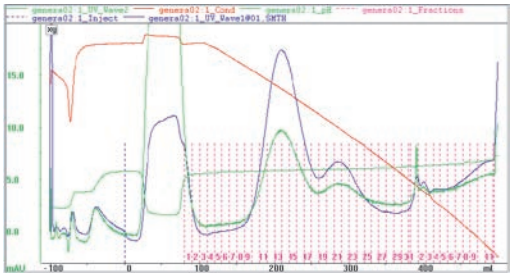
Cation exchange chromatography



Conditions
Column: HiLoad 16/10 SP Sepharose™ Fast Flow.
Column volume: 20 ml.
Detection: UV at 280 nm.
Buffer: BufferPrep CIEX, pH range 3–7.5.
Running pH: 4.5.
Gradient: 0→ 1.0 M NaCl.
Gradient vol: 20 column volumes.
Flow rate: 150 cm/h (5 ml/min)
Sample volume: 150 ml.
Sample injected via Superloop.
Active fractions pooled: 10–14.

Step two

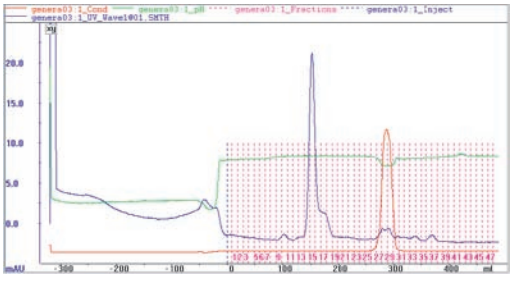
Hydrophobic interaction chromatography



Conditions
Column: HiLoad 16/10 Phenyl Sepharose 6 Fast Flow (high sub).
Column volume: 20 ml.
Detection: UV at 280 nm.
Buffer A: 1.0 M ammonium sulphate in 30 mM phosphate, pH 6.0.
Buffer B: 30 mM phosphate, pH 6.0.
Gradient: 1.0 → 0 M ammonium sulphate.
Gradient vol: 20 column volumes.
Flow rate: 150 cm/h (5 ml/min).
Sample volume: 50 ml.
Sample injected via Superloop.
Active fractions pooled: 11–16.

Step three

Size exclusion chromatography



Conditions
Column: HiLoad 26/60 Superdex 75 prep grade.
Column volume: 320 ml.
Detection: UV at 280 nm.
Buffer: 150 mM NaCl in 20 mM Phosphate, pH 7.0.
Sample volume: 8 ml[1].
Flow rate: 50 cm/h (4.4 ml/min).
Sample injected via Superloop.

[1] The pooled fractions from step two were concentrated ~8 times before injection.

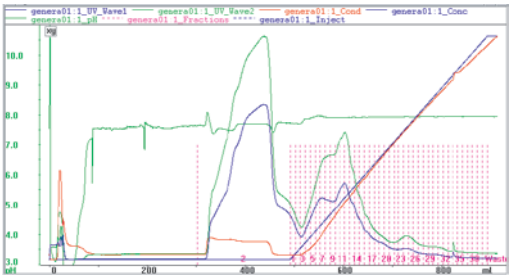
Example 2

Streptomyces α-amylase was recombinantly produced in *E. coli* and purified according to the Standard Purification Protocol on page 28. However, with CIEX at pH 4.5, all α-amylase activity eluted in the flow-through fraction so AIEX was used in step one instead.

The recombinant α-amylase has a molecular mass of 49.2 kD, an isoelectric point of ~6 and is secreted by the host.

Step one

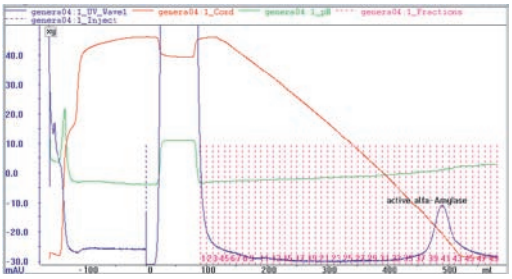
Anion exchange chromatography



Conditions
Column: HiLoad 16/10 Q Sepharose Fast Flow.
Column volume: 20 ml.
Detection: UV at 280 nm.
Buffer: BufferPrep AIEX pH mixture 5–9.5.
Running pH: 8.0.
Gradient: 0 → 1.0 M NaCl.
Gradient vol: 20 column volumes.
Flow rate: 150 cm/h (5 ml/min).
Sample volume: 120 ml.
Sample injected via Superloop.
Active fractions pooled: 10–15.

Step two

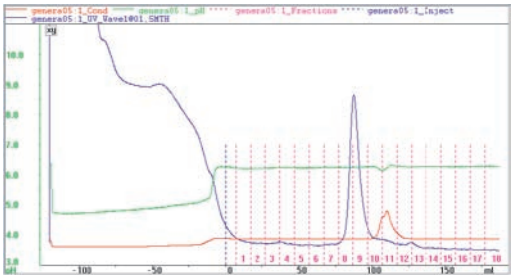
Hydrophobic interaction chromatography



Conditions
Column: HiLoad 16/10 Phenyl Sepharose 6 Fast Flow (high sub).
Column volume: 20 ml.
Detection: UV at 280 nm.
Buffer A: 1.0 M ammonium sulphate in 50 mM phosphate, pH 6.0.
Buffer B: 50 mM phosphate, pH 6.0.
Gradient: 1.0 → 0 M ammonium sulphate.
Gradient vol: 20 column volumes.
Flow rate: 150 cm/h (5 ml/min).
Sample volume: 120 ml.
Sample injected via Superloop.
Active fractions pooled: 40–41.

Step three

Size exclusion chromatography



Conditions
Column: HiLoad 16/60 Superdex 75 prep grade.
Column volume: 120 ml.
Detection: UV at 280 nm.
Buffer: 150 mM NaCl in 20 mM phosphate, pH 7.0.
Sample volume: 3 ml[1].
Flow rate: 50 cm/h (1.7 ml/min).
Sample injected via Superloop.

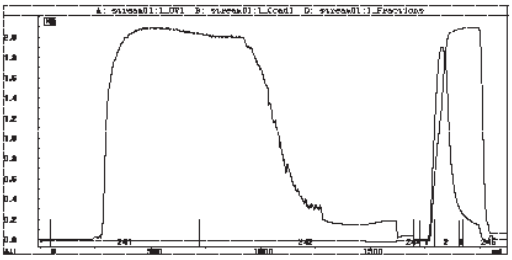
[1] The pooled fractions from step two were concentrated ~7 times before injection.

Example 3

Annexin V is a calcium-dependent anticoagulant present in the human placenta. In the example below, recombinant annexin V produced in *E. coli* was purified according to the Standard Purification Protocol on page 28, modified by exchanging the packed bed in step one with expanded bed adsorption on STREAMLINE™ DEAE. This modification allows unclarified lysate to be applied to the column. Annexin V has a molecular mass of 34 kD, and an isoelectric point of 4.9 and is located in the cytoplasm of the host.

Step one

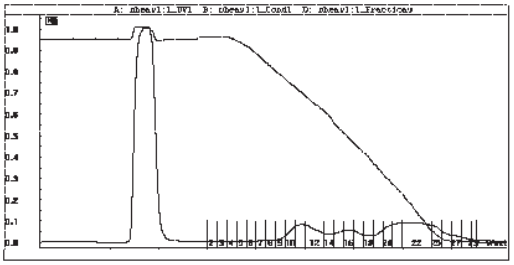
Expanded bed adsorption



Conditions
Medium: STREAMLINE DEAE.
Column: STREAMLINE 50.
Packed bed volume: 300 ml.
Detection: UV at 280 nm.
Adsorption buffer: 30 mM ammonium acetate, pH 5.5.
Elution buffer: 1.0 M NaCl in 30 mM ammonium acetate, pH 5.5.
Expanded bed flow rate: 300cm/h.
Desorption flow rate: 100 cm/ h.
Sample volume: 800 ml.
Sample applied via external pump.

Step two

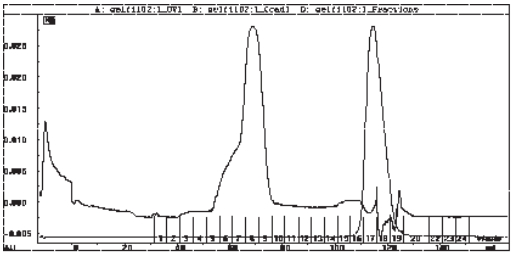
Hydrophobic interaction chromatography



Conditions
Column: HiLoad 16/10 Phenyl Sepharose 6 Fast Flow (high sub).
Column volume: 20 ml.
Detection: UV at 280 nm.
Buffer A: 1.0 M ammonium sulphate in 20 mM phosphate, pH 6.0.
Buffer B: 20 mM phosphate, pH 6.0.
Gradient: 1.0 → 0 M ammonium sulphate
Gradient vol: 10 column volumes.
Flow rate: 150 cm/h (2.5 cm/ min).
Sample volume: 30 ml.
Sample injected via Superloop.
Active fractions pooled: 10–14.

Step three

Size exclusion chromatography



Conditions
Column: HiLoad 16/60 Supedex 75 prep grade.
Column volume: 120 ml.
Buffer: 150 mM NaCl in 20 mM phosphate, pH 7.0.
Sample volume: 6 ml.
Flow rate: 50 cm/h (1.7 ml/min).
Sample injected via Superloop.

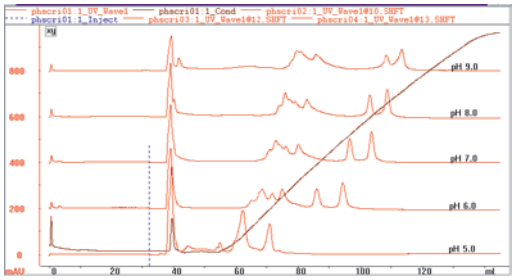
12.2. Optimization of individual protein purification steps

Optimization of ion exchange chromatography pH scouting

Example 1

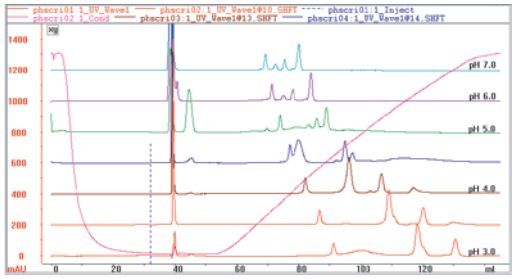
To illustrate the versatility of the pH scouting facility of ÄKTA*explorer 100*, the pH Optimization Protocol (page 35) was used to separate a mixture of chymotrypsinogen A (pI 9.0), horse heart cytochrome C (pI 9.4), lysozyme (pI 11.0), transferrin (pI 6.0), ovalbumin (pI 4.7) and β-lactoglobulin (pI 5.1) on AIEX and CIEX at various pH values. BufferPrep was used to automatically prepare the eluents and the series of experiments was carried out unattended.

AIEX, pH range 5.0 to 9.5



Conditions
Column: Mono Q HR 10/10.
Column volume: 8 ml.
Detection: UV at 280 nm.
Buffer: BufferPrep, AIEX pH range 5–9.5.
Gradient: 0 → 0.5 M NaCl.
Gradient vol: 10 column volumes.
Flow rate: 4.0 ml/min.
Sample volume: 500 µl.

CIEX, pH range 3.0 to 7.0

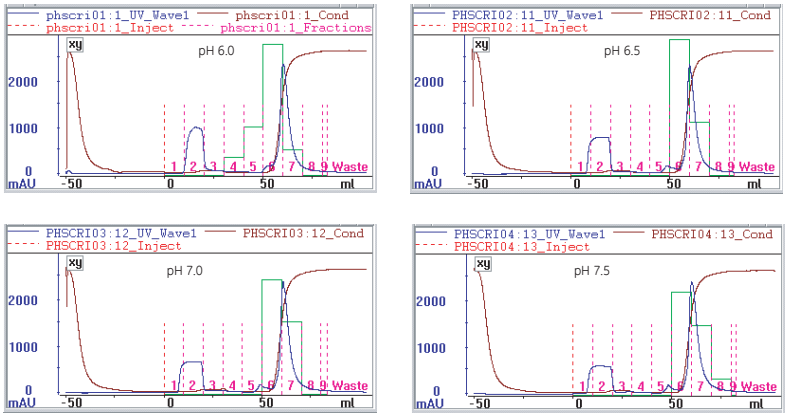


Conditions
Column: Mono S HR 10/10.
Column volume: 8 ml.
Detection: UV at 280 nm.
Buffer: BufferPrep, CIEX pH range pH 3–7.5.
Gradient: 0 → 1.0 M NaCl.
Gradient vol: 10 column volumes.
Flow rate: 4.0 ml/min.
Sample volume: 500 µl.

