Media characteristics

Composition: sulfopropyl (SP) or quaternary amino (Q) groups coupled to highly cross-linked 6% agarose via chemically stable ether bonds.

Product	Functional group	pH stability*	Mean particle size
Q Sepharose High Performance	$-CH_2N^+(CH_3)_3$	Long term: 2–12 Short term: 1–14	34 µm
SP Sepharose High Performance	$-CH_2CH_2CH_2SO_3^-$	Long term: 4–13 Short term: 3–14	34 µm

**Long term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance.*

Short term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. All ranges are estimates based on the experience and knowledge within Amersham Biosciences.

Chemical stability

For daily use, Sepharose High Performance media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), 70% ethanol, 1 M acetic acid, 30% acetonitrile and with additives such as non-ionic detergents.

Sepharose High Performance can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.



Avoid cationic detergents with SP Sepharose High Performance. Avoid anionic detergents with Q Sepharose High Performance. Avoid oxidizing agents.

Storage

For column storage, wash with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Include 0.2 M sodium acetate in the 20% ethanol solution for columns packed with SP Sepharose High Performance. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at +4° C to +30° C in 20% ethanol. Do not freeze.



To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

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Sepharose Fast Flow: purification with good resolution and easy scale-up

- Use Sepharose Fast Flow media for purification of proteins.
- Use Sepharose Fast Flow for capture or intermediate purification steps that require good resolution (flows up to 300 cm/h).
- Use a weak ion exchanger such as DEAE, CM or ANX Sepharose Fast Flow, if a strong ion exchanger (substituted with Q, S or SP) does not give the required selectivity.
- Run Sepharose Fast Flow columns on systems such as ÄKTAdesign, FPLC System and HPLC or systems using peristaltic pumps. Appendix 4 gives guidance on how to select the most suitable ÄKTAdesign system.

Sepharose Fast Flow media are based on a matrix of 90 µm particles made from 6% agarose and highly cross-linked for chemical and physical stability. ANX Sepharose 4 Fast Flow (high sub) is based on 4% agarose to form a medium that maintains a high binding capacity when separating large molecules such as thyroglobulin (M_r = 650 000), particularly suitable for large scale production when total binding capacity becomes economically significant.

Sepharose Fast Flow matrices are substituted with a range of ion exchange groups (Q, DEAE, ANX, SP and CM) giving the opportunity to test and use different selectivities (see Chapter 1 for an explanation of strong and weak ion exchangers). Ion exchangers containing strong ion exchange groups (Q and SP) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application.

Ion exchangers containing weak ion exchange groups (DEAE, CM and ANX) offer alternative selectivities, but over a narrower pH working range. Figure 55 illustrates how the selectivity of Sepharose Fast Flow media changes according to the anion exchange group.

Particle size and bed volumes remain stable, despite changes in ionic strength or pH, to ensure fast separations at high flow rates with good resolution. Methods can be easily scaled up from columns such as HiTrap Q FF (1 ml, prepacked with Q Sepharose Fast Flow) through to large scale columns such as FineLINE. The performance of Sepharose Fast Flow media is well documented and there are many examples of the smooth transfer from the laboratory to pilot scale and on to production.

Reference lists highlighting the use of Sepharose Fast Flow media are available at *www.chromatography.amershambiosciences.com*

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Columns:	a) HiTrap ANX Sepharose 4 Fast Flow (high sub) 1 ml b) HiTrap Q XL 1 ml c) HiTrap Q Sepharose Fast Flow 1 ml d) HiTrap DEAE Sepharose Fast Flow 1 ml
Sample:	0.4 mg conalbumin (pI=6.3), 0.8 mg α-lactoglobulin (pI=5.8), 1.2 mg soya bean trypsin inhibitor (pI=4.5) dissolved in 2 ml start buffer
Sample volume:	2 ml
Start buffer:	20 mM Tris-HCl pH 7.4
Elution buffer:	20 mM Tris-HCl, 0.5 M NaCl pH 7.4
Flow:	1 ml/min (150 cm/h)
Gradient:	0% elution buffer (25 CV), 0-80% elution buffer (40 CV) Wash: 5 ml start buffer Elution: 40 ml, linear gradient, 0–80 % elution buffer

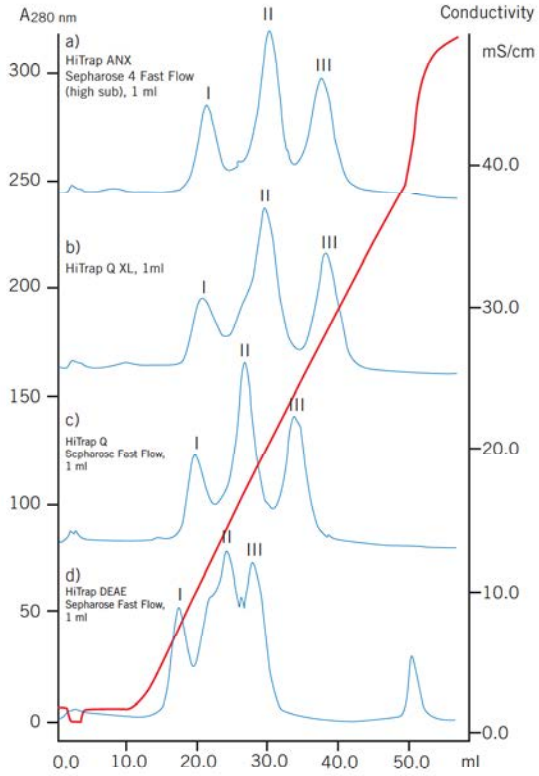


Fig. 55. Separation of conalbumin (I), α-lactalbumin (II) and soya bean trypsin inhibitor (III) on a range of anion exchange HiTrap columns demonstrates the difference in selectivity according to the anion exchange group.

Purification options



Fig. 56. Sepharose Fast Flow media, with a range of selectivities, are available prepacked in HiTrap and HiPrep columns and in media packs.

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Product	Binding capacity per column or per ml medium	Recommended working flow*	Maximum flow*	Working pH range**	Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar*
Strong anion exchangers					
HiTrap Q FF, 1 ml	3 mg (thyroglobulin, M_r 669 000) 120 mg (HSA, M_r 68 000) 110 mg (α–lactalbumin, M_r 14 300)	up to 1 ml/min	4 ml/min	2–12	0.3/43
HiTrap Q FF, 5 ml	15 mg (thyroglobulin, M_r 669 000) 600 mg (HSA, M_r 68 000) 550 mg (α–lactalbumin, M_r 14 300)	up to 5 ml/min	20 ml/min	2–12	0.3/43
HiPrep 16/10 Q FF, 20 ml	60 mg (thyroglobulin, M_r 669 000) 2400 mg (HSA, M_r 68 000) 2200 mg (α–lactalbumin, M_r 14 300)	2–10 ml/min	10 ml/min	2–12	0.15/22
Q Sepharose Fast Flow	3 mg/ml (thyroglobulin, M_r 669 000) 120 mg/ml (HSA, M_r 68 000) 110 mg/ml, (α–lactalbumin, M_r 14 300)	50–400 cm/h	750 cm/h	2–12	0.3/43
Strong cation exchangers					
HiTrap SP FF, 1 ml	50 mg (bovine COHb, M_r 69 000) 50 mg (human IgG, M_r 160 000) 70 mg (ribonuclease A, M_r 13 700)	up to 1 ml/min	4 ml/min	4–13	0.3/43
HiTrap SP FF, 5 ml	250 mg (bovine COHb, M_r 69 000) 250 mg (human IgG, M_r 160 000) 350 mg (ribonuclease A, M_r 13 700)	up to 5 ml/min	20 ml/min	4–13	0.3/43
HiPrep 16/10 SP FF, 20 ml	1000 mg (bovine COHb, M_r 69 000) 1000 mg (human IgG, M_r 160 000) 1400 mg (ribonuclease A, M_r 13 700)	2–10 ml/min	10 ml/min	4–13	0.15/22
SP Sepharose Fast Flow	50 mg/ml (bovine COHb, M_r 69 000) 50 mg/ml (human IgG, M_r 160 000) 70 mg/ml (ribonuclease A, M_r 13 700)	50–400 cm/h	750 cm/h	4–13	0.3/43
Weak anion exchangers					
HiTrap DEAE FF, 1 ml	100 mg (α–lactalbumin, M_r14 300) 110 mg (HSA, M_r 68 000)	up to 1 ml/min	4 ml/min	2–9	0.3/43

continues on following page

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Product	Binding capacity per column or per ml medium	Recommended working flow*	Maximum flow*	Working pH range**	Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar*
HiTrap DEAE FF, 5 ml	500 mg (α–lactalbumin, M_r 14 300) 550 mg (HSA, M_r 68 000)	up to 5 ml/min	20 ml/min	2–9	0.3/43
HiPrep 16/10 DEAE FF, 20 ml	2000 mg (α–lactalbumin, M_r 14 300) 2200 mg (HSA, M_r 68 000)	2–10 ml/min	10 ml/min	2–9	0.15/22
DEAE Sepharose Fast Flow	100 mg/ml (α–lactalbumin, M_r 14 300) 110 mg/ml (HSA, M_r 68 000)	50–400 cm/h	750 cm/h	2–9	0.3/43
HiTrap ANX FF (high sub), 1 ml	43 mg (BSA, M_r 67 000) 5 mg (thyroglobulin, M_r 669 000)	up to 1 ml/min	4 ml/min	2–9	0.3/43
HiTrap ANX FF (high sub), 5 ml	215 mg (BSA, M_r 67 000) 25 mg (thyroglobulin, M_r 669 000)	up to 5 ml/min	20 ml/min	2–9	0.3/43
HiPrep 16/10 ANX FF (high sub), 20 ml	860 mg (BSA, M_r 67 000) 100 mg (thyroglobulin, M_r 669 000)	2–10 ml/min	10 ml/min	2–9	0.15/22
ANX Sepharose 4 Fast Flow (high sub)	43 mg/ml (BSA, M_r 67 000) 5 mg/ml (thyroglobulin, M_r 669 000)	50–300 cm/h	400 cm/h	2–9	0.1/14
Weak cation exchangers					
HiTrap CM FF, 1 ml	50 mg (ribonuclease A, M_r 13 700)	up to 1 ml/min	4 ml/min	6–10	0.3/43
HiTrap CM FF, 5 ml	250 mg (ribonuclease A, M_r 13 700)	up to 5 ml/min	20 ml/min	6–10	0.3/43
HiPrep 16/10 CM FF, 20 ml	1000 mg (ribonuclease A, M_r 13 700)	2–10 ml/min	10 ml/min	6–10	0.15/22
CM Sepharose Fast Flow	50 mg/ml medium (ribonuclease A, M_r 13 700)	50–400 cm/h	750 cm/h	6–10	0.3/43

**See Appendix 5 to convert linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa.*

***Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

****Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

Use prepacked HiTrap columns (1 ml or 5 ml) for media selection, method scouting, group separations, small scale purification, sample concentration or clean-up. Connect up to 3 HiTrap columns in series to scale-up.