Example 2

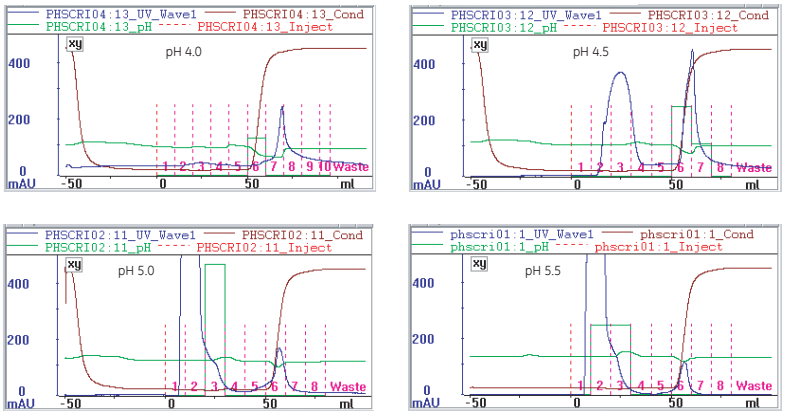
Alkaline phosphatase occurs naturally in *E. coli* cytoplasm. In the experiments below, this enzyme from clarified *E. coli* extract was used to illustrate the optimization of running pH in ion exchange chromatography. This enzyme has a molecular mass of 80 kD and an isoelectric point of 5.1 to 5.4.

Q Sepharose Fast Flow, pH range 6.0 to 7.5



Conditions
Column: HiLoad 16/10 Q Sepharose Fast Flow.
Column volume: 20 ml.
Detection: UV at 280 nm.
Buffer: BufferPrep, AIEX pH range 5–9.5.
Gradient: 0 → 1.0 M NaCl.
Gradient volume: 10 column volumes.
Flow rate: 5 ml/min.
Sample volume: 10 ml.

SP Sepharose Fast Flow, pH range 4.0 to 5.5



Conditions
Column: HiLoad 16/10 SP Sepharose Fast Flow.
Column volume: 20 ml.
Detection: UV at 280 nm.
Buffer: BufferPrep CIEX recipe pH 3–7.5.
Gradient: 0 → 1.0 M NaCl.
Gradient volume: 10 column volumes.
Flow rate: 5 ml/min.
Sample volume: 10 ml.

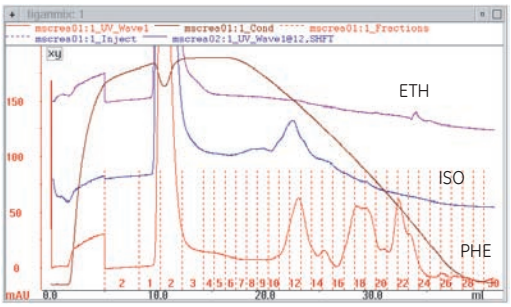
With SP Sepharose Fast Flow, adsorption is observed at pH 4.5. Note that some activity is lost at pH 4.0.
On Q Sepharose Fast Flow, complete adsorption is observed at pH ≥6.5.

Due to the risk of loosing activity at pH 4.5, SP Sepharose was considered less suitable and Q Sepharose at pH 6.5 was chosen for further experiments.

Optimization of hydrophobic interaction chromatography

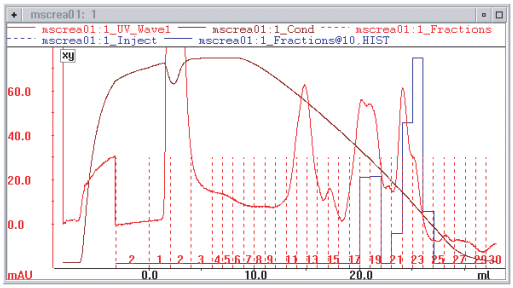
In the experiments below, partially purified *E. coli* alkaline phosphatase was used to illustrate the optimization of hydrophobic interaction chromatography by media screening and scouting the following parameters; gradient, binding buffer, flow rate and sample load. *E. coli* alkaline phosphatase has a molecular mass of 80 kD and an isoelectric point of 5.1 to 5.4.

Media screening



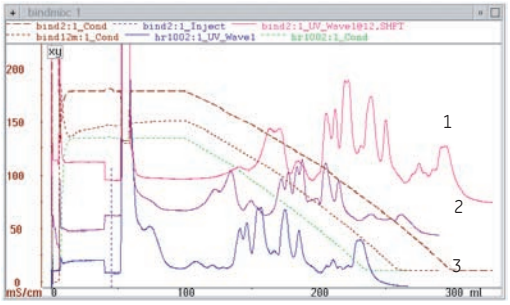
Conditions
Columns: HIC test kit consisting of
1. RESOURCE 15ETH.
2. RESOURCE 15ISO.
3. RESOURCE 15PHE.
Column volume: 1 ml
Buffer A: 2.0 M ammonium sulphate in 100 mM phosphate, pH 7.0
Buffer B: 100 mM phosphate, pH 7.0
Gradient: 1.2 → 0 M ammonium sulphate
Gradient vol: 20 column volumes.
Flow rate: 1.0 ml/ min.
Sample volume: 1.0 ml.

SOURCE 15PHE was considered the optimal medium.



Conditions
See above.

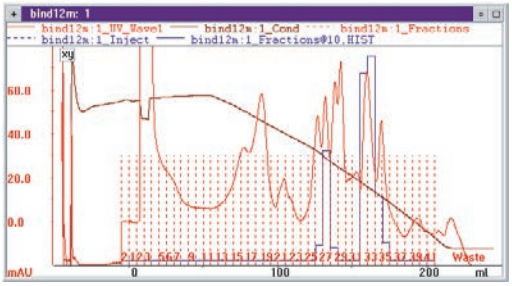
Binding buffer scouting



Conditions
Medium: SOURCE 15PHE.
Column: HR 10/10.
Column volume: 8 ml.
Buffer A: 2.0 M ammonium sulphate in 100 mM phosphate, pH 7.0
Buffer B: 100 mM phosphate, pH 7.0
Gradients:
1. 1.5 → 0 M ammonium sulphate.
2. 1.2 → 0 M ammonium sulphate.
3. 1.0 → 0 M ammonium sulphate.
Gradient volumes[1]**:**
1. 25 column volumes.
2. 20 column volumes.
3. 17 column volumes.
Flow rate: 2.0 ml/min.
Sample volume: 7.0 ml.
Sample injected via Superloop.

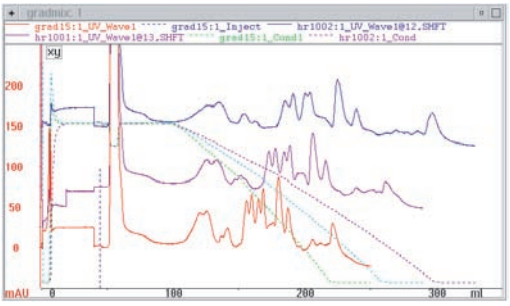
[1] All gradients are of the same slope

1.2 M ammonium sulphate was considered the best compromise between resolution and amount of non-adsorbed protein background.



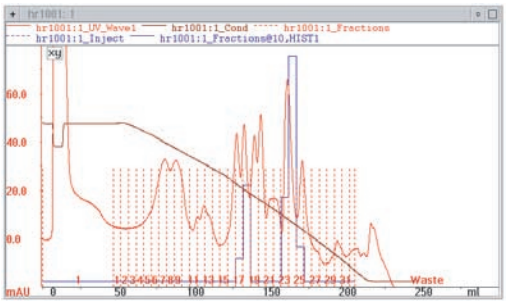
Conditions
See above.

Gradient volume scouting



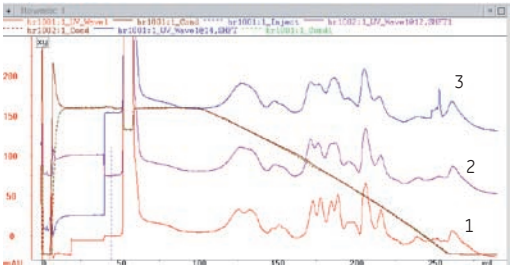
Conditions
Medium: SOURCE 15PHE.
Column: HR 10/10.
Column volume: 8 ml.
Buffer A: 2.0 M ammonium sulphate in 100 mM phosphate, pH 7.0
Buffer B: 100 mM phosphate, pH 7.0
Gradient: 1.2 → 0 M ammonium sulphate.
Gradient volumes:
1. 15 column volumes.
2. 20 column volumes.
3. 25 column volumes.
Flow rate: 2.0 ml/min.
Sample volume: 7 ml.
Sample injected via Superloop.

A gradient volume of 20 column volumes was considered to provide sufficient resolution.



Conditions
See above.

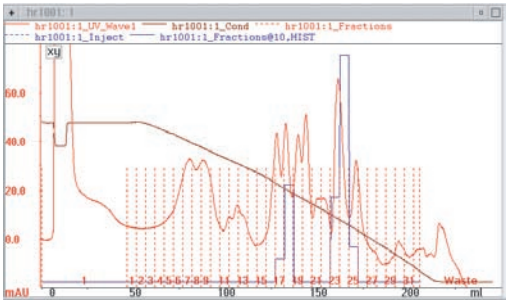
Flow rate scouting



Conditions
Column: HR 10/10.
Medium: SOURCE 15PHE.
Column volume: 8 ml.
Buffer A: 1.2 M ammonium sulphate in 100 mM phosphate, pH 7.0
Buffer B: 100 mM phosphate, pH 7.0
Gradient: 1.2 → 0 M ammonium sulphate.
Gradient volume: 20 column volumes.
Flow rates:
1. 2.0 ml/min.
2. 4.0 ml/min.
3. 6.0 ml/min.

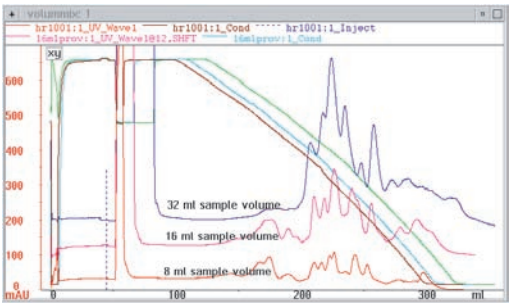
Sample volume: 7.0 ml.
Sample injected via Superloop.

2 ml/min was chosen as the highest flow rate giving acceptable resolution among those tested.



Conditions
See above.

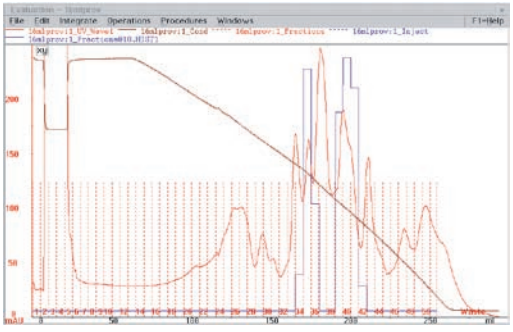
Sample load scouting



Conditions
Medium: SOURCE 15PHE.
Column: HR 10/10.
Column volume: 8 ml.
Buffer A: 1.2 M ammonium sulphate in 100 mM phosphate, pH 7.0
Buffer B: 100 mM phosphate, pH 7.0
Gradient: 1.2 → 0 M ammonium sulphate.
Gradient volume: 20 column volumes.
Flow rate: 2.0 ml/min.
Sample volumes:
1. 8 ml.
2. 16 ml.
3. 32 ml.
Sample injected via SuperLoop.

Resolution was considered acceptable at a sample load of 8 ml.

Final method



Conditions
See above.