Use prepacked HiPrep columns (20 ml) for method development, group separations, larger scale purification, sample concentration or clean-up. Connect several HiPrep columns in series to increase binding capacity.

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For column packing:

Column	Volume	Bed height
Tricorn 10/100	up to 8 ml	up to 10 cm
Tricorn 10/150	up to 12 ml	up to 15 cm
Tricorn 10/200	up to 16 ml	up to 20 cm
XK 16/20	up to 30 ml	up to 15 cm
XK 26/20	up to 80 ml	up to 15 cm
XK 26/40	up to 196 ml	> 15 cm
XK 50/20	up to 274 ml	up to 14 cm
XK 50/30	up to 559 ml	up to 28.5 cm

Select a production column such as BPG or Chromaflow for larger volumes.

Purification examples

Media scouting

Using 1 ml HiTrap columns the most suitable matrix and charged group for a separation can be quickly and easily selected before optimization and scale-up. In Figure 57 a comparison of elution profiles for the same sample separated under identical conditions on three different media illustrates the differences in selectivity and resolution that can result from changing the charge group and the particle size. The most suitable medium can be selected and conditions optimized according to the requirements for the separation, for example to isolate a single, well-resolved peak or to maximize resolution between several peaks of interest.

Begin by scouting on the strong ion exchangers (Q, S or SP) in order to find the greatest differences in charge between the molecules of interest.

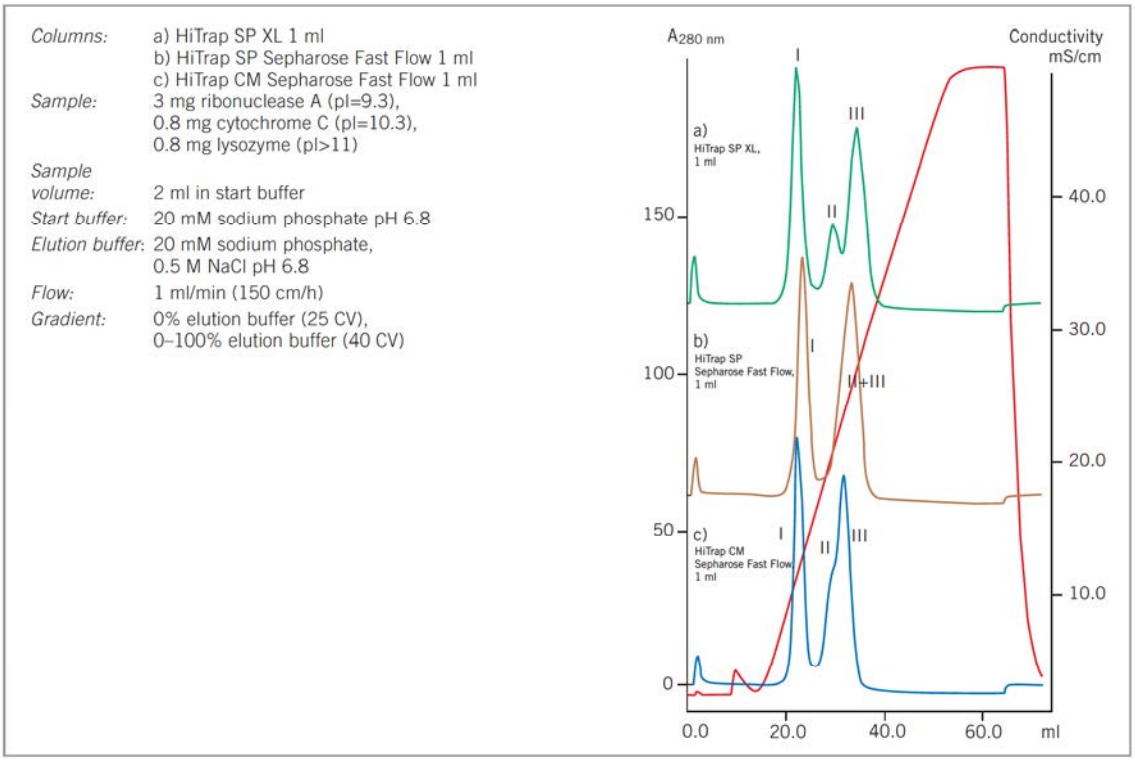


Fig. 57. Media scouting: separation of ribonuclease A (I), cytochrome C (II) and lysozyme (III) on HiTrap CM Sepharose Fast Flow 1 ml, HiTrap SP Sepharose Fast Flow 1 ml and HiTrap SP XL 1 ml.

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Capture

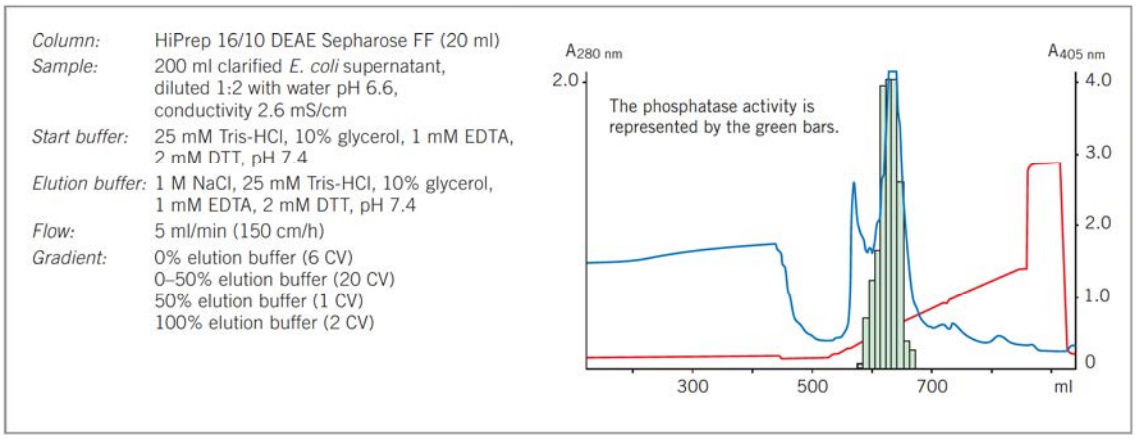


Fig. 58. A HiPrep 16/10 DEAE Sepharose Fast Flow column is used as the capture step to concentrate rPhosphatase and remove most of the contaminants.

Scaling-up

Figure 59 shows the ease with which separations can be scaled up on columns prepacked with Sepharose Fast Flow media. Beginning with a 1 ml HiTrap column the reproducibility of the separation has been maintained through a 20-fold scale-up.

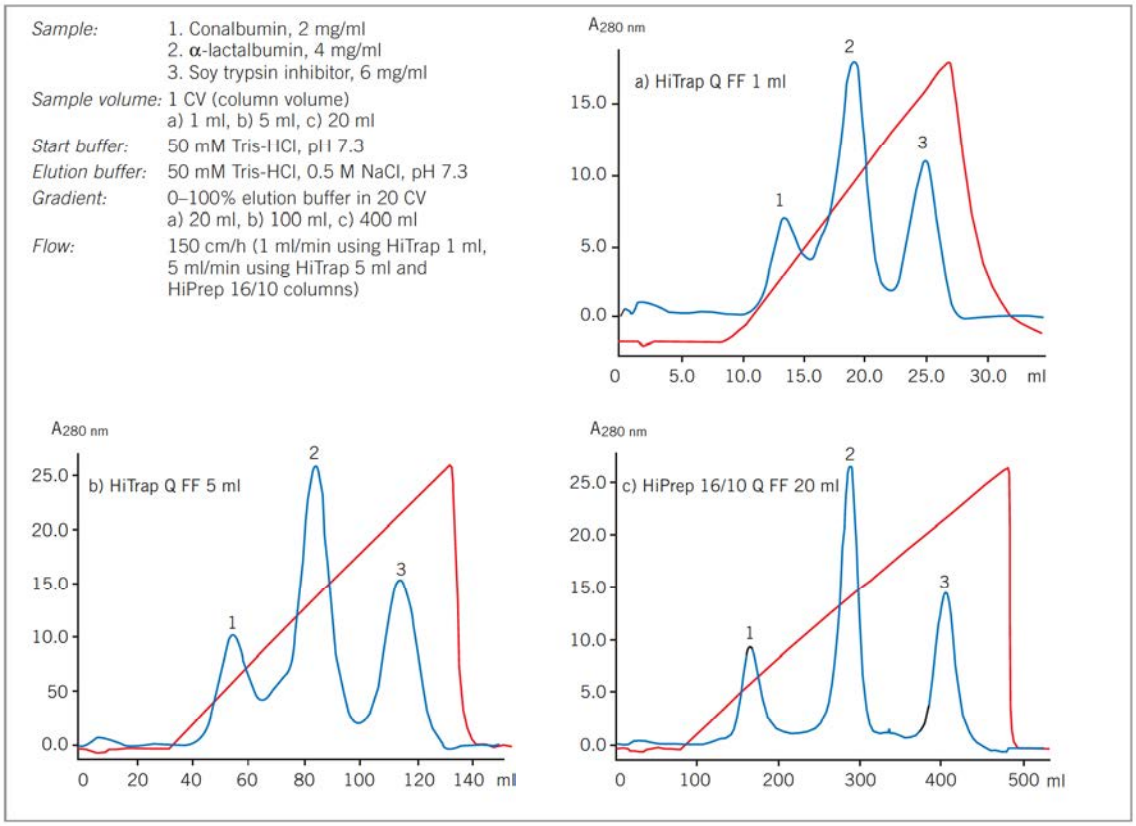
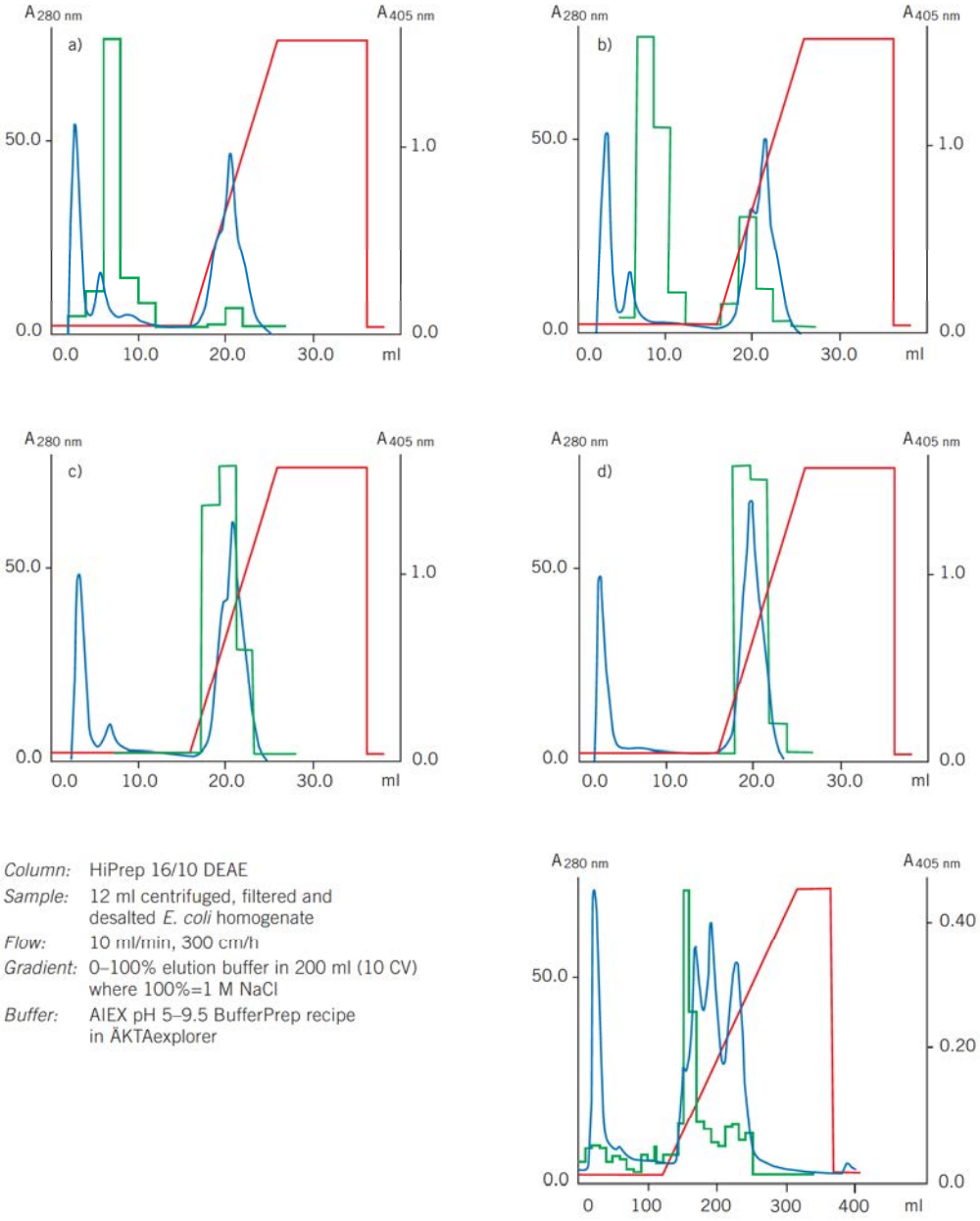


Fig. 59. 5-fold and 20-fold scale-up using prepacked Q Sepharose Fast Flow columns.

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Column: HiTrap DEAE, 1 ml
Sample: 1 ml centrifuged, filtered and de salted *E. coli* homogenate
Flow: 2 ml/min, 310 cm/h
Gradient: 0–100% elution buffer in 10 ml (10 CV) where 100 %=1 M NaCl
Buffer: AIEX pH 5–9.5 BufferPrep recipe in ÄKTAexplorer

Alkaline phosphatase assay
1) Pipette 100 µl sample (fractions) + 100 µl substrate (SIGMA FAST pNPP substrate tablet set, N2270, prepared according to the manufacturer's protocol) into micro-titrer plate. Blank = 100 µl substrate + 100 µl buffer.
2) Incubate for 1 hr in the dark at room temperature.
3) Read the absorbance at 405 nm.



Column: HiPrep 16/10 DEAE
Sample: 12 ml centrifuged, filtered and desalted *E. coli* homogenate
Flow: 10 ml/min, 300 cm/h
Gradient: 0–100% elution buffer in 200 ml (10 CV) where 100%=1 M NaCl
Buffer: AIEX pH 5–9.5 BufferPrep recipe in ÄKTAexplorer

Fig. 60. Optimization and scale up on DEAE Sepharose Fast Flow.

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Optimizing pH

When the most suitable medium has been selected for a separation, conditions can be optimized further by adjusting parameters such as pH. Figure 61 shows how increasing the pH on a column prepacked with CM Sepharose Fast Flow (HiPrep 16/10 CM FF) significantly improved resolution of a mixture of model proteins.

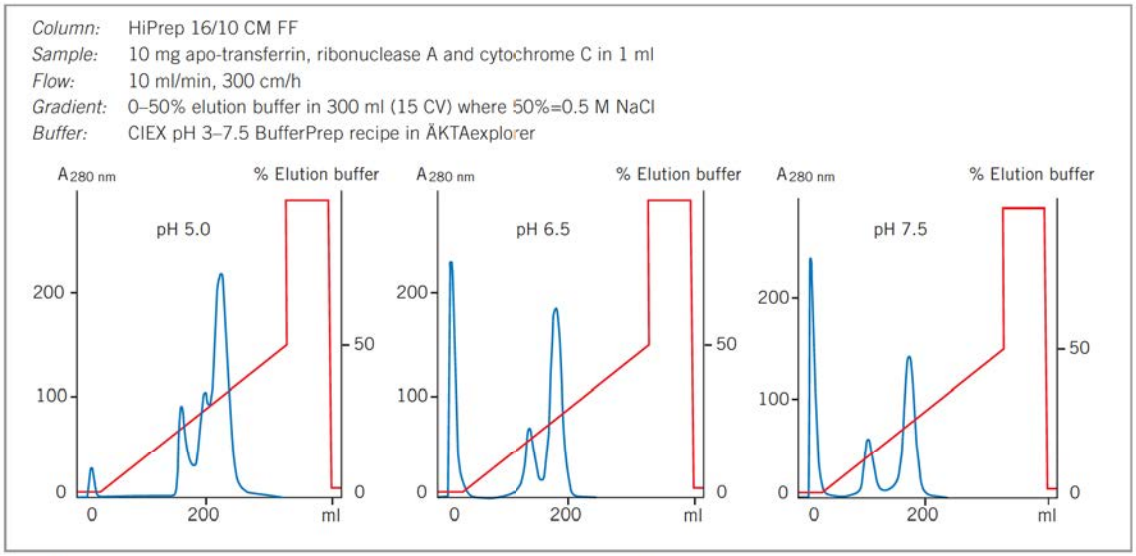


Fig. 61. Selecting optimal pH for separation of standard proteins on HiPrep 16/10 CM FF.

Sample concentration

It can be an advantage to concentrate a sample prior to gel filtration in order to minimize sample volume and facilitate a rapid, high resolution size separation. HiTrap columns offer a convenient, ready to use solution for sample concentration. Table 7 on page 89 gives examples of the high concentration factors achieved when concentrating proteins from very dilute starting material using HiTrap columns prepacked with Sepharose HP medium. Similar results can be achieved with HiTrap columns prepacked with Sepharose Fast Flow or Sepharose XL media.

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions using filters of 1 µm or less. To avoid formation of air bubbles in a packed column, maintain buffers and columns at a constant temperature before and during a run.

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The pH of the start buffer should be at least 0.5–1 pH unit above the pI of the target substance when using an anion exchanger (Q, DEAE or ANX) and 0.5–1 pH unit below the pI of the target substance when using a cation exchanger (SP, CM). See Appendix 2 for recommendations on volatile and non-volatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

– **anion exchange (Q)**
start buffer: pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

– **cation exchange (SP)**
start buffer: pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

If selectivity is not satisfactory when using a strong ion exchanger (Q or SP), try a weak ion exchanger (DEAE, ANX or CM) instead.

Users of ÄKTAdesign systems with BufferPrep functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8 or cation exchange chromatography at pH 6.

First time use or after long term storage

1. To remove ethanol, wash with 1 column volume of distilled water at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 2 ml/min (HiPrep 20 ml), or at 50 cm/h for Sepharose Fast Flow packed in larger columns. This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 column volumes of start buffer at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml) or 5 ml/min (HiPrep 20 ml).
3. Wash with 5 column volumes of elution buffer, same flow as step 2.
4. Wash with 5 column volumes of start buffer, same flow as step 2.
5. Run a blank elution before applying sample.

Separation by gradient elution

Flow: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 150 cm/h for Sepharose Fast Flow packed in larger columns. Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 column volumes of 1 M NaCl (100%B) to elute any remaining ionically-bound material.
6. Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

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Separation by step elution

Flow: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 150 cm/h for Sepharose Fast Flow packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Elute with 5 column volumes of start buffer + NaCl at chosen ionic strength.
5. Repeat step 4 at higher ionic strengths until the target protein(s) has been eluted.
6. Wash with 5 column volumes of a high salt solution (1 M NaCl in start buffer) to elute any remaining ionically-bound material.
7. Re-equilibrate 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.



Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.



If ionic detergents have been used, wash the column with 5 column volumes of distilled water, followed by 2 column volumes 2 M NaCl. Re-equilibrate with at least 10 column volumes of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove non-ionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.



Refer to Chapter 2 for advice on optimizing the separation.



Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3. Note that this does not apply to HiTrap or HiPrep columns.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.



It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

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The following procedure should be satisfactory to remove common contaminants:

1. Wash with at least 2 column volumes of 2 M NaCl at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 40 cm/h with a ccntact time of 1–2 hours for Sepharose Fast Flow packed in larger columns.
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 1) until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins, refer to Appendix 1.

Media characteristics

Composition:

- sulfopropyl (SP), carboxymethyl (CM), quaternary amino (Q) or diethylaminoethyl (DEAE) groups coupled to highly cross-linked 6% agarose via chemically stable ether bonds.
- diethylaminopropyl (ANX) group coupled to highly cross-linked 4% agarose via chemically stable ether bonds.