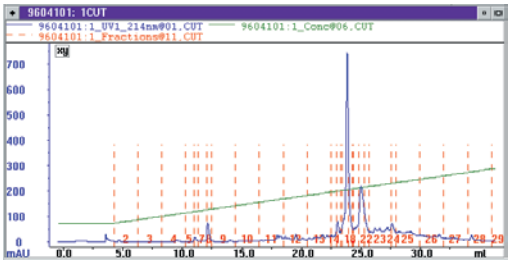
12.3 Purification of peptides

12.3.1 Synthetic peptides

Example 1

Peptide synthesis can often be carried out with great precision, leaving just a few by-products such as synthetic failures and modified peptides as the major contaminants. However, these products are often structurally closely related to the target peptide and high resolution techniques are required to remove them. Combining two chromatography steps with different selectivities is often a much simpler way of obtaining the necessary purity than optimizing a one-step method. Running pH often has a large influence on the selectivity in RPC. In the example below, RPC at pH 7.0 was combined with RPC at pH 2.5 using the same chromatography medium. In contrast to step one, the eluent system of step two is volatile and the final peptide product can thus be directly lyophilised. The purity check was carried out on a different RPC medium with yet another selectivity. The target peptide is a 30-mer with the following sequence:
EPPTA AREKQ YLINS QQKSL LQPGE KLVSD.

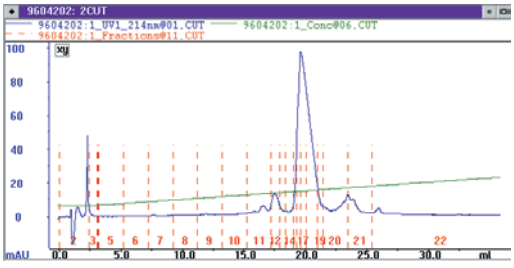
Step one RPC at pH 7.0



Instrument: ÄKTApurifier.

Conditions
Sample: 0.6 mg of crude material.
Column: Sephasil Peptide C18 5 µm ST 4.6/100.
Column volume: 1.66 ml.
Detection: UV at 214 nm.
Eluent A: 10 mM phosphate, pH 7.0.
Eluent B: 75 % acetonitrile in 9 mM phosphate.
Running pH: 7.0.
Gradient: 10–40 % B in 20 column volumes.
Flow rate: 1 ml/min.

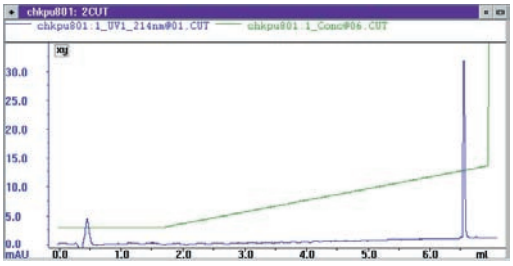
Step two RPC at pH 2.5



Instrument: ÄKTApurifier.

Conditions
Sample: Fractions 17–18 from step one.
Column: Sephasil Peptide C18 5 µm ST 4.6/100.
Column volume: 1.66 ml.
Detection: UV at 214 nm.
Eluent A: 0.06 % TFA.
Eluent B: 84 % acetonitrile in 0.055 % TFA.
Running pH: 2.5.
Gradient: 25–35 % B in 20 column volumes.
Flow rate: 1 ml/min.

Purity check



Conditions
Sample: Fractions 16–18 from step two.
Column: µRPC C2/C18 SC.
Column volume: 0.35 ml.
Detection: UV at 214 nm.
Eluent A: 0.06 % TFA.
Eluent B: 84 % acetonitrile in 0.055 % TFA.
Running pH: 2.5.
Gradient: 20–40 % B in 15 column volumes.
Flow rate: 0.15 ml/min.

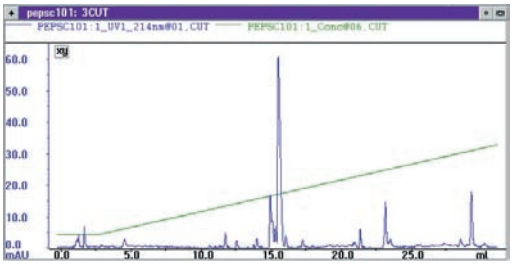
Purity of the target peptide in the crude material: 33 %.
Purity of the target peptide after purification: 99 %.

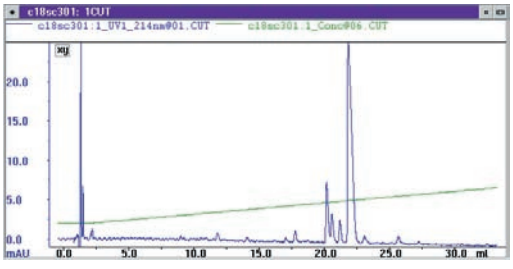
Example 2

This example of purification of synthetic peptides uses the one-step approach. To optimize the conditions, test runs were performed at pH 2.5 and 7.0 at different gradient slopes.
The best resolution was obtained in run 4 and the preparative run was consequently performed under the corresponding conditions.
The purity check was carried out on a RPC medium with a different selectivity.
The target peptide was a 10-mer with the following sequence:
YADKI TEDLK.

Optimization

RPC at pH 2.5

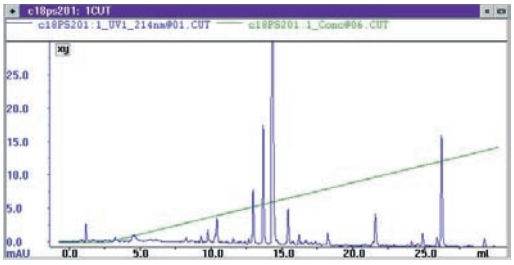


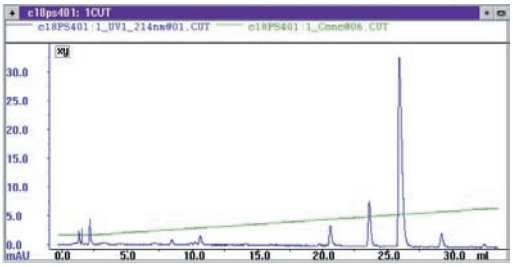


Instrument: ÄKTApurifier.

Conditions
Sample: 20 µg of crude material.
Column: Sephasil Peptide C18 5 µm ST 4.6/100.
Column volume: 1.66 ml.
Detection: UV at 214 nm.
Eluent A: 0.06 % TFA.
Eluent B: 84 % acetonitrile in 0.055 % TFA.
Running pH: 2.5.
Gradients:
1) 0–60 % in 20 column volumes.
2) 10–30 % B in 20 column volumes.
Flow rate: 1 ml/min.

RPC at pH 7.0



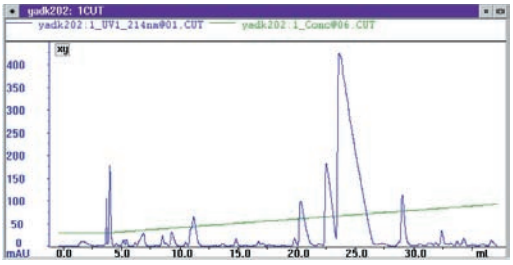


Instrument: ÄKTApurifier.

Conditions
Sample: 20 µg of crude material.
Column: Sephasil Peptide C18 5 µm ST 4.6/100.
Column volume: 1.66 ml.
Detection: UV at 214 nm.
Eluent A: 10 mM phosphate, pH 7.0.
Eluent B: 75 % acetonitrile in 9 mM phosphate.
Running pH: 7.0.
Gradient: 1) 0–60 % B in 20 column volumes.
2) 5–20 % B in 20 column volumes.
Flow rate: 1 ml/min.

Preparative run

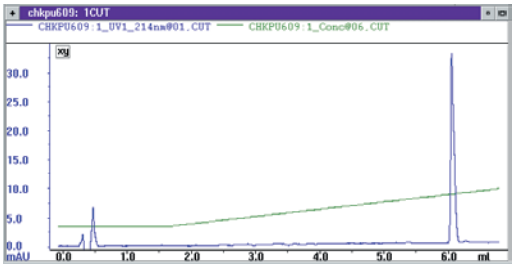
RPC at pH 7.0.



Instrument: ÄKTApurifier.

Conditions
Sample: 1.0 mg of crude material.
Column: Sephasil Peptide C18 5 µm ST 4.6/100.
Column volume: 1.66 ml.
Detection: UV at 214 nm.
Eluent A: 10 mM phosphate, pH 7.0.
Eluent B: 75 % acetonitrile in 9 mM phosphate.
Running pH: 7.0.
Gradient: 5–20 % B in 20 column volumes.
Flow rate: 1 ml/min.

Purity check



Instrument: ÄKTApurifier.

Conditions
Sample: Peak fractions.
Column: µRPC C2/C18 SC.
Column volume: 0.35 ml.
Detection: UV at 214 nm.
Eluent A: 0.06 % TFA.
Eluent B: 84 % acetonitrile in 0.055 % TFA.
Running pH: 2.5.
Gradient: 10–30 % B in 15 column volumes.
Flow rate: 0.15 ml/min.

Purity of the target peptide in the crude material: 52 %.
Purity of the target peptide after purification: 99 %.

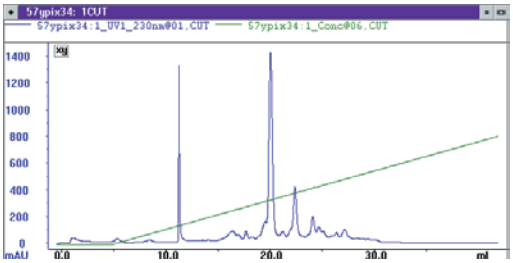
Example 3

Charged protein fragments can be separated by ion exchange chromatography. This technique thus constitutes a good complement to RPC. In this example, the synthetic peptide was sufficiently soluble only above pH 7 and unstable at higher pH values. This rules out the use of silica based RPC media so cation exchange chromatography at pH 7.0 was tried as an alternative. In example 4 below, the same peptide was run on a RESOURCE RPC column, which is stable at pH ≤12.

Note the difference in selectivity between the AIEX and the RPC runs.

The target peptide had the following sequence:
ISPDG HEYIY (PO_3)VDP MQLPY.

AIEX at pH 7.0



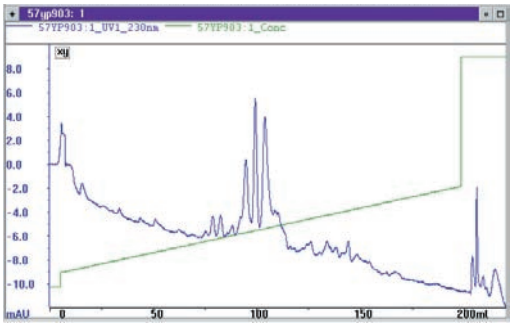
Instrument: ÄKTApurifier.

Conditions
Sample: 200 µg of crude synthetic peptide.
Column: Mono Q HR 5/5.
Detection: UV at 230 nm.
Eluent A: 30 % acetonitrile in 5 mM sodium phosphate, pH: 7.0.
Eluent B: Eluent A containing 0.75 mol/l NaCl.
Gradient: 0–75 % B in 40 column volumes.
Flow rate: 1 ml/min.

Example 4

RESOURCE RPC was chosen to purify the synthetic peptide from example 3 above.

The experiment was carried out at pH 9.0 as a compromise between solubility, stability and resolution. Peptide sequence:
ISPDG HEYIY (PO_3)VDP MQLPY.

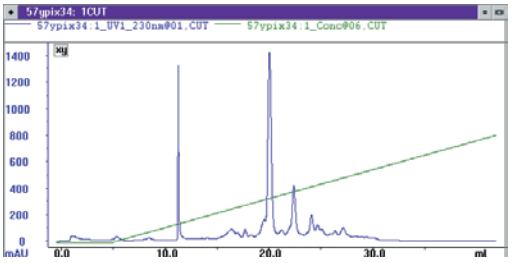


Instrument: ÄKTApurifier.

Conditions
Sample: 600 µg of crude peptide.
Column: RESOURCE RPC.
Column volume: 3 ml.
Detection: UV at 214 nm.
Eluent A: 0.1 M NH_4HCO_3.
Eluent B: 60 % acetonitrile.
Running pH: 9.0.
Gradient: 10–40 % B in 60 column volumes.
Flow rate: 1.3 ml/min.

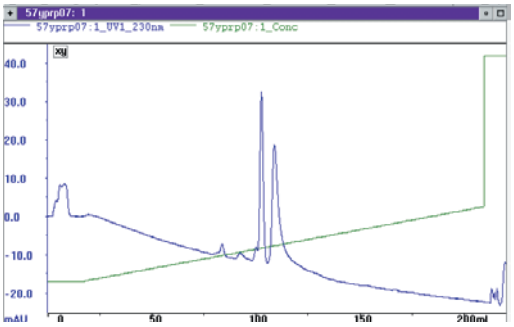
Example 5

Two truncated peptides were found besides the full length product in the crude preparation after synthesis of the peptide ISPDG HEYIY (PO_3)VDP MQLPY. When analysing the results in examples 3 and 4 above by MS, it turned out that the IEX experiment was able to remove one of the truncated forms, while the RPC experiment removed the other. Thus by combining the two techniques it was possible to obtain the target peptide completely free from any truncated forms.



Instrument: ÄKTApurifier.

Conditions
Sample: 200 µg of crude synthetic peptide.
Column: Mono Q HR 5/5.
Detection: UV at 230 nm.
Eluent A: 30 % acetonitrile in 5 mM phosphate, pH 7.0.
Eluent B: Elent A containing 0.75 mol/l NaCl.
Gradient: 0–75 % in 40 column volumes.
Flow rate: 1 ml/min.



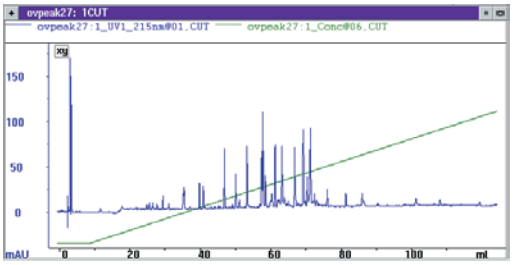
Instrument: ÄKTApurifier.

Conditions
Sample: 100 µg of main peak from step one diluted 1:5 in eluent A.
Column: RESOURCE RPC 3ml.
Detection: UV at 230 nm.
Eluent A: 0.1 M NH_4HCO_3, pH 8.9.
Eluent B: 60 % acetinitrile.
Gradient: 10–40 % B in 60 column volumes.
Flow rate: 1 ml/min.

12.3.2 Protein fragments

Example 1

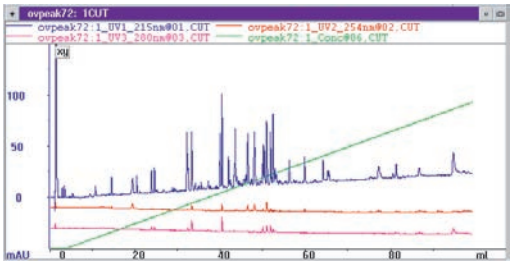
Reduced and pyridylethylated ovalbumin was digested with modified trypsin and standard conditions were used to separate the resulting fragments.

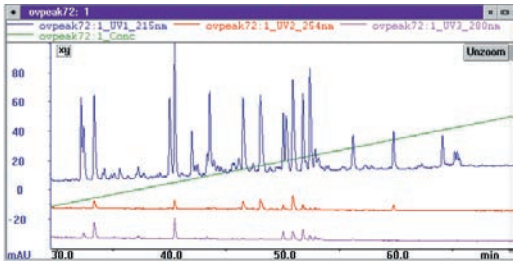


Instrument: ÄKTApurifier.
Conditions
Sample: Reduced and pyridylethylated ovalbumin digested with modefied trypsin, 1 nmol.
Column: Sephasil Peptide C18 5 µm ST 4.6/250.
Column volume: 4.16 ml.
Detection: UV at 214 nm.
Eluent A: 0.06 % TFA.
Eluent B: 84 % acetonitrile in 0.055 % TFA.
Running pH: 2.5.
Gradient: 0–75 % B in 30 column volumes.
Flow rate: 1.0 ml/min.

Example 2

Reduced and pyridylethylated ovalbumin was digested with Lys-C specific *Achromobacter lyticus* protease. The pyridylethylation will tag all free SH-groups within the protein and thus indicate which fragments contain cysteine residues by absorption at 250 nm. Standard conditions were then used to separate the fragments obtained.





Instrument: ÄKTApurifier.

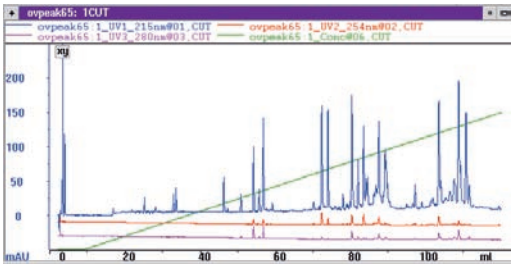
Conditions
Sample: Reduced and pyridylethylated ovalbumin with modefied trypsin, 1 nmol.
Column: µRPC C2/C18 SC.
Column volume: 0.35 ml.
Detection: UV at 214 nm, 254 nm and 280 nm.
Eluent A: 0.06 % TFA.
Eluent B: 84 % acetonitrile in 0.055 % TFA.
Running pH: 2.5.
Gradient: 0–75 % B in 30 column volumes.
Flow rate: 1.0 ml/min.

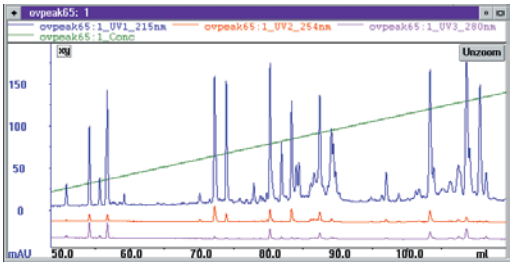
Magnification of the chromatogram above.

Example 3

Here the Lys-C digest of example 2 was separated under the same conditions as above except for the column. It is clear that the two columns show rather different selectivities.

Note that the cysteine-containing fragments (the 254 nm trace) elute in different orders in the two experiments.





Magnification of the chromatogram above.

Instrument: ÄKTApurifier.

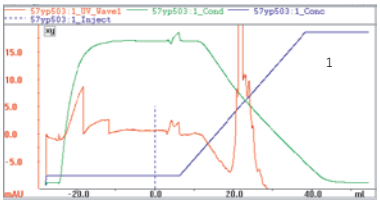
Conditions
Sample: Reduced and pyridylethylated ovalbumin digested with modefied trypsin, 1 nmol.
Column: Sephasil Peptide C18 5 µm ST 4.6/250.
Column volume: 4.16 ml.
Detection: UV at 214 nm, 254 nm and 280 nm.
Eluent A: 0.06 % TFA.
Eluent B: 84 % acetonitrile in 0.055 % TFA.
Running pH: 2.5.
Gradient: 0–75 % B in 30 column volumes.
Flow rate: 1.0 ml/min.

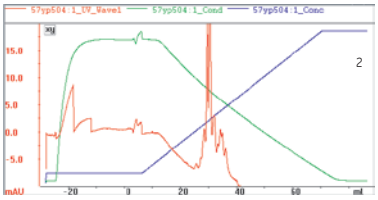
12.3.3 Example of optimization of individual peptide purification steps

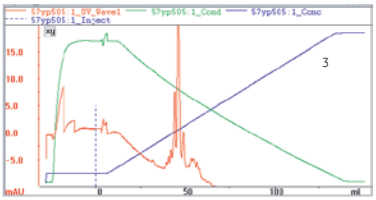
RPC at alkaline conditions

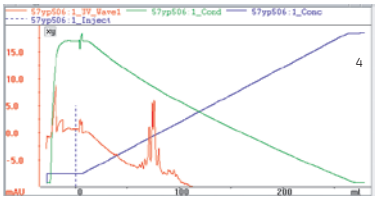
The experiment below illustrates the separation of synthetic peptides at alkaline conditions. This target peptide has the following sequence: ISPDGHEYIY(PO_3)VDPMQLPY, and is sufficiently soluble only above pH 7. However, the peptide is unstable at higher pH values and the experiment was carried out at pH 9.0 as a compromise between solubility, stability and resolution.

Gradient scouting

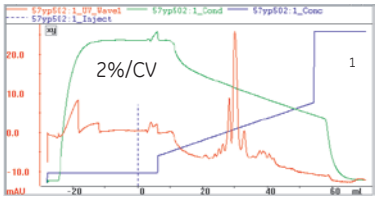


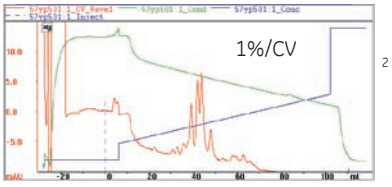


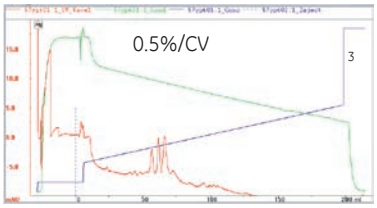




Conditions
Column: RESOURCE RPC.
Column volume: 3 ml.
Eluent A: 0.1 M NH_4HCO_3.
Eluent B: 60 % acetonitrile.
Running pH: 9.0.
Gradients:
1. 5–85 % B in 10 column volumes.
2. 5–85 % B in 20 column volumes.
3. 5–85 % B in 40 column volumes.
4. 5–85 % B in 80 column volumes.
Flow rate: 1.3 ml/min (1.0 cm/min).
Sample amount: 90 µg.







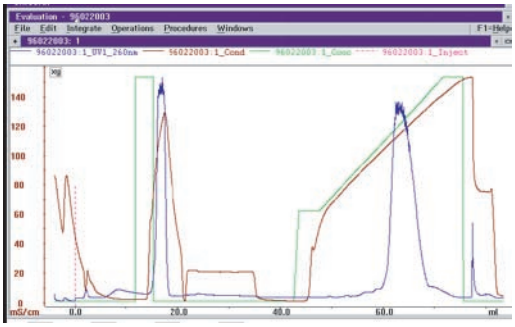
Conditions
Column: RESOURCE RPC.
Column volume: 3 ml.
Running pH: 9.0.
Eluent A: 0.1 M NH_4HCO_3.
Eluent B: 60 % acetonitrile.
Gradients:
1. 15–45 % B in 15 column volumes.
2. 15–45 % B in 30 column volumes.
3. 15–45 % B in 60 column volumes.
Flow rate: 1.3 ml/min (1.0 cm/min).
Sample amount: 90 µg.

The experiment eluted with a gradient of 15–45 % B in 60 column volumes clearly provides the best resolution. Cutting off the extreme ends of the gradient helps reduce the experimental time.

12.4 Purification of synthetic oligonucleotides

12.4.1 Phosphorothioate oligonucleotides

The example below shows the purification of a 25-mer phosphorothioated oligonucleotide according to the Standard Purification Protocol for synthetic oligonucleotides (page 87). The first figure shows the result from the purification of the 25-mer and the second shows a 30-fold scale-up of the purification.



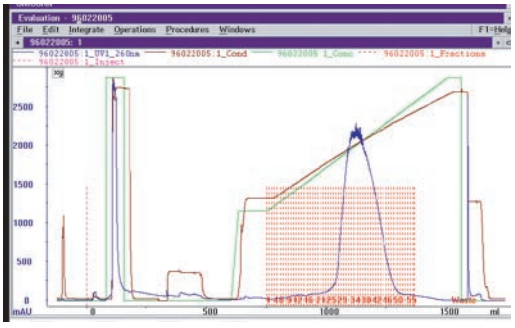
Purity: 97.6 %

Conditions
Column: RESOURCE Q
Column volume: 1 ml.
Eluents:
1. 2 M NaCl in 10 mM NaOH (removal of non-tritylated material).
2. 0.4 % TFA (for de-tritylation).

3 a. 10 mM NaOH.
3 b. 2 M NaCl in 10 mM NaOH (gradient elution of de-tritylated material).

4. 2 M NaCl in 30 % isopropanol (for regeneration).

Flow rate: 1.8 ml/min.
Sample: 7.4 mg tritylated 25-mer synthesis raw product.



Purity: 98.4 %

Conditions
Medium: SOURCE 15Q
Column: FineLine Pilot 35
Column volume: 30 ml.
Eluents:
1. 2 M NaCl in 10 mM NaOH (removal of non-tritylated material).
2. 0.4 % TFA (for de-tritylation).