Product	Functional group	pH stability*	Mean particle size
Q Sepharose Fast Flow	$-CH_2N^+(CH_3)_3$	Long term: 2–12 Short term: 1–14	90 µm
SP Sepharose Fast Flow	$-CH_2CH_2CH_2SO_3^-$	Long term: 4–13 Short term: 3–14	90 µm
DEAE Sepharose Fast Flow	$-O-CH_2CHOHCH_2N^+H(CH_2CH_3)_2$	Long term: 2–13 Short term: 1–14	90 µm
ANX Sepharose 4 Fast Flow	$-OCH_2CHOHCH_2OCH_2CHOHCH_2N^+H(CH_2CH_3)_2$	Long term: 3–13 Short term: 2–14	90 µm
CM Sepharose Fast Flow	$-O-CH_2COO^-$	Long term: 4–13 Short term: 2–14	90 µm

**Long term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance.*

Short term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. All ranges are estimates based on the experience and knowledge within Amersham Biosciences.

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Chemical stability

For daily use, Sepharose Fast Flow media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), with additives such as non-ionic detergents, 70% ethanol, 1 M acetic acid and 30% isopropanol.

Sepharose Fast Flow can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.



Avoid cationic detergents with SP or CM Sepharose Fast Flow. Avoid anionic detergents with Q, DEAE or ANX Sepharose Fast Flow. Avoid oxidizing agents.

Storage

For column storage, wash with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Include 0.2 M sodium acetate in the 20% ethanol solution for SP Sepharose Fast Flow. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at +4° C to +30° C in 20% ethanol. Do not freeze.



To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

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Sepharose XL – for selected proteins that require very high binding capacity to increase productivity, easy scale-up

Use Sepharose XL media for purification of proteins when improved binding capacity compared to other Sepharose media has been confirmed for the selected protein.

Use Sepharose XL at the beginning of a purification scheme for initial capture when a high binding capacity and rapid separation is required for a selected protein from clarified samples.

Run columns packed with Sepharose XL on systems such as ÄKTAdesign, FPLC System and HPLC or systems using peristaltic pumps. Appendix 4 gives guidance on how to select the most suitable ÄKTAdesign system.

Purify viruses or viral vectors using Q Sepharose XL virus licensed.

Sepharose XL media are based on a matrix of 90 µm particles, made from 6% agarose and highly cross-linked for chemical and physical stability, substituted with quaternary ammonium (Q) or sulfopropyl (SP) groups. The ionic groups are bound to long, flexible dextran chains which have been coupled to the agarose. This increases the exposure of the Q or SP groups thereby raising the binding capacity to a very high level without restricting the passage of charged molecules. The strong ion exchange groups maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application. Particle size and bed volumes remain stable, despite changes in ionic strength or pH, to ensure fast separations at high flow rates with good resolution.

Purification options



Fig. 62. Q and S Sepharose XL are available in prepacked HiTrap and HiPrep columns, in media packs and in the Ion Exchange Selection Kit.

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Product	Binding capacity per column or per ml medium	Recommended working flow*	Maximum flow*	Working pH range**	Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar*
Strong anion exchangers					
HiTrap Q XL, 1 ml	>130 mg (BSA, M_r 67 000)	up to 1 ml/min	4 ml/min	2–12	0.3/43
HiTrap Q XL, 5 ml	>650 mg (BSA, M_r 67 000)	up to 5 ml/min	20 ml/min	2–12	0.3/43
HiPrep 16/10 Q XL, 20 ml	>2600 mg (BSA, M_r 67 000)	2–10 ml/min	10 ml/min	2–12	0.15/22
Q Sepharose XL and Q Sepharose XL virus licensed****	>130 mg/ml (BSA, M_r 67 000)	300–500 cm/h	700 cm/h	2–12	0.3/3
Strong cation exchangers					
HiTrap SP XL, 1 ml	>160 mg (lysozyme, M_r 14 500)	up to 1 ml/min	4 ml/min	4–13	0.3/43
HiTrap SP XL, 5 ml	>800 mg (lysozyme, M_r 14 500)	up to 5 ml/min	20 ml/min	4–13	0.3/43
HiPrep 16/10 SP XL, 20 ml	>3200 mg (lysozyme, M_r 14 500)	2–10 ml/min	10 ml/min	4–13	0.15/22
SP Sepharose XL	>160 mg/ml (lysozyme, M_r 14 500)	300–500 cm/h	700 cm/h	4–13	0.3/43

**See Appendix 5 to convert linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa.*

***Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

****Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

***** Important information: Separating viral particles with Q Sepharose XL may require a license under United States patent 6,537,793 B2 and foreign equivalents owned by Gencell SAS. Such a license is not included with the purchase of Q Sepharose XL, but is included with the purchase of Q Sepharose XL virus licensed products. Purchasers of Q Sepharose XL virus licensed products are granted a free limited license under US patent 6,537,793 B2 and foreign equivalents owned by Gencell SAS to separate viral particles solely through use of the product purchased.*



Use prepacked HiTrap columns (1 ml or 5 ml) for media selection, method scouting, group separations, small scale purification, sample concentration or clean-up. Connect up to 3 HiTrap columns in series to scale-up.



Use prepacked HiPrep columns (20 ml) for method development, group separations, larger scale purification, sample concentration or clean-up. Connect several HiPrep columns in series to increase binding capacity.



For column packing:

Column	Volume	Bed height
Tricorn 10/100	up to 8 ml	up to 10 cm
Tricorn 10/150	up to 12 ml	up to 15 cm
Tricorn 10/200	up to 16 ml	up to 20 cm
XK 16/20	up to 30 ml	up to 15 cm
XK 26/20	up to 80 ml	up to 15 cm
XK 26/40	up to 196 ml	> 15 cm
XK 50/20	up to 274 ml	up to 14 cm
XK 50/30	up to 559 ml	up to 28.5 cm

Select a production column such as BPG or Chromaflow for larger volumes.

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Purification examples

Media selection

Using 1 ml HiTrap columns the most suitable matrix and charged group for a separation to be selected quickly and easily. In Figure 63 a comparison of elution profiles for the same sample separated under identical conditions on three different media illustrates the differences in selectivity and resolution that can result from changing the charge group and matrix. The most suitable medium can be selected and conditions optimized according to the requirements for the purification. In this example Sepharose XL resolves the three components and optimization of elution conditions could further improve the resolution. However, any of these media would be suitable if the aim was to isolate the first major peak (ribonuclease A).

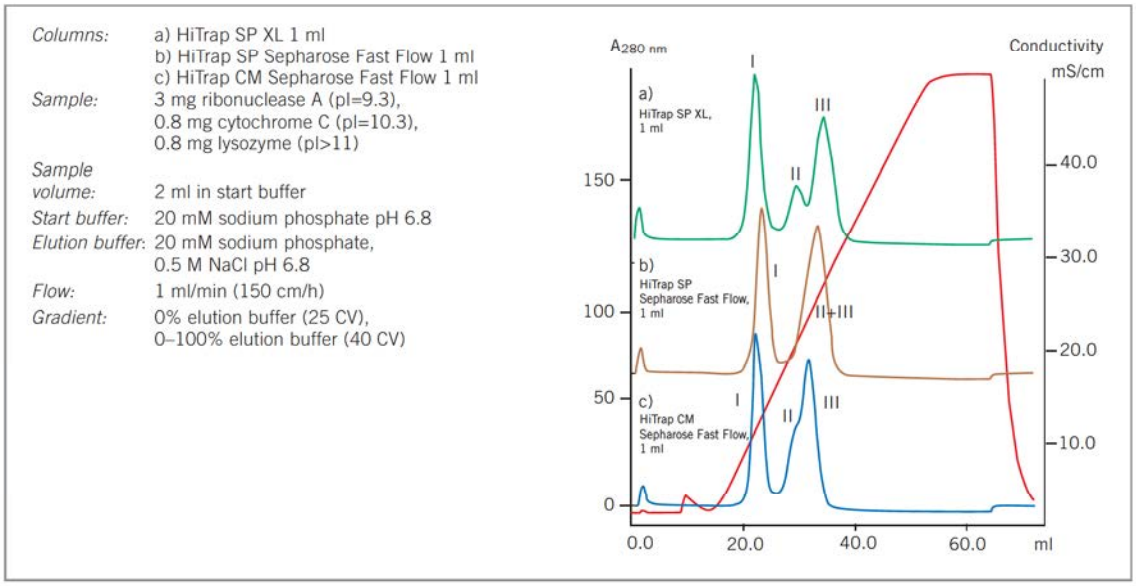


Fig. 63. Media scouting: separation of ribonuclease A (I), cytochrome c (II) and lysozyme (III) on a range of anion exchange HiTrap columns.

Capture

Capture of alkaline phosphatase from a clarified lysate of *E. coli* using a HiTrap Q XL 1 ml column is shown in Figure 64. Separation was monitored at A_{280} nm and phosphatase activity assayed by a spectrophotometric method at A_{405} nm

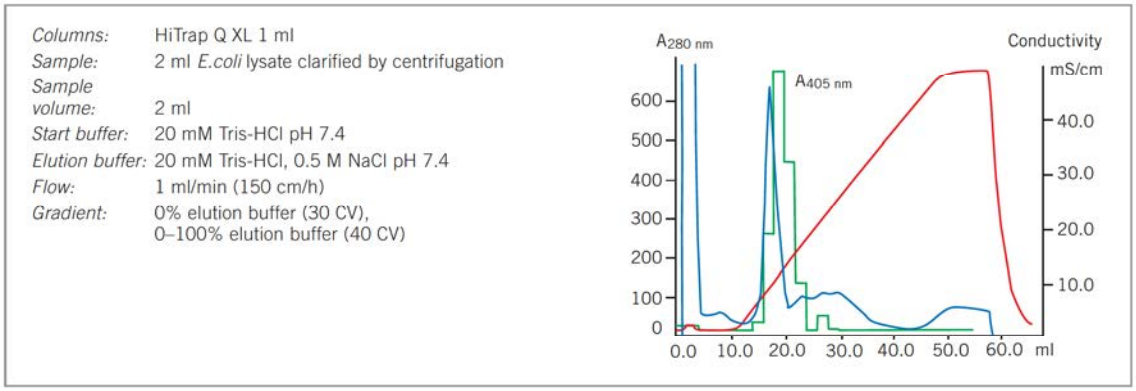


Fig. 64. Clarified *E. coli* lysate on HiTrap Q XL, absorbance values at 450 nm relate to phosphatase activity in eluted fractions.

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Capture and scale-up

Figure 65 shows a pilot scale purification performed on a Sepharose XL ion exchanger. The separation was developed on Q Sepharose XL packed in an XK 16/20 column in order to select optimal pH and to determine maximum binding capacity available. Adding $CaCl_2$ to the sample precipitated DNA and so increased the binding capacity for the target protein. Final loading was reduced to 75% of the maximum capacity and the result verified before scaling-up to the larger column.

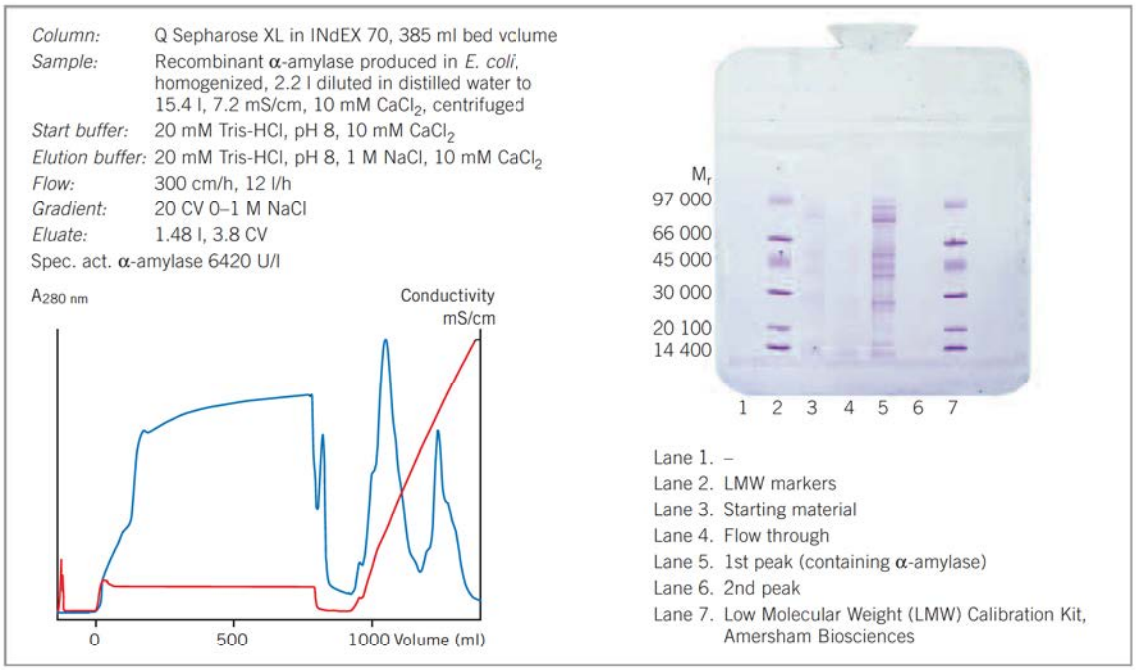


Fig. 65. Capture of recombinant α-amylase from *E. coli* on Q Sepharose XL pilot scale column together with SDS-PAGE (Phast System, Coomassie Brilliant Blue staining) of starting material and eluted fractions (reduced).

Viral purification

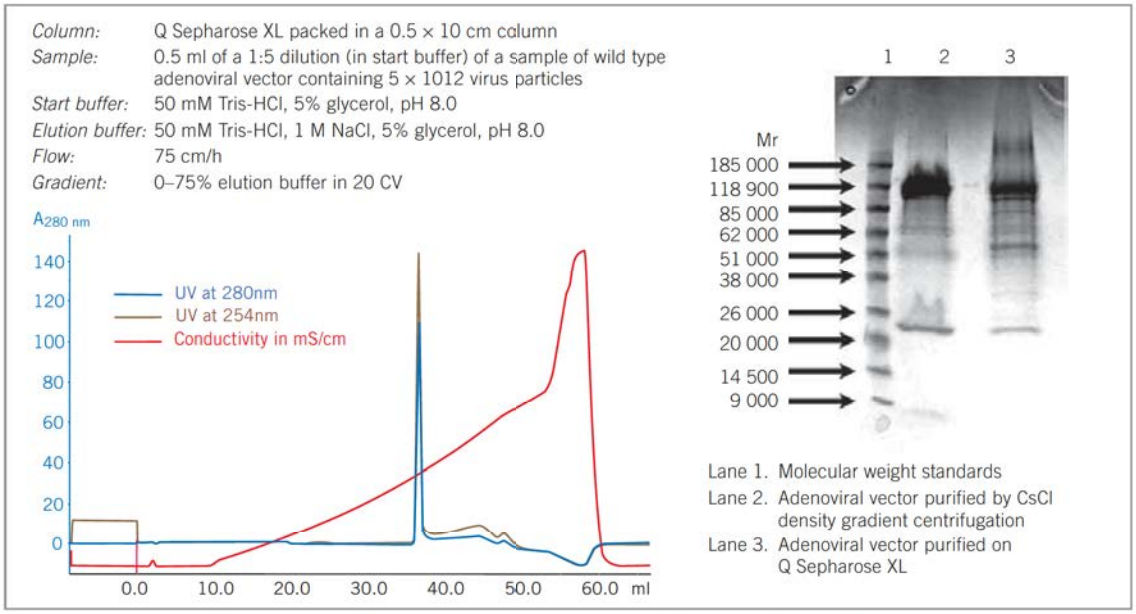


Fig. 66. Q Sepharose XL virus licensed provides an alternate to traditional cesium chloride density gradient centrifugation used for purification of viruses and viral vectors.

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Sample concentration

It can be an advantage to concentrate a sample prior to gel filtration in order to minimize sample volume and facilitate a rapid, high resolution size separation. HiTrap columns offer a convenient, ready to use solution for sample concentration. Table 7 on page 89 gives examples of the high concentration factors achieved when concentrating proteins from very dilute starting material using HiTrap columns prepacked with Sepharose HP medium. Similar results can be achieved with HiTrap columns prepacked with Sepharose Fast Flow or Sepharose XL media.

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.



Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.



Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions using filters of 1 µm or smaller. To avoid formation of air bubbles in a packed column, maintain buffers and columns at a constant temperature before and during a run.



The pH of the start buffer should be at least 0.5–1 pH unit above the pI of the target substance when using an anion exchanger (Q) and 0.5–1 pH unit below the pI of the target substance when using a cation exchanger (S). See Appendix 2 for recommendations on volatile and non-volatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

– **anion exchange (Q)**
  start buffer: pH 8.0
  elution buffer: start buffer including 1 M NaCl, pH 8.0

– **cation exchange (SP)**
  start buffer: pH 6.0
  elution buffer: start buffer including 1 M NaCl, pH 6.0



Users of ÄKTAdesign systems with BufferPrep functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8 or cation exchange chromatography at pH 6.

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First time use or after long term storage

1. To remove ethanol, wash with 1 column volume of distilled water at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml), or at 50 cm/h for Sepharose XL packed in larger columns. This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 column volumes of start buffer at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 150 cm/h for Sepharose XL packed in larger columns.
3. Wash with 5 column volumes of elution buffer, same flow as step 2.
4. Wash with 5 column volumes of start buffer, same flow as step 2.
5. Run a blank elution before applying sample.

Separation by gradient elution

Flow: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 150 cm/h for Sepharose XL packed in larger columns. Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 column volumes of 1 M NaCl (100%B) to elute any remaining ionically-bound material.
6. Re-equilibrate 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Flow: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 150 cm/h for Sepharose XL packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Elute with 5 column volumes of start buffer + NaCl at chosen ionic strength.
5. Repeat step 4 at higher ionic strengths until the target protein(s) has been eluted.
6. Wash with 5 column volumes of a high salt solution (1 M NaCl in start buffer) to elute any remaining ionically-bound material.
7. Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.



Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.



If ionic detergents have been used, wash the column with 5 column volumes of distilled water, followed by 2 column volumes 2 M NaCl. Re-equilibrate with at least 10 column volumes of start buffer until the UV baseline, eluent pH and/or conductivity are stable.

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Organic solvents such as ethanol can be used to remove non-ionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

The following procedure should be satisfactory to remove common contaminants:

1. Wash with at least 2 column volumes of 2 M NaCl at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 40 cm/h with a contact time of 1–2 hours for Sepharose XL packed in larger columns.
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 1) until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins, refer to Appendix 1.

Media characteristics

Composition: sulfopropyl (SP) or quaternary amino (Q) groups attached via chemically stable ether bonds to long, flexible dextran chains that are covalently coupled to highly cross-linked 6% agarose.

Product	Functional group	pH stability*	Mean particle size
Q Sepharose XL	$-CH_2N^+(CH_3)_3$	Long term: 2–12 Short term: 2–14	90 µm
SP Sepharose XL	$-CH_2CH_2CH_2SO_3^-$	Long term: 4–13 Short term: 3–14	90 µm

**Long term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance.*

Short term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. All ranges are estimates based on the experience and knowledge within Amersham Biosciences.

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Chemical stability

For daily use, Sepharose XL media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), with additives such as non-ionic detergents, 70% ethanol, 1 M acetic acid and 30% isopropanol.

Sepharose XL can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.



Avoid cationic detergents with SP Sepharose XL. Avoid anionic detergents with Q Sepharose XL. Avoid oxidizing agents.

Storage

For column storage, wash with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Include 0.2 M sodium acetate in the storage solution for SP Sepharose XL. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at +4° C to +30° C in 20% ethanol. Do not freeze.



To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

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Sepharose Big Beads: purification from crude, viscous samples at large scale

Use Sepharose Big Beads for purification of proteins from crude, viscous samples.

Use Sepharose Big Beads when handling large volumes of crude or viscous samples that must be bound rapidly and when resolution is less important.

Use Sepharose Big Beads for capture steps, when viscosity and back-pressure may limit the throughput attainable with ion exchangers of smaller particle size.

Run columns packed with Sepharose Big Beads on systems such as ÄKTAdesign, FPLC System and HPLC or systems using peristaltic pumps. Appendix 4 gives guidance on how to select the most suitable ÄKTAdesign system.

Sepharose Big Beads are ion exchangers designed for large-scale industrial applications. The media are based on 100–300 µm, cross-linked 6% agarose particles, substituted with quaternary ammonium (Q) or sulfopropyl (SP) groups. The large particle size, together with a high degree of cross-linking for extreme physical and chemical stability, ensures that high flow rates can be maintained when processing very viscous samples. For example, a flow of 500 cm/h can be maintained in an industrial process at viscosities up to 2.5 times the viscosity of water. More dilute samples can be run at 1000 cm/h. Particle size and bed volumes remain stable, despite changes in ionic strength or pH. The strong ion exchange groups (Q and SP) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application.

Figures 67 and 68 show the excellent flow characteristics and typical binding capacities for Big Bead media.