3 a. 10 mM NaOH.
3 b. 2 M NaCl in 10 mM NaOH (gradient elution of de-tritylated material).

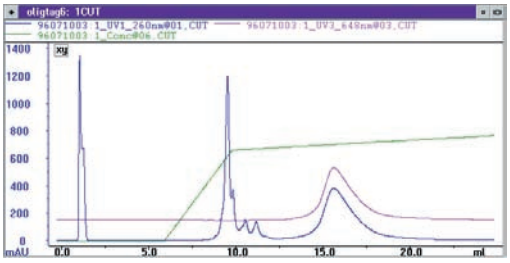
4. 2 M NaCl in 30 % isopropanol (for regeneration).

Flow rate: 48 ml/min.
Sample: 222 mg tritylated 25-mer raw product.

12.4.2 Labelled oligonucleotides

Silica-based RPC media are unstable above neutrality. RESOURCE RPC was therefore used to purify a 5'-Cy5-labelled oligonucleotide. Unlike unlabelled oligonucleotides, Cy5-labelled ones absorb UV light at 648 nm. It is thus easy to differentiate the labelled olignucleotide from unlabelled material by concomitant UV monitoring at 260 and 648 nm.

In the experiment below, a 5'-Cy5-labelled 20-mer oligonucleotide was separated from unlabelled material by RPC.



Final purity of the pooled 5'-Cy5-labelled material: >95 %.

Instrument: ÄKTApurifier.

Conditions
Sample: Approximately 0.5 mg of crude material.
Column: RESOURCE RPC 1 ml.
Detection: UV at 260 nm (blue trace) and visible at 648 nm (red trace).
Eluent A: 5 % acetonitrile in 0.1 M TEAA.
Eluent B: 34 % acetonitrile in 0.1 M TEAA.
Gradient: 0–53 % B in 4 CV, then 53–63 % B in 10 CV.
Flow rate: 1 ml/min.

Appendix I – Tips and hints

I.1 Sample stability

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

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

It is obvious that a stability test should be carried out as a safety precaution before designing a purification protocol. The list below may be used as a basis for such a stability test:

- Test pH stability in steps of one pH unit between pH 2 and pH 9.
- Test salt stability with NaCl and NH_4SO_2 in the steps of 0.5 M between 0 to 4 M and 0 to 2 M respectively.
- Test the stability towards ACN and MeOH in 10 % steps between 0 and 50 %.
- Test the temperature stability in 10 °C steps between 4 and 40 °C.
- Test the stability and occurrence of proteolytic activity by leaving an aliquot of the sample at room temperature overnight.

Centrifuge each sample and measure activity and UV absorbance at 280 nm in the supernatant.

I.2 Sample pre-treatment and storage

I.2.1 Requirements on the starting material

Before the onset of any chromatography step, the sample has to be rendered separable by freeing it from components that may harm the function of the column or may reduce the stability of the sample. The table below lists harmful components usually found in starting materials and the methods for their elimination:

Contaminant	Harmful effect	Preventive action
Particulate matter	Cloggs column.	• Filter through 0.45–0.22 µm membrane or glass filter. • Centrifuge 10–15 minutes at 10 000 g. • Cell homogenate: Centrifuge 30 min at 4 000–50 000 g.
Lipids	• Blocks media ligands. • May cause aggregation. • May cause non-specific adsorption.	• Centrifuge 10–15 minutes at 10 000 g. • If compatible with target molecule stability: Extract with an organic solvent.
Nucleic acids (NA)	• High viscosity. • Blocks media ligands.	• Precipitate NA by addition of streptomycin sulphate, poly-ethyleneimine, protamine sulphate or manganese salts. • Degrade NA by adding nucleases.
Proteases	Degrades target molecule.	• Add protease inhibitors. • Remove proteases by affinity chromatography. • Rapidly perform 1st purification step. • Work at low temperature. • Select protease deficient *E. coli* strains.

I.2.2 Conditioning the sample for a particular purification step

Before applying the sample to a particular purification step, it has to be conditioned to meet the methodological requirements of that step. The order of purification steps in the purification protocol used determines whether conditioning can be done simply, by adjusting sample pH or adding a certain salt, or if buffer exchange techniques have to be applied. The table on the next page lists the required conditions for the different chromatography techniques.

	Chromatographic techniques			
Sample condition	**IEX** (gradient-eluted)	**HIC** (gradient-eluted)	**RPC** (gradient-eluted)	**SEC** (isocratic)
Volume	No limitation.	No limitation.	No limitation.	1–5 % of column volume. Concentrate by: • Step-eluted IEX or RPC. • Ultrafiltration. • Freeze-drying.
Amount	10–20 % of total loading capacity. Dilute with "eluent A" to proper sample conc.	10–20 % of total loading capacity. Dilute with "eluent A" to proper sample conc.	10–20 % of total loading capacity. Dilute with "eluent A" to proper sample conc.	Limited only by sample viscosity. (See below)
Viscosity	Max 4cP corresp. to < 5 % protein conc.	Max 4cP corresp. to < 5 % protein conc.	Max 4cP corresp. to < 5 % protein conc.	Max 4cP corresp. to < 5 % protein conc.
pH/salt	Corresp. to that of "eluent A". Adjust with conc. "eluent A" or perform buffer exchange on SEC medium.	Salt type and content corresp. to that of "eluent A". Adjust with conc. "eluent A".	Salt content not critical. Adjust with conc. "eluent A".	Limited only by matrix and sample stability.
Organic solvent	Up to 30 % (e.g. ACN) acceptable to reduce non-specific adsorption.		Corresp. to that of "eluent A". Adjust with conc. "eluent A". If necessary reduce by freeze-drying.	Limited only by matrix and sample stability.

I.2.3 Sample storage conditions

Conditions that keep the target molecule stable and active depend on the particular sample. However, some general precautions may be taken, as listed below:

Short term storage (max. 24 hours)

- *Avoid conditions close to stability limits.* Precipitation and denaturation are slow processes with no sharp onset thresholds.
- *Keep refrigerated and in a closed vessel.* This will lower the risk of bacterial contamination and breakdown due to residual proteolytic activity.

Extended storage (days)

- *Avoid conditions close to stability limits.* (See above)
- *Add a bacteriostatic agent when possible.*
- *Keep refrigerated and in a closed vessel.* (See above)

Long-term storage

- *Avoid conditions close to stability limits.* (See above)
- *Keep frozen or freeze-dried.* Although being good long-term storage methods, repeated freeze-drying/re-dissolving or freezing/thawing often leads to losses of biological activity.

I.2.4 Optimizing recovery

Most purification techniques do not provide 100 % recovery and, as seen in table below, the overall recovery in a purification protocol is indeed influenced by the total number of steps.

	Over-all recovery after n steps				
Recovery per step (%)	**n = 1**	**n = 2**	**n = 3**	**n = 4**	**n = 5**
99	99.0	98.0	97.0	96.1	95.1
95	95.0	90.3	85.7	81.5	77.4
90	90.0	81.0	72.9	65.6	59.0
80	80.0	64.0	51.2	41.0	32.8
70	70.0	49.0	34.3	24.0	16.8

This is one of the major reasons for keeping an eye on the recovery in each step in a purification protocol and keeping the number of steps as low as possible. In most cases, such losses depend either on non-specific adsorption effects, on conditions chosen too close to the stability limit of the sample, or on the removal of a stabilising factor.

For HIC, the choice of conditions and medium is often a balance between the use of ligands that bind the target molecule just strongly enough not to harm it and employing a salt concentration that promotes binding but does not precipitate the sample. Knowing the stability range of the sample is thus quite important. Keeping the actual separation time as short as possible is also a good safety precaution.

In general, peptides are slightly more hydrophobic than proteins and when run on IEX or SEC with aqueous eluents, losses due non-specific adsorption may occur. However, the addition of 20–40 % acetonitrile will in most cases counteract such effects.

Besides leading to excessive losses, non-specific adsorption may lower the loading capacity of the column over time. To help prevent from such phenomena, it is recommended to perform cleaning-in-place (CIP) at regular intervals. Listed below are some measures to be taken when recovery suffers:

- Always keep within the stability limits of the sample.
- Keep the number of purification steps as low as possible.
- Scout for running conditions which give maximal recovery with acceptable resolution.
- Scout for ACN or MeOH additions (up to 30 %) to eliminate losses caused by hydrophobic interaction.
- Try the addition of EDTA.
- Try to saturate the non-specific adsorption sites by flushing the column with a non-target protein, e.g. albumin.
- Use oxygen-depleted eluents to avoid any oxidation, especially at high running pH values.
- Change to another matrix within the same technique.
- Change to another technique which gives acceptable recoveries.

I.3 Means of evaluating purification results

UV detection alone is, in most cases, unable to identify the different components of a protein or peptide sample. It can, however, be used as a simple and practical method to continuously monitor the protein or the peptide content in the eluate from chromatography columns.

To be able to follow the target molecule through the different steps of a purification protocol, one has to rely on off-line techniques. Thus a fraction collector has to be employed even in analytical situations such as scouting, in order to "freeze" the results and to enable the transfer of small aliquots of the relevant fractions to the off-line detection device.

The techniques commonly used to evaluate the results of purification experiments are described below.

Techniques for the determination of target protein content

- *Enzyme activity assays*. These are often spectrophotometric and rather easy to perform.
- *Bio-assays*. These can be rather unprecise and time-consuming and should for this reason be avoided if possible.
- *RIA , ELISA*. Though quite time-consuming and dependent on the availability of specific antibodies, these techniques are widely used.
- *Immuno-electrophoretic techniques*. Depending on the specificity of the available antibodies, these techniques can be used to detect and quantify both the target molecule and the contaminants. They are, however, rather time-consuming and require hands-on experience to work well.
- *Fluorometry*. This technique is very sensitive and easy to perform, but requires the target molecule to contain an fluorescent chromophor.
- *Mass spectrometry*. MS can provide very accurate Mr determinations, but requires volatile solvents, not too complex samples and is restricted to Mr < 100 000.

I.3.1 Techniques for the determination of total protein content

None of the methods used for determining total protein content provide very high accuracy. Still, they are sufficiently accurate to allow calculation of useful specific activity or purity values.

- *UV-absorption.* The absorption at 280 nm reflects the content of tyrosine and tryptophane and is commonly used for proteins. Peptides, however, may lack these amino acids and absorption which measures the content of double bonds at 214 nm is used instead.
- *Colourimetry* according to Lowry.
- *Dye-binding* according to Bradford.

I.3.2 Techniques for the determination of target peptide content

- *Analytical RPC*. This technique provides very high resolution and does not require staining to visualise the separated components. However, when the target molecule is a peptide, RPC has in most cases already been used as part of the purification protocol. In such cases, it is advisable to apply different running conditions to provide maximum resolution.
- *RIA, ELISA*. Though quite time-consuming and dependent on the availability of specific antibodies, these techniques are widely used.
- *Immuno-electrophoretic techniques*. Depending on the specificity of the available antibodies, these techniques can be used to detect and quantify both the target molecule and the contaminants. They are, however, rather time-consuming and require hands-on experience to work well.
- *Capillary zone electrophoresis*. This technique is ideal for peptides, since it provides very high resolution and does not require staining to visualise the separated components.
- *Mass spectrometry*. MS is very suitable for peptide analysis. It provides very accurate Mr determinations, but requires volatile solvents, and not too complex samples.

I.3.3 Techniques for the determination of complexity

- Analytical IEX (applicable to both proteins and peptides).
- Analytical RPC (applicable primarily to peptides and smaller proteins).
- SDS-electrophoresis (applicable to proteins and larger peptides).
- Isoelectric focusing (applicable to proteins only).
- Capillary zone electrophoresis (applicable to both proteins and peptides).

I Appendix – tips and hints

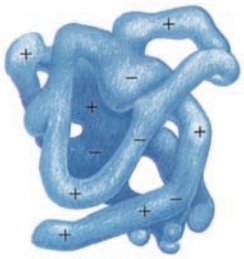
I.3 Means of evaluating purification results

Appendix II – A brief theoretical background to the chromatography techniques

II.1 Properties of target molecules used for separation

The possibility to separate proteins and peptides relies on differences in their physical properties. In most cases, the native structure of the target molecule is to be conserved during purification and only properties associated with the surface of the target molecule are available for separation.