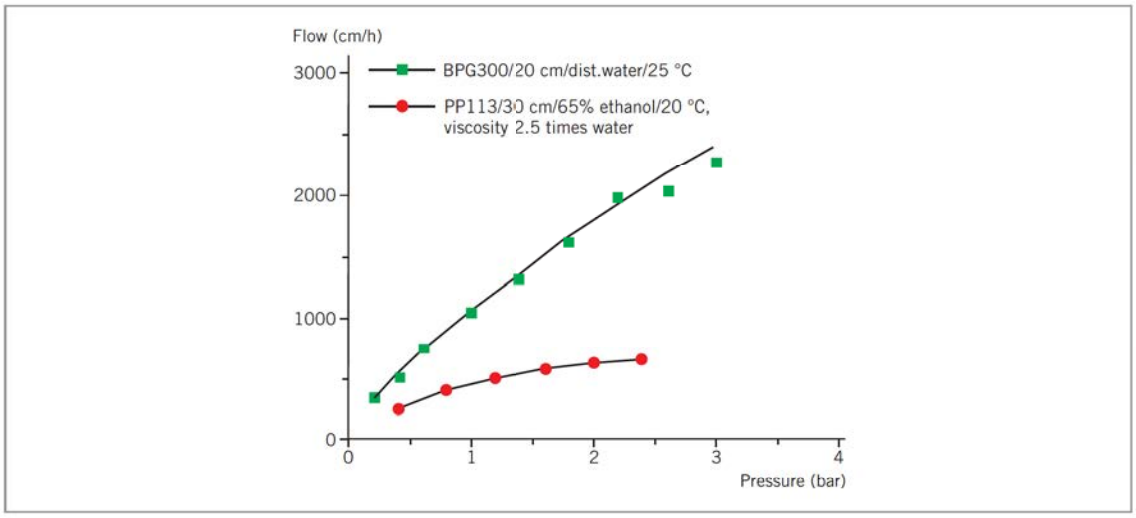


Fig. 67. Sepharose Big Beads allow high flow rates with high viscosity samples.

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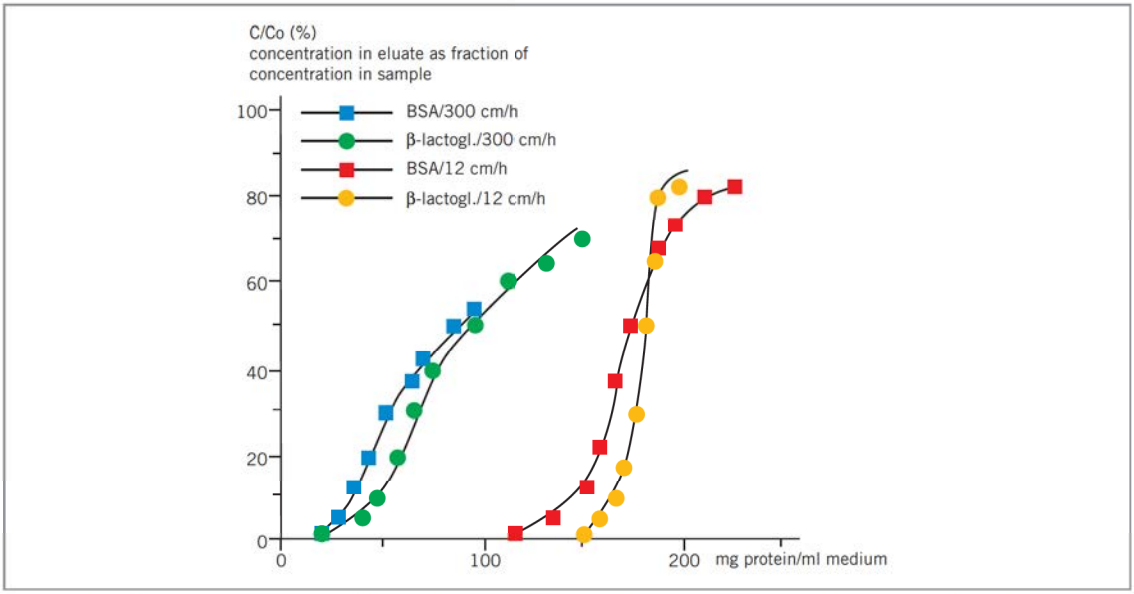


Fig. 68. Typical binding capacities of SP Sepharose Big Beads. Binding capacity measured in acetate pH 5 for bovine serum albumin and formate pH 4.1 for β-lactoglobulin at linear flows of 12 and 300 cm/h.

Purification options

Product	Binding capacity/ml medium	Recommended working flow*	Maximum flow*	Working pH range**	Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar*
Strong anion exchangers					
Q Sepharose Big Beads	tested for each specific application	up to 300 cm/h	1800 cm/h	2–12	0.3/43
Strong cation exchanger					
SP Sepharose Big Beads	tested for each specific application	up to 300 cm/h	1800 cm/h	4–13	0.3/43

**See Appendix 5 to convert linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa.*

***Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

****Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

For column packing during method development, particularly when handling crude, viscous samples:

XK 16/20	up to 30 ml	up to 15 cm
XK 26/20	up to 80 ml	up to 15 cm
XK 26/40	up to 196 ml	> 15 cm
XK 50/20	up to 274 ml	up to 14 cm
XK 50/30	up to 559 ml	up to 28.5 cm

Select a production scale column such as BPG or Chromaflow for larger volumes. Sepharose Big Beads can be packed in large scale columns by applying constant pressure between 1–3 bar, by slurry sedimentation followed by adaptor compression, or by suction packing. Follow the instructions supplied with the medium.

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Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions and method optimization are given in Chapter 2. See Appendix 2 for recommendations on volatile and non-volatile buffer systems.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Refer to Chapter 2 and Appendix 1 for recommendations and advice.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions through 1 µm filters. To avoid formation of air bubbles in a packed column, maintain buffers and columns at a constant temperature before and during a run.

First time use or after long term storage

1. Wash with 5 column volumes of distilled water at 300 cm/h.
2. Wash with 5 column volumes of start buffer, same flow as step 1.
3. Wash with 5 column volumes of elution buffer, same flow as step 1.
4. Wash with 5 column volumes of start buffer, same flow as step 1.
5. Run a blank elution before applying sample.

Gradient or step elution

Conditions for a large scale purification using Sepharose Big Beads will be determined during method development and relate to the specific application. Refer to Chapter 2 for advice on optimizing a separation. Typical separation flow rates should be 200–500 cm/h.

If ionic detergents have been used, wash the column with 5 column volumes of distilled water, followed by 2 column volumes 2 M NaCl. Re-equilibrate with at least 10 column volumes of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove non-ionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3.

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Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.



It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

The following procedure should be satisfactory to remove common contaminants:

1. Wash with at least 2 column volumes of 2 M NaCl at 40 cm/h for a contact time of 1–2 hours.
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 1) until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins, refer to Appendix 1.

Media characteristics

Composition: sulfopropyl (SP) or quaternary amino (Q) groups coupled to highly cross-linked 6% agarose via chemically stable ether bonds.

Product	Functional group	pH stability*	Mean particle size
Q Sepharose Big Beads	$-CH_2N^+(CH_3)_3$	Long term: 2–12 Short term: 2–14	200 µm
SP Sepharose Big Beads	$-CH_2CH_2CH_2SO_3^-$	Long term: 4–13 Short term: 3–14	200 µm

**Long term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance.*

Short term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. All ranges are estimates based on the experience and knowledge within Amersham Biosciences.

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Chemical stability

For daily use, Sepharose Big Beads media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), with additives such as non-ionic detergents, 70% ethanol, 1 M acetic acid, 30% acetonitrile and 30% isopropanol.

Sepharose Big Beads can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.



Avoid cationic detergents with SP Sepharose Big Beads. Avoid anionic detergents with Q Sepharose Big Beads. Avoid oxidizing agents.

Storage

For column storage, wash with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Include 0.2 M sodium acetate in the storage solution for SP Sepharose Big Beads. For small scale columns, degas the ethanol/water mixture thoroughly, for large scale columns ensure that an air trap is included before the column. Add storage solution at a low flow rate, checking the back pressure as the column equilibrates. Alternatively, store at neutral pH in buffer containing 20% ethanol or in 0.01 M NaOH.

Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Store unused media at +4° C to +30° C in 20% ethanol. Do not freeze.

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Chapter 4
Ion exchange in a Purification Strategy (CIPP)

To ensure efficient, reproducible purification giving the required degree of purity, it is beneficial to develop a multi-step process using the purification strategy of Capture, Intermediate Purification and Polishing (CIPP), shown in Figure 69.

CIPP is used in both the pharmaceutical industry and in the research laboratory to ensure faster method development, a shorter time to pure product and good economy. This chapter gives a brief overview of this approach which is recommended for any multi-step protein purification. The *Protein Purification Handbook* from Amersham Biosciences is an ideal guide for planning efficient and effective protein purification strategies. An important first step for any purification is correct sample preparation and this is covered in more detail in Appendix 1 and Chapter 2.

Ion exchange chromatography (IEX) plays a significant and highly flexible role in most multi-step purification schemes. If a specific affinity medium is not available or if little is known about the target molecule, IEX is recommended as the first step to consider for any purification. The technique can be used for capture, intermediate purification or polishing, according to the demands of the specific application. Since IEX offers different selectivities (using anion or cation exchangers) and since the pH of the purification can be modified to alter the charge characteristics of the sample components, it is possible to use the technique more than once in the same purification scheme. In addition IEX can be used with step-wise elution for a rapid capture step or with gradient elution to achieve the highest resolution in a polishing step.

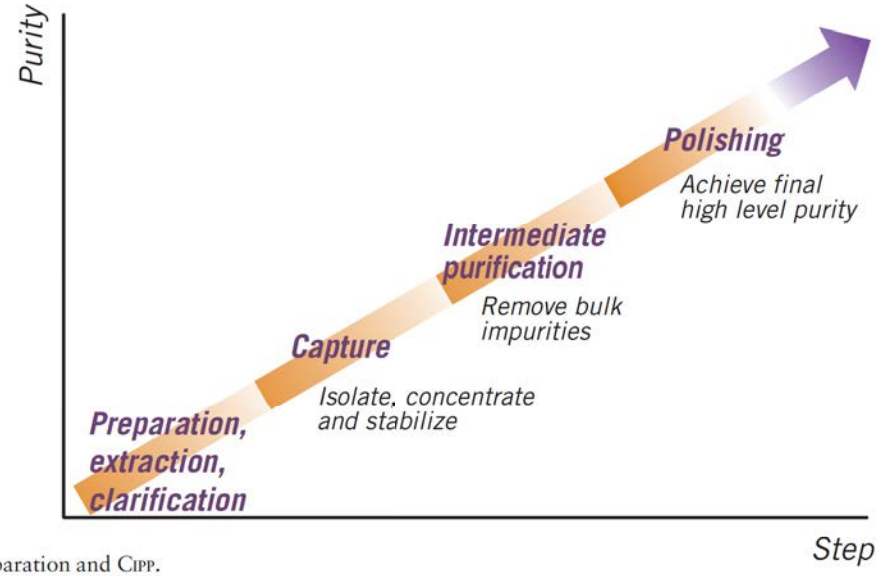


Fig. 69. Preparation and CIPP.

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Applying CIPP

Imagine the purification has three phases: Capture, Intermediate Purification and Polishing.

Assign a specific objective to each step within the purification process.

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

In the *capture phase* the objectives are to *isolate*, *concentrate and stabilize* the target product. The product should be concentrated and transferred to an environment that will conserve potency/activity.

During the *intermediate purification phase* the objectives are to *remove most of the bulk impurities*, such as other proteins and nucleic acids, endotoxins and viruses.

In the *polishing phase* most impurities have already been removed. The objective is to *achieve final purity* by removing any remaining trace impurities or closely related substances.

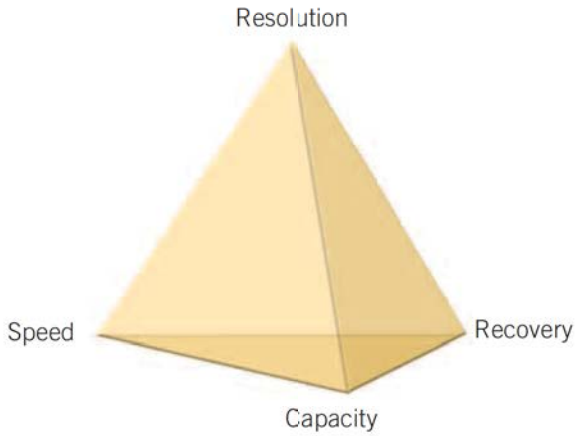
The optimal selection and combination of purification techniques for *Capture, Intermediate Purification and Polishing* is crucial for an efficient purification.

Selection and combination of purification techniques

Proteins are purified using purification techniques that separate according to differences in specific properties, as shown in Table 8.

Table 8. Protein properties used during purification.

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



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

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

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

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

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

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

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

A guide to the suitability of each purification technique for the stages in CIPP is shown in Table 9.

Table 9. Suitability of purification techniques for CIPP.

Technique	Main features	Capture	Intermediate	Polishing	Sample start condition	Sample end condition
IEX	high resolution high capacity high speed	★★★	★★★	★★★	low ionic strength sample volume not limiting	high ionic strength or pH change **concentrated sample**
HIC	good resolution good capacity high speed	★★	★★★	★	high ionic strength sample volume not limiting	low ionic strength **concentrated sample**
AC	high resolution high capacity high speed	★★★	★★★	★★	specific binding conditions sample volume not limiting	specific elution conditions **concentrated sample**
GF	high resolution using Superdex™ media		★	★★★	limited sample volume (<5% total column volume) and flow rate range	buffer exchanged (if required) diluted sample
RPC	high resolution		★	★★★	sample volume usually not limiting additives may be required	in organic solvent, risk loss of biological activity

Minimize sample handling between purification steps by combining techniques to avoid the need for sample conditioning. The product should be eluted from the first column in conditions suitable for the start conditions of the next column (see Table 9).

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Ammonium sulfate, often used for sample clarification and concentration (see Appendix 1), leaves the sample in a high salt environment. Consequently HIC, which requires high salt to enhance binding to the media, becomes the ideal choice as the capture step. The salt concentration and the total sample volume will be significantly reduced after elution from the HIC column. Dilution of the fractionated sample or rapid buffer exchange using a desalting column will prepare it for the next IEX or AC step.

Gel filtration is a non-binding technique unaffected by buffer conditions, but with limited volume capacity. GF is well suited for use after any of the concentrating techniques (IEX, HIC, AC) since the target protein will be eluted in a reduced volume and the components from the buffer will not affect the gel filtration process.

Selection of the final strategy will always depend upon specific sample properties and the required level of purification. Logical combinations of techniques are shown in Figure 70.

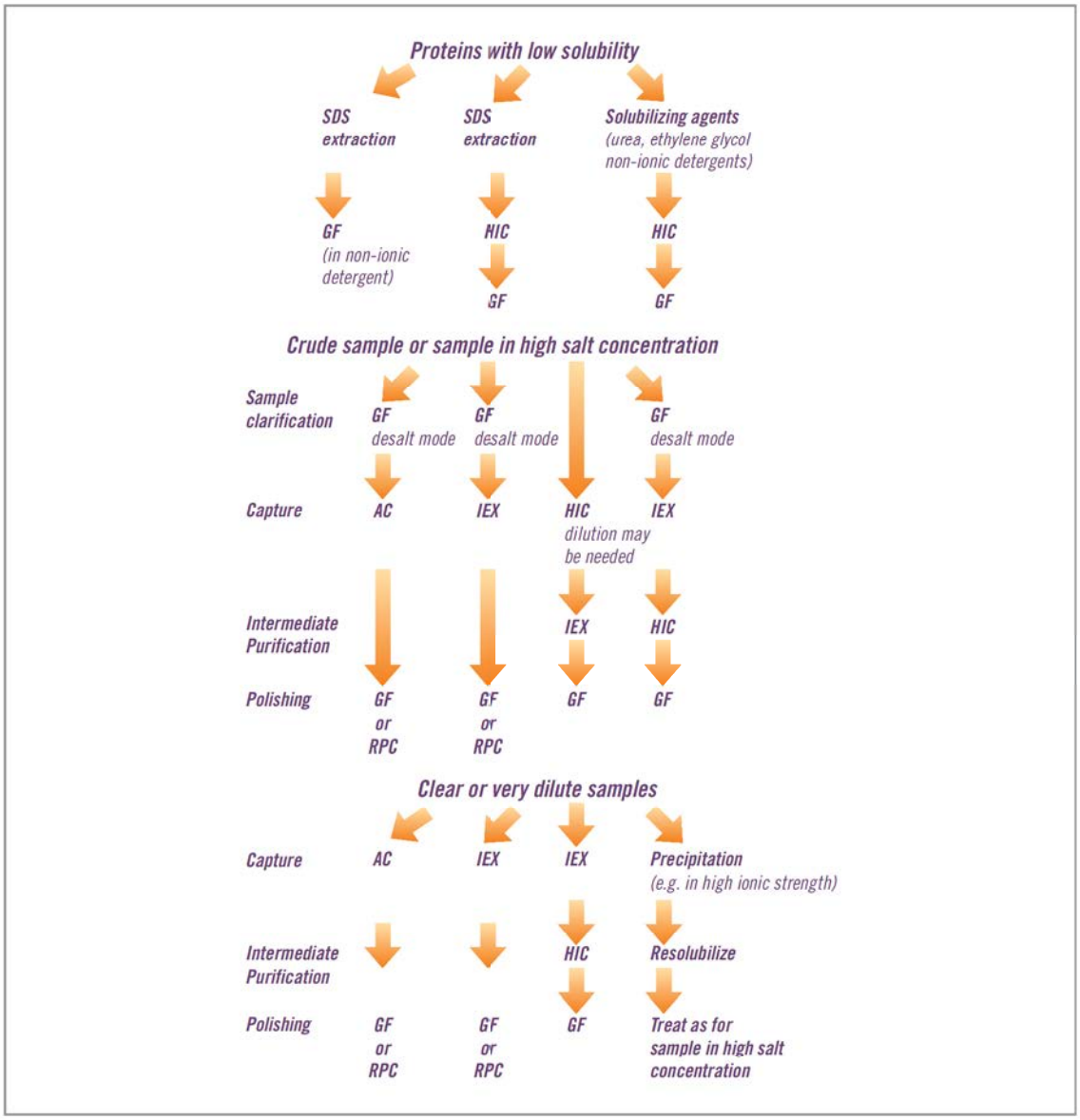


Fig. 70. Logical combinations of chromatography techniques.

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For any capture step, select the technique showing the most effective binding to the target protein while binding as few of the contaminants as possible, i.e. the technique with the highest selectivity and/or capacity for the target protein.

A sample is purified using a combination of techniques and alternative selectivities. For example, in an IEX-HIC-GF strategy, the capture step selects according to differences in charge (IEX), the intermediate purification step according to differences in hydrophobicity (HIC) and the final polishing step according to differences in size (GF).

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

Consider the use of both anion and cation exchange chromatography to give different selectivities within the same purification strategy.

Ion exchange as a capture step

When IEX chromatography is used as a capture step, the objective is to quickly adsorb the protein(s) of interest from the crude sample and isolate them from critical contaminants such as proteases and glycosidases. The target protein(s) are concentrated and transferred to an environment which will conserve potency/activity. Removal of other critical contaminants may also be achieved by careful optimization of pH and elution conditions.

The focus is on capacity and speed in a capture step. It may be advisable to compromise on the potential resolution that can be achieved by an IEX separation in order to maximize the capacity and/or speed of the separation in this first step.

IEX media for capture steps should offer high speed and high capacity.

1. Sepharose Fast Flow (90 µm particle size) – good resolution for crude mixtures at any scale using flows up to 300 cm/h and offering a wide range of selectivities.
2. Sepharose XL (90 µm particle size) – high capacity, good resolution for capture of selected proteins at laboratory and process scale using flows up to 300 cm/h.
3. Sepharose Big Beads (200 µm particle size) – for viscous samples that preclude the use of IEX media with smaller particle size, using flows up to 300 cm/h, or for fast separations of very large sample volumes when resolution is of less importance, using flows up to 1000 cm/h.

If only milligram quantities of product are needed and the capture step will not be scaled up, use high performance media such as MonoBeads or MiniBeads according to the capacity required. Also, if the starting material is reasonably clean, a single step purification on MonoBeads may be sufficient to achieve required purity at laboratory scale.

Select start conditions that avoid adsorption of contaminants and so help to maximize the binding capacity for the target protein(s). This will facilitate a fast, simple step elution of the concentrated target protein(s).