Surface net charge, hydrophobicity and size form the basis of the most commonly used purification techniques, namely ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), reversed phase chromatography (RPC) and size exclusion chromatography (SEC).



Affinity chromatography makes use of more specific properties of a target molecule that associate with certain ligands. Such ligands are often analogues of molecules that are involved in the biological function of the target molecule e.g. enzyme substrates and inhibitors. Ligands like lectins and antibodies that recognise certain structural features are also used. Sometimes a specific binding property, an affinity handle, can be built into the target molecule and used for its purification. The poly-His handle which will associate with metal chelator ligands is an example of this.

II.2 Sources of proteins and peptides

Three main sources of proteins and peptides can be distinguished, namely:

- Natural sources i.e. tissues or body fluids from living matter.
- Recombinant sources i.e. genetically manipulated cells.
- Synthetic sources.

Natural sources are in most cases very complex and low in the content of the target molecule. Starting from a natural source thus nearly always means that large volumes of extracts have to be handled and that a multi-step purification protocol has to be applied to remove all contaminants. Care has to be taken to remove particulate matter and nucleic acids and to minimise protease activity in order to arrive at a stable chromatographable solution.

Recombinant sources are normally much richer in the target molecule and access to raw material is more or less unlimited. Three principally different locations of the expressed target protein occur:

- Cytoplasmic location, which requires disintegration of the whole cell and results in the release of nucleic acids, particulate matter and some thousand host cell proteins.

  Sometimes the target molecule occurs in the form of insoluble inclusion bodies in the cytoplasm. Inclusion bodies consist almost exclusively of the expressed target molecule and are easily isolated by centrifugation. However, their solubilisation and renaturation is often complicated.

- Periplasmic location, i.e. located in the space between the outer and the inner membrane. This location reduces the number of contaminating proteins to some hundred.

- Secreted into the surrounding culture medium. Only some ten proteins are secreted from *E. coli* and the background of contaminants depends heavily on the composition the culture medium. Since the concentration of the target molecule is normally very low, water constitutes one of the major contaminants.

Although less starting material has to be handled, recombinantly produced proteins require multi-step protocols for their purification. As with natural sources, particulate matter and nucleic acids have to be removed and protease activity has to be minimised in order to arrive at a stable chromatographable solution.

Peptide synthesis avoids the presence of "living matter contaminants". Instead the raw product contains non-peptide impurities like cleaved-off protecting groups and scavengers. Furthermore, failure peptides due to chemical side reactions and incomplete synthesis may occur. Non-peptide impurities are in most cases easily removed. Failure peptides, however, often deviate very little in chromatographic properties from the target molecule and can only be eliminated by high resolution techniques. The table opposite summarises some important characteristics of the different sources.

Source	Content of target molecule	Complexity	Type of contaminants	Pre-chromatography treatment	Purification strategy
NATURAL	Often low	High (thousands of proteins)	Solids Host proteins Nucleic acids Proteases	**Remove:** Solids Nucleic acids **Inhibit or remove:** Proteases	**1) Capture chromatography** to concentrate and reduce the number of contaminants. **2) High resolution chromatography** to remove the "difficult" ones. **3) Polishing chromatography** to remove polymeric and denatured forms
RECOMBINANT **– Cytoplasmic**	Normally high	High (thousands of proteins)	Cell debris Host proteins Nucleic acids Proteases	**Remove:** Solids Nucleic acids **Inhibit or remove:** Proteases	**Same as for Natural source**
– Inclusion bodies	High	Low (mainly target protein)		**Isolate by centrifugation.** **Solubilise and renature**	**High resolution chromatography** to eliminate denatured forms
– Periplasmic	Normally high	Moderate (~hundred host proteins)	Host proteins Proteases	**Remove solids**	
– Secreted	Low	Low (mainly culture medium proteins)	Culture medium Proteases Host proteins	**Remove solids** **Inhibit or remove proteases**	**1) Capture chromatography** to concentrate and reduce the number of contaminants. If necessary: **2) High resolution chromatography** to remove the difficult" ones. **3) Polishing chromatography** to remove polymeric and denatured forms
SYNTHETIC	High	Low (mainly failures)	Cleaved off protection groups Scavengers Failure peptides	**Before freeze-drying:** Remove chemicals and acids	**High resolution chromatography** to eliminate failure peptides

II.3 Basis of chromatography techniques

II.3.1 Basis of Ion Exchange Chromatography (IEC)

IEC uses matrix-bound charged groups to reversibly adsorb oppositely charged sample molecules. With proteins and peptides, desorption is brought about either by a change in the pH or by an increase in the salt concentration of the eluent.

Charge properties of ion exchangers

The charged groups on the ion exchanger can either be negatively or positively charged. Negatively charged ion exchangers adsorb cations while positively charged ones adsorb anions. Hence they are named cation exchangers (CIEX) and anion exchangers (AIEX) respectively.

Depending on the pKa value of the charged group, the ion exchangers are further divided into strong and weak. Strong ion exchangers are fully charged over the total pH range normally applicable to these molecules. For protein and peptide purification, the weak ion exchangers are nowadays less frequently used since they provide no essential advantage over the strong ones.

Note that strong or weak in this sense does not reflect the binding strength between the charged groups on the ion exchangers and the molecules in the solution.

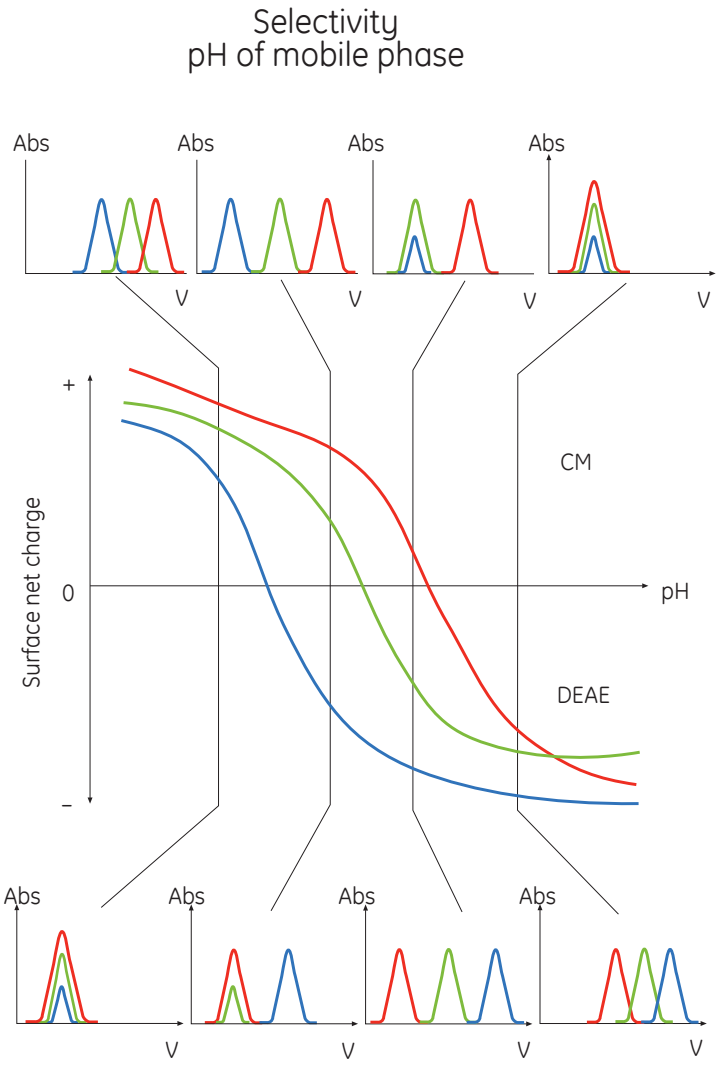
pH and selectivity

The higher the net charge of a protein or a peptide the stronger it will adsorb to an ion exchanger. Protein and peptide net charge, however, depends on the amino acid content:

Amino acid	Type of group	pKa of side chain
Aspartic acid	Carboxyl	4.5
Glutamic acid	Carboxyl	4.6
Histidine	Imidazole	6.2
Cysteine	Thiol	9.1–9.5
Tyrosine	Phenol	9.7
Lysine	Amino	10.4
Arginine	Guanido	12.0
α-amino group	Carboxyl	6.8–7.9
α-carboxylic group	Amino	3.5–4.3

Proteins and peptides are thus ampholytic, i.e. their net charges are either positive or negative depending on pH.

The figure below explains the effect of pH on the selectivity in ion exchange chromatography with proteins and peptides. Three curves are shown which represent the net charge variation with pH for three different proteins. At the top and bottom of the diagram, chromatograms are shown that represent the type of resolution obtained at the four pH values, as represented by the vertical lines. The chromatograms at the top represent cation exchange, while those at the bottom represent anion exchange.



At the lowest pH value (the vertical line at the far left) all three proteins are positively charged and adsorb only to the cation exchanger. They elute in the order of net charge when a salt gradient is applied. The higher the net charge, the later they elute. With the anion exchanger, all proteins pass the through column unretarded and no separation is achieved.

At the second lowest pH value (second vertical line from the left) protein C has switched to a negative net charge, while protein A and B are still positively charged but to a lesser degree. With the cation exchanger, protein A and B are still adsorbed while protein C passes right through. With the anion exchanger, only protein C is adsorbed.

At the second highest pH value (third vertical line from the left) only protein A remains positively charged while the other two proteins carry net negative charges. The cation exchanger now adsorbs only protein A, while the two others pass right through the column. By contrast, the anion exchanger allows protein A to pass through unadsorbed, while proteins B and C elute according to net charge.

At the highest pH value (vertical line at the far right) all the three proteins carry negative net charges and remain unadsorbed with the cation exchanger. With the anion exchanger, all three proteins are adsorbed and eluted according to their respective net negative charges.

Thus by varying the pH of the eluent, the selectivity in ion exchange chromatography can be influenced.

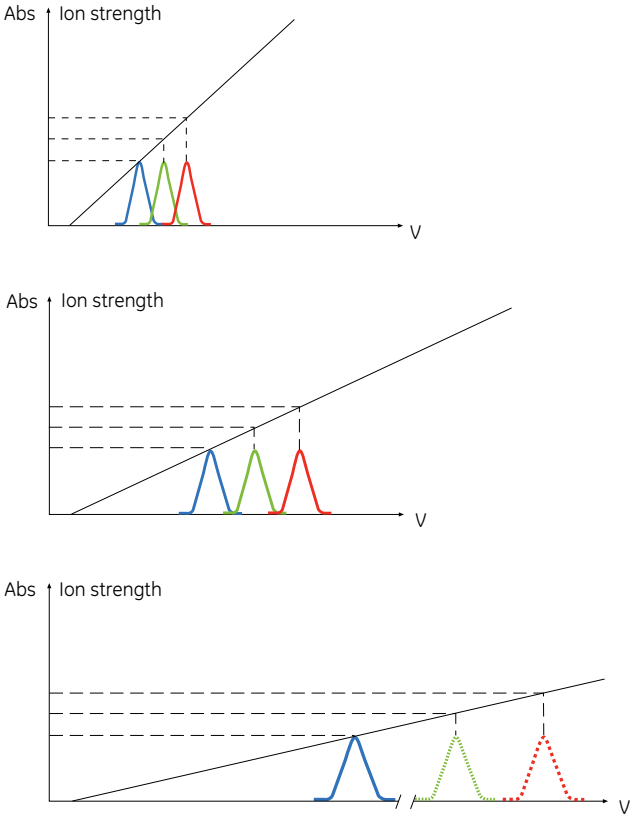
Salt concentration and selectivity

The strength with which a protein or a peptide is adsorbed by an ion exchanger depends not only on the net charge of the sample molecule, but also on the effect of other ions. By competing with the sample molecule for the charges on the ion exchanger, salt ions modify the strength with which a sample molecule is adsorbed.

Plotting binding strength vs. NaCl concentration for a protein gives rise to steep sigmoidal adsorption curves. It may be rather difficult to find the single salt concentration that elutes the target molecule at a good resolution. To simplify the adaptation of running conditions to the purification problem at hand, salt gradients are used to elute the sample components.

The gradient serves to compress the chromatogram so that most of the sample components can be eluted in a reasonable time. Sample components are eluted according to their respective binding strengths (net charges), and the distance between them depends on the slope of the gradient; the steeper the gradient, the closer the sample molecules will elute. On the other hand, the steeper the gradient, the sharper the eluted peaks. This latter effect, however, will not compensate for the loss in the spread of the peaks, and resolution will tend to decrease with increased gradient slope.

Optimizing the gradient

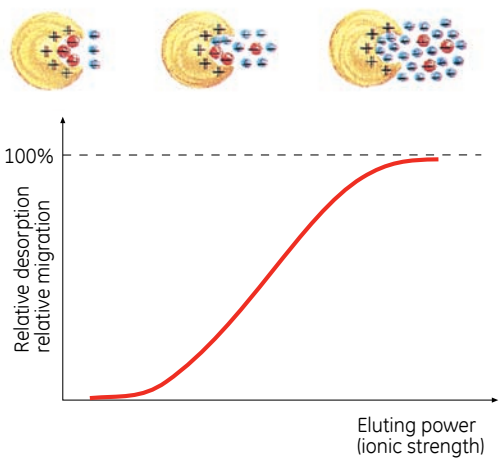


The effect of the gradient on an adsorbed protein or peptide is to make this "slide" down its adsorption curve as the salt concentration increases (see top figure on next page).

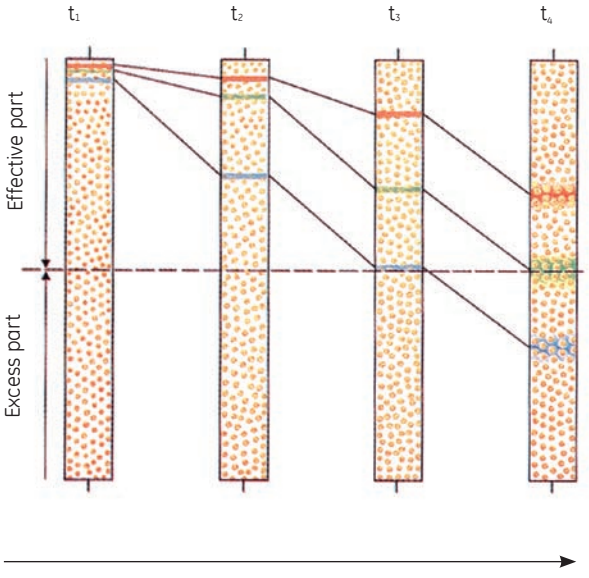
The migration velocity of the protein or peptide gradually increases with increasing salt concentration and will eventually reach that of the eluent. When this happens, the protein or peptide has reached its final position within the gradient. When all sample components have reached their final positions, maximum resolution has been reached with the gradient applied. Further transport down the column will only result in extra band-broadening. Thus, optimal column length depends on the gradient slope used (see bottom figure on next page)

The shallower the gradient, the longer the column needed to reach maximum resolution.

Selectivity
Effect of competing salt ions



Band broadening effects
Gradient and column length



Priority of parameters when optimizing ion exchange chromatography

pH and type of ion exchanger are the two most powerful parameters to influence selectivity in ion exchange chromatography. The pH/net charge curves vary quite considerably in their relative positions at different pH values and even sometimes cross at certain points! The second most powerful parameter is the gradient slope and only in third place comes the flow rate.

The list below gives the different steps of an optimization protocol for ion exchange chromatography:

- *Scout for optimal type of ion exchanger and running pH by testing AIEX and CIEX at different pH values within the pH stability range of the sample.*

  AIEX will, in most cases, be advantageous for acidic target molecules, since a large number of the contaminants are probably less acid and can be made to pass the column unadsorbed. For the same reasons CIEX will, in most cases, be advantageous for basic target molecules.

- *Scout for the steepest gradient that provides acceptable resolution at the pH selected above.*

- *Scout for the highest flow rate that provides acceptable resolution under the conditions selected above.*

In most cases, the first two steps above suffice to reach an acceptable resolution. Especially with labile samples, however, a short separation time may be crucial for recovery and step three above then becomes important.

If the scouting facilities of ÄKTAexplorer are not used, the following rules may be used to arrive at good resolution:

Choice of IEX type:

- *For acidic target molecules, select AIEX.*
  A large number of the contaminants are probably less acid than the target molecule and can be made to pass through the column unadsorbed.

- *For basic target molecules, select CIEX.*
  A large number of the contaminants are probably more acid than the target molecule and can be made to pass through the column unadsorbed.

- *For neutral or slightly acidic target molecules, test both AIEX and CIEX.*
  With neutral or slightly acidic target molecules, it is hard to guess if the bulk of the contaminants have pI values higher or lower than that of the target molecule.

Choice of running pH

- *With AIEX, select running pH to be at least one pH unit higher than the pI of the target molecule*. This is to ascertain proper adsorption of the target molecule.
- *With CIEX, select running pH to be at least one pH unit lower than the pI of the target molecule*. This is to ascertain proper adsorption of the target molecule.

Choice of gradient slope

- *A gradient from 0 to 1.0 M NaCl over 20 column volumes* is in most cases considered a good compromise.

II.3.2 Basis of hydrophobic chromatography (HIC)

HIC utilises matrix-bound hydrophobic ligands to adsorb proteins, which carry hydrophobic areas on their surfaces.

Hydrophobic interaction (HI) depends on the way water molecules interact with each other and with molecules surrounded by them. Water molecules appear in loosely organised clusters held together by hydrogen bonding. These clusters continuously and quickly interchange water molecules. The higher the temperature, the less stable are the clusters. When a hydrophobic (non-polar) molecule is immersed in water, the water molecules cannot interact directly with the hydrophobic molecule, but form a "shell" of highly organised water around the hydrophobic molecule. Thermodynamically this is unfavourable and the hydrophobic molecules aggregate to minimise the surface and the water shell. HI is entrophy-driven, since the system strives to minimise the amount of ordered water structures. Anything that influences the tendency of bulk water molecules to form ordered structures will also influence HI.

Means of influencing selectivity in HIC

Type of HI-promoting salt, pH, and temperature will all influence binding in HIC. The most important parameter used to influence selectivity, however, is the choice of ligand type.

– Type of ligand

Listed below are the commonly used ligands in order of binding strength:

- Ether
- Isopropyl
- Butyl
- Octyl
- Phenyl

The difference between first four ligands is quantitative rather than qualitative. The phenyl ligand, on the other hand, offers another type of selectivity, mainly because of its possibility to form additional bonds.

– pH

Although present, the influence of pH in HIC is far less than in IEC.

The effect seems less pronounced in the pH range 5 to 8.5. Though worthwhile, the pH effects are seldom considered in practice.

– Eluent composition

Listed below in the order of strength are the most commonly used HI promoting salts:

- NaCl
- $(NH_4)_2SO_4$
- Na_2SO_4

Besides influencing binding strength, selectivity will vary somewhat with the type of salt used. $(NH_4)_2SO_4$ is the most commonly used salt in HIC.

Changing the polarity properties of the eluent by adding ethylene glycol or iso-propanol will weaken binding strength, but also influence selectivity to some degree.

– Temperature

Unlike in IEC, increased temperatures will strengthen HI.

Optimizing HIC

The different steps used to optimize HIC are listed below:

- *Determine the stability window of the sample* (see page 125).
- *Scout for media* (ligand) type that requires the lowest salt concentration for adsorbing the target protein, while leaving a maximum of the contaminants unbound. (Mind the stability window of the sample!)
- *Scout for maximum gradient slope* giving acceptable resolution.
- *Scout for maximum flow rate* giving acceptable resolution.

II.3.3 Basis of reversed phase chromatography (RPC)

Reversed phase chromatography utilises the solubility properties of the sample molecules by partitioning them between a non-polar or hydrophobic chromatographic medium (the stationary phase) and eluents (the mobile phase) with varying polar properties. The process is, in a way, analogous to the organic chemist's two phase partitioning in a separation funnel. The sample distributes between the stationary and the mobile phases in proportions determined by its hydrophobicity or non-polar properties, the hydrophobic nature of the stationary phase and the hydrophilic or polar properties of the mobile phase. Hydrophobic molecules bind to the stationary phase at aqueous conditions and are eluted in order of hydrophobicity by a gradient of increasing organic solvent content. Relatively hydrophilic macromolecules like proteins and peptides seem to adsorb to rather than dissolve in the hydrophobic hydrocarbon layer of the stationary phase and their retardation seems to be determined by hydrocarbon content rather than hydrocarbon chain length.

In contrast to IEC, gradient elution in RPC is not always simple and straightforward, indicating that more than one force is involved. Molecules with secondary and tertiary structures may undergo conformational changes when exposed to RPC eluents, which in turn may lead to altered adsorption properties. For instance, increased concentrations of organic solvents will strengthen ionic interactions and hydrogen bonds and thus increase the stability of secondary structures. The intramolecular hydrophobic bonds that stabilise tertiary structures will, on the other hand, be weakened, which may lead to de-stabilisation of tertiary structures.

Many proteins will indeed denature under RPC conditions. With peptides, on the other hand, RPC works extremely well, probably because of their low content of tertiary structure. In fact, today RPC is the main technique used to purify peptides.

Selectivity in reversed phase chromatography

There are surprisingly many parameters that influence the selectivity in RPC. The type and density of the bonded hydrophobic phase has a profound influence on the selectivity, as has the type of organic solvent used, pH and the use of ion pairing agents. The general resolution obtained with RPC is very high and a few standard solvent systems normally suffice for most purposes.

– pH and selectivity

Most RPC media use porous silica particles to support the hydrocarbon stationary phase. Silica, however, is not stable at pH values over 7. This has made chromatographers refrain from running RPC at neutral or basic pH values, even though the charge characteristics, and hence the RPC selectivity, of a protein or a peptide will be quite different from those at acidic pH values. Moreover, residual silanol groups with pKa values around 6 may cause non-specific adsorption and/or tailing in the pH range 6 to 7. Polymer-based RPC media are stable up to pH 11 and do not contain weakly acidic groups. They can thus be used even in the pH range 6 to 11, which encounters the pKa values of histidine, cysteine, tyrosine and lysine i.e. where rather drastic changes in net hydrophobicity are to be expected as a function of running pH.

– Ion pairing agents and selectivity

A very commonly used way to increase the hydrophobicity of charged peptides is to add ion pairing agents to the mobile phase. These will combine with the charged groups of the target molecule and thereby suppress their influence on the overall hydrophobicity. Since most peptides are slightly basic, ion pairing agents are often acids. Below is listed some commonly used ion pairing agents:

- *Trifluoroacetic acid (TFA),* suppresses amino group charges and sets eluent pH to around 2 where all acidic amino acid side chain charges are undissociated.
- *Phosphoric acid.* Acts in the same manner as TFA, but renders the ion pair slightly less hydrophobic.
- *Triethylaminophosphate (TEAP),* suppresses both negative and positive charges and sets eluent pH to around neutrality.

Optimizing resolution in RPC

Below is listed the steps used to optimize RPC:

- *Scout for RPC media* providing the best resolution.
- *Scout for eluent pH and ion pairing agent* providing the best resolution.
- *Scout for maximum gradient slope* providing acceptable resolution.
- *Scout for maximum flow rate* providing acceptable resolution.

Difference between HIC and RPC

HIC media contain discrete hydrophobic ligands bound to a hydrophilic base matrix. RPC media, on the other hand, contain a more or less continuous layer of hydrophobicity consisting of hydrocarbon chains. For proteins, this difference in ligand coverage means a rather big difference in the "quantity" of interaction:

- HIC ligands can only interact with discreet hydrophobic patches on the surface of the sample molecule and require high salt conditions to bring about adsorption.
- RPC hydrophobic layers will interact with much larger parts of the sample molecule and adsorption is strong even in pure water.
- The elution of an RPC column requires increasing concentrations of an organic solvent.
- The elution of a HIC column is simply brought about by decreasing the concentration of the HI promoting salt.

Using HI for the chromatography of proteins is always a balance between obtaining enough interaction and damaging the protein. The strong interaction of RPC often leads to de-naturation and irreversible binding of proteins either during the adsorption phase or under elution. In HIC, however, adsorption is much less drastic and the risk for denaturation on that ground is substantially lower. On the other hand, the high salt concentrations used may cause precipitation.

II.3.4 Basis of size exclusion chromatography (SEC)

The basis of size exclusion chromatography can be looked upon as a matter of different apparent column volumes for macromolecules of different sizes. SEC media contain pores that behave as gates, permitting smaller molecules to pass easily, while excluding larger ones. For a single bead, this gating effect is not a step function, but rather a continuous decrease in accessibility for molecules of increasing sizes. A matrix containing such pores will be highly penetrable for small molecules, while "medium-sized" molecules can only partly penetrate into its interior and larger molecules cannot penetrate at all. When such media are packed into chromatography columns, the accessible column volume will be different for small and large molecules. On a molecular level, the column can be divided into three functionally different volumes:

- The volume occupied by the solid part of the medium.
- The liquid volume within the pores of the medium.
- The liquid volume between the particles in the column.

The two latter spaces are filled with liquid, while the solid part only acts as the supporting backbone for the pores. All sample molecules, regardless of size, have access to the liquid between the particles and none of them has access to the backbone space.

The access to the volume in the pores, however, will depend on their respective sizes.

Consequently, for large molecules that cannot penetrate any pores at all, the apparent column volume will be equal to the space between the beads. For small molecules with full access to the pores, the apparent column volume equals the sum of the volume within the pores and the volume between the particles. However, for molecules with sizes intermediate to those which are completely excluded and those with full access to the pores, the apparent column volume will be somewhere in between that of the large and the small molecules, depending on molecular size.

Consequently, after having passed the column, the sample components will leave the column in order of decreasing molecular sizes. Thus no molecule can elute earlier than it takes to pass through the space between the beads (the void volume) and no molecule can elute later than it takes to pass through the volume of the pores plus the volume between the particles i.e. slightly less than one column volume. To prevent excessive broadening of the sample peaks during their passage through the column, flow rates has to be low enough to allow the sample molecules to fully equilibrate between the pores and the mobile liquid between the beads and high enough to minimise diffusion along the column.

Selectivity in SEC

When elution volume is plotted against log Mr in size exclusion chromatography, reasonably linear selectivity curves are obtained. It has been shown that it is the hydrodynamic volumes of the sample molecules rather than their molecular weights that determine the degree of access to the pores.

For proteins, especially when globular in shape, Mr serves as a good substitute for hydrodynamic volume and only small individual deviations from the selectivity curve are found.

With smaller proteins and peptides, however, deviations are more frequent. One reason for this may be that even small structural irregularities represent a larger relative difference for small molecules as compared to larger molecules. Resolution in size exclusion chromatography depends on the ratio of molecular sizes. To fully resolve two molecules at least a twofold difference in Mr is required. Thus monomers and dimers are normally fully resolved while dimers and trimers are resolved, although not with full baseline separation.

Non-specific adsorption in SEC

SEC media in general show very little non-specific adsorption effects. However, the amount of "backbone" material increases as the pores are made smaller and effects that can be overlooked with media for proteins are more pronounced with media for peptides. Peptides are in general more hydrophobic than proteins and may thus deviate from linear selectivity because of non-specific adsorption. In fact, the addition of acetonitrile to the eluent seems to have a marked influence on retention volumes for a large number of peptides. Many peptides show a minimum in retention volume at approximately 30 % acetonitrile in the eluent, which points to a rather complex mechanism behind this phenomenon. However, when the scope of the SEC experiment is to separate peptides rather than to estimate molecular weights, the effect of adding acetonitrile to the eluent may be included in the optimization measures.

Eluents compatible with SEC

As long as keeping within the stability window of the sample, any eluent compatible with the chemical stability of the medium may be used in SEC.

A unique property inherent in the working principle of SEC is that low molecular weight components like buffer salts etc. will elute after approximately one column volume. This forms the basis for buffer exchange with SEC media. When e.g. the solvent conditions in the fractions from one purification step are not compatible with the next step, this eluent can be exchanged for "buffer A" of the next step by passing the fractions through a SEC medium equilibrated with "buffer A". If a SEC medium capable of excluding the sample molecules but leaving the buffer salts to elute at one column volume is chosen, sample volumes up to 25 % of that of the column can be loaded.

Optimizing resolution in SEC

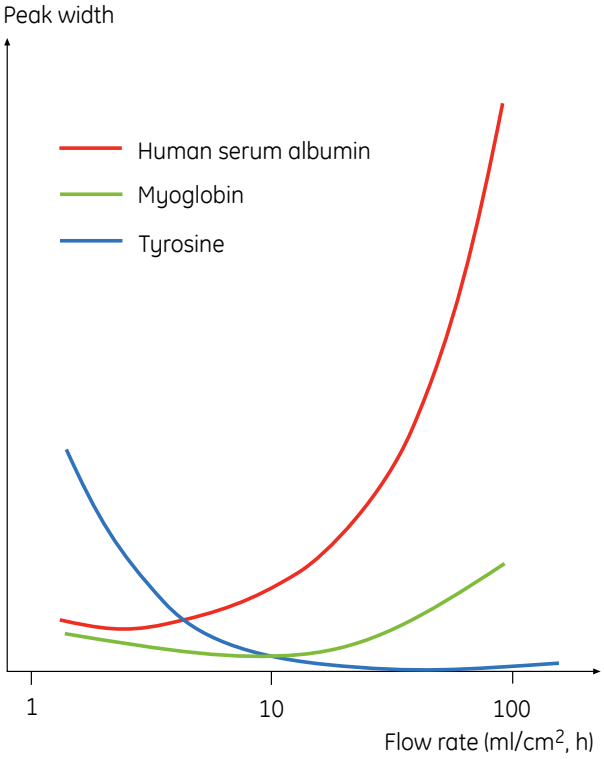
Size exclusion is an isocratic technique which means that no concentrating effect on the sample can be achieved. Unlike isocratic techniques in general, the separation here takes place within one column volume. The major means of optimizing size exclusion is by the choice of matrix.

The selectivity and the bead size of the medium are the two most influential parameters.

Best resolution is normally obtained in the middle part of the selectivity curve. Bead sizes around 10 µm give sharp peaks, but also give rise to back pressures that may inhibit the use of large columns. The flow rate chosen can have a rather profound influence on the peak shape. With large molecules like proteins, it is important to keep flow rate rather low or peaks will broaden.

Due to their larger diffusion coefficients, peptides can be run a lot faster.

Choosing the flow rate



Below is listed the steps used in optimizing SEC:

- *Select a SEC medium* that is compatible with the eluents to be used and where the target molecule Mr falls into the linear part of the selectivity curve.
- *Select the smallest bead size* that, with column size in mind, allows a proper flow rate.
- *Scout for maximum flow rate* providing acceptable resolution.

Appendix III – Glossary

α; The symbol for the selectivity factor. (*See selectivity, below*).

AC; *Affinity chromatography.*

ACN; *Acetonitrile.* An eluent component used in RPC or added to other chromatography techniques to suppress hydrophobic interactions.

AIEX; *Anion exchange chromatography.* A technique that separates molecules according to net negative charge.

Band broadening; The continuos widening of chromatographic peaks as they move down the column. Band broadening is enhanced by:

1) Badly packed columns.
2) Large bead sizes.
3) Flow rates that are not adapted to the equilibration process between the matrix and the mobile phase on one hand, and the axial diffusion on the other.
4) Partially blocked columns.

Block; A group of variables included in chromatography methods.

Capacity factor; The constant ($k' = V_e - V_0)/V_0$; representing the distribution of a molecule between the stationary and the mobile phase in chromatography.

Capture step; The first step of a purification protocol. The aim with the capture step is to concentrate the sample and to remove the bulk of contaminants.

CIEX; *Cation exchange chromatography*. A technique that separates molecules according to net positive charge.

Efficiency; A variable that describes how well band broadening effects are counteracted. Efficiency is normally expressed as the number of theoretical plates or plate count per meter.

Gel filtration; A synonym for size exclusion chromatography. A technique that separates molecules according to size.

HIC; *Hydrophobic interaction chromatography*. A technique that separates molecules according to hydrophobicity.

IEX; *Ion exchange chromatography*. A technique that separates molecules according to net charge.

Inter step conditioning; Measures to adapt a sample to the next step in a multi-step purification protocol.

Inter step treatment; See Inter step conditioning!

Intermediate step; A purification step intermediate in a multi-step purification protocol. The aim of an intermediate purification step is to separate molecules with close chromatographic properties.

Ion pairing agent; An additive used in RPC to manipulate the hydrophobicity of sample molecules.

Iso-electric point; The pH value at which the positive charges balances the negative charges of a proteins or a peptide i.e. where the net charge is zero.

k'; The symbol for the capacity factor. (See *capacity factor,* above!)

Loading capacity; The maximum amount of a certain sample that can be loaded onto a certain chromatography column. Two types of loading capacity measures are used:
1) The dynamic loading capacity which equals the maximum amount of the sample that can be completely adsorbed to the column.
2) The practical loading capacity which measures the amount of the sample that can be applied to the column without influencing the peak width.

Mass purity; The purity of a sample expressed in the amount of target molecule divided by the total amount of contaminants. Mass purity is often given as %.

Matrix; 1) Chromatography medium.
2) Composition of a sample, the target molecule excluded.

Molecular mass; A synonym for molecular weight.

Monomer; A single entity of a molecule.

MS; *Mass spectrometry*, A technique that measures molecular mass. MS can be used to determine molecular masses up 100 000. Three types can be distinguished:
1) *Fast atom bombardment (FAB) MS*. This technique cannot be used for continous monitoring.
2) *Electrospray ionisation (ESI) MS*. This technique can be used to directly monitor chromatographic eluates on-line.
3) *Matrix-assisted laser assisted desorption ionisation time of flight MS (MALDI TOF).* This technique cannot be used on-line with a chromatography system, but is the most widely used MS techniques for macromolecules.

Natural peptides; Peptides originating from living matter.

Natural source; The source of biomolecules stemming from living matter.

Polishing step; The final step in a multi-step purification protocol. This step aims at removing polymers and denatured forms of the target molecule.

Polymer; A complex consisting of many single entities of a molecule.

Primary structure; The sequence of amino acids constituting the backbone of a protein or a peptide.

Quaternary structure; The three-dimensional structure consisting of the subunits necessary to form a complete protein.

Recombinant source; The source for biomolecules produced by recombinantly modified cells.

Recovery; The amount of target molecule obtained after purification. The recovery is normally expressed as the amount of target molecule in the starting material divided by the amount of target molecule in the end product.

Resolution; A variable that describes the degree of separation in a chromatography experiment. Resolution is expressed as the distance between the centers of two peaks divided by the mean peak width of the two peaks.

RPC; *Reversed phase chromatography*. A technique that separates molecules according to hydrophobicity.

Sample stability window; The conditions under which a certain biomolecule retains its full stability.

Scouting; A name for the iterative process by which the optimal value of a certain chromatography variable is established.

Screening; A name for a process by which an optimal chromatographic medium is selected.

SEC; *Size exclusion chromatography*. A synonym for gel filtration. This technique separates molecules according to size.

Secondary structure; The way in which the amino acid backbone of proteins and peptides are folded "back on itself". Two major types of secondary structures are normally encountered:
1) Helical structures.
2) Sheet-like structures.

Selectivity; The way in which molecules are eluted in relation to each other. The selectivity may be expressed as the *selectivity factor* α= k'1 / k'2 (*See also capacity factor above*.).

Size exclusion chromatography; See *SEC above*.

SSW; See *Sample stability window* above.

TEAA; *Triehtylammoniumacetic acid*. TEAA is used as anion pairing agent in RPC.

Template; A pre-made set-up of method variables needed to create a certain chromatography method.

Tertiary structure; The way in which secondary structures of a certain peptide or protein are held together to form a functionally correct subunit.

TFA; *Trifluoroacetic acid*. TFA is used as an ion pairing agent in RPC.

Yield; The amount of end product obtained after purification. The yield is often expressed in the amount of end product obtained divided by amount of target molecule in the starting material (%). (See also *Recovery, above*.)

Concluding remark

ÄKTAdesign represents a new concept in the area of biomolecule purification. It includes instrumentation, prepacked columns, chromatography software and method templates for most purification needs and focuses on dependability and ease of use.

The purpose of this handbook is to assist the user in applying methods tailored for his individual needs and it is our hope that it will contribute to making your purification work straightforward and convenient.

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