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Figure 71 shows optimization of a capture step used for purification of a recombinant enzyme, deacetooxycephalosporin C synthase (DAOCS). Since this enzyme is oxygen-sensitive, it was important to rapidly remove the most harmful contaminants from the relatively unstable target protein. The isoelectric point of DAOCS (pI = 4.8) made an anion exchanger the most suitable choice. Columns from the HiTrap IEX Selection Kit were screened to select the most suitable medium (results not shown) before optimizing the separation on a larger HiPrep 16/10 Q XL column. A detailed description of the entire purification process for DAOCS can be found in Application Note 18-1128-91, available from Amersham Biosciences.

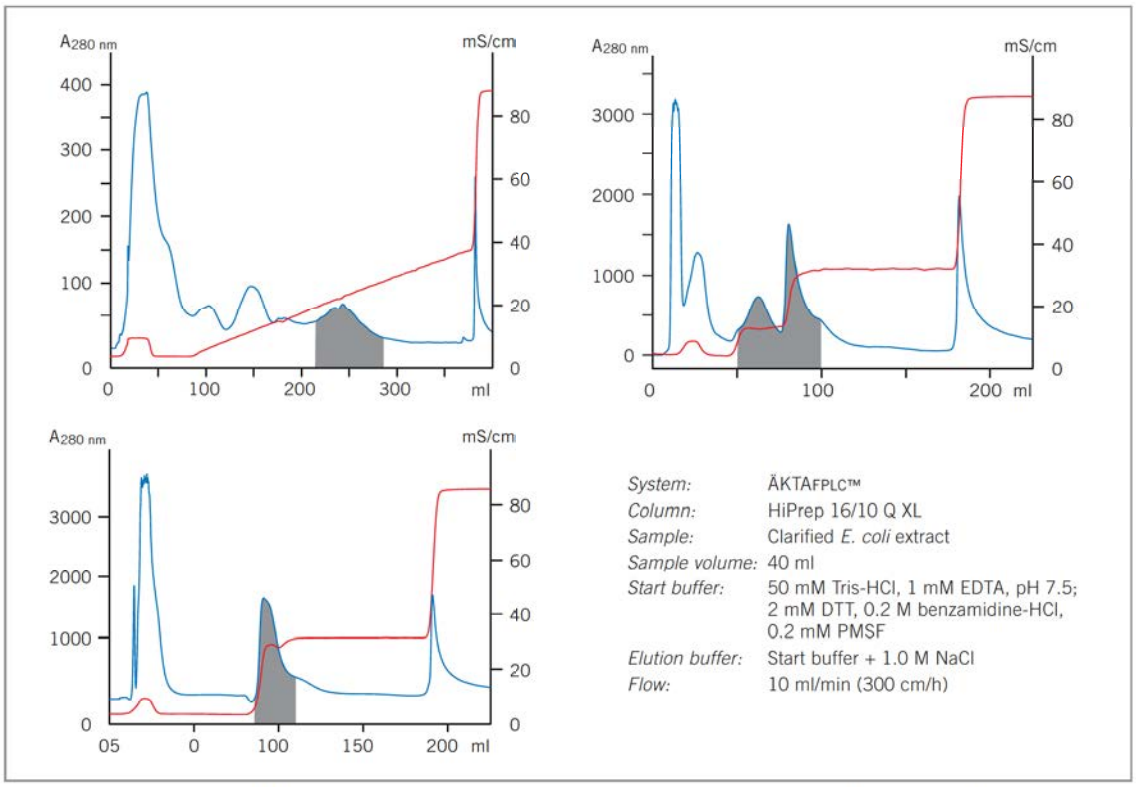


Fig. 71. Capture step using IEX and optimization of conditions. The elution position of DAOCS is shaded.

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Ion exchange for intermediate purification

When IEX is used for intermediate purification, the objective is to remove most of the significant impurities such as proteins, nucleic acids, endotoxins and viruses.

In a typical intermediate purification step, speed is less critical since sample volume has been reduced and damaging contaminants have been removed during capture. Focus is on capacity and resolution in order to maintain productivity (amount of target protein processed per column in unit time) and to achieve as high selectivity (purity) as possible. Consequently, a gradient elution will usually be required.

Use a technique with a selectivity that is complementary to that used in the capture step.

IEX media for intermediate purification should offer high capacity and high resolution with a range of complementary selectivities:

1. Sepharose High Performance (34 µm particle size) – high resolution using flows up to 150 cm/h.
2. SOURCE 15 (15 µm particle size) – high throughput, high resolution for laboratory or large-scale applications using flows up to 1800 cm/h.
3. SOURCE 30 (30 µm particle size) – an alternative to SOURCE 15 for large-scale applications when flows up to 2000 cm/h can be used.
4. Sepharose Fast Flow (90 µm particle size) – fast separations, good resolution using flows up to 300 cm/h, broad range of selectivities.

If only milligram quantities are required and the intermediate purification step will not be scaled-up, use MonoBeads or MiniBeads according to the capacity required.

Optimize the selectivity of the medium to ensure high binding capacity and to maximize resolution. Use continuous gradient or multi-step elution conditions.

Figure 72 shows a process scheme developed for purification of epidermal growth factor (EGF), expressed as an extra-cellular product from *Saccharomyces cerevisiae*. As shown the scheme was developed and optimized at laboratory scale before being transferred to large scale production. Purity and yield were maintained when the process was scaled up (Figure 73). Starting material was a clarified supernatant supplied by Chiron-Cetus Corp., Emeryville, CA, USA. Q Sepharose High Performance provides a high resolution intermediate purification step after capturing the sample by HIC and prior to polishing by GF on Superdex 75 prep grade. Note the decrease in particle size to further improve resolution as the purification moves from a capture step using a Sepharose Fast Flow matrix to the Sepharose High Performance matrix.

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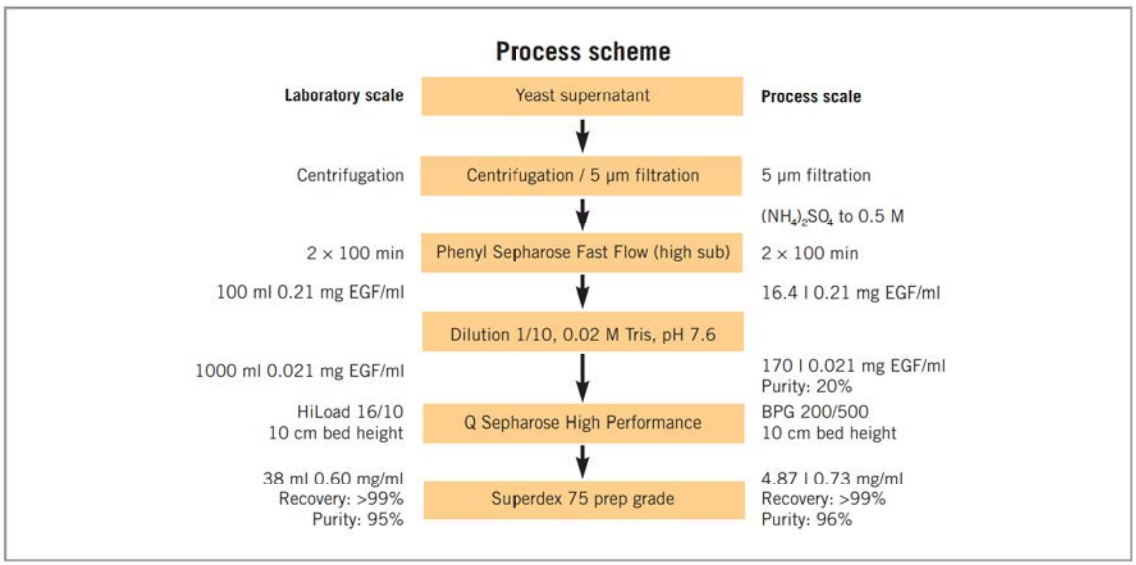


Fig. 72. Process scheme for purification of EGF.

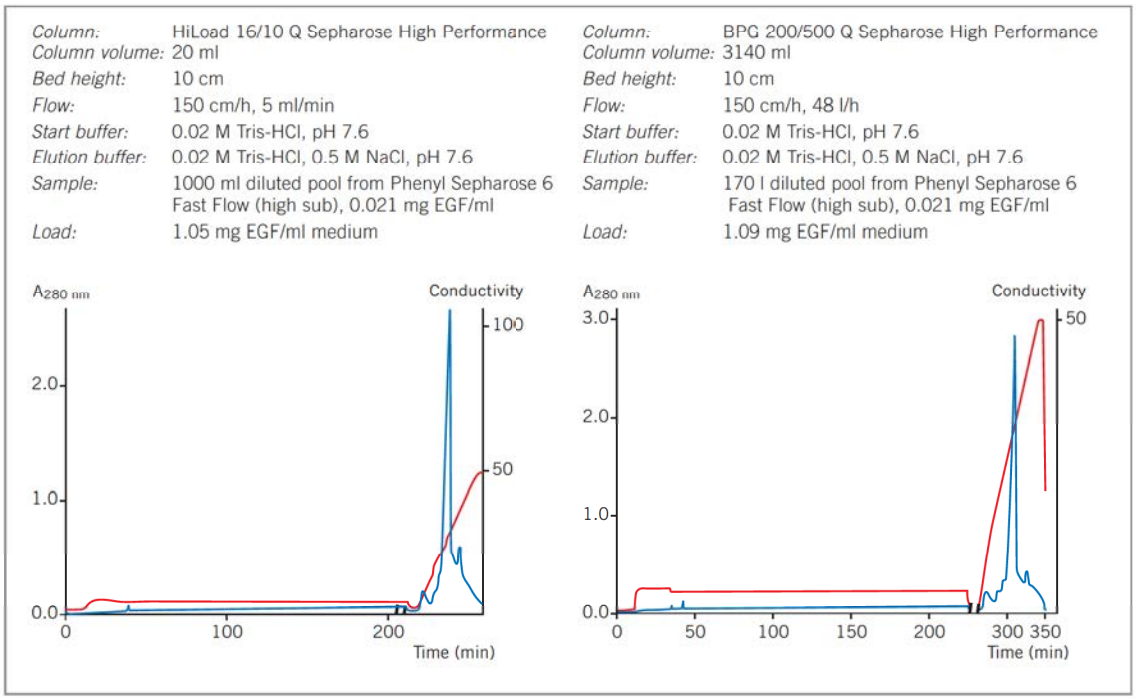


Fig. 73. Elution pattern, purity and yield are maintained when scaling-up from a HiLoad column to a BPG column.

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Ion exchange as a polishing step

When IEX is used for polishing, most impurities have been removed except for trace amounts or closely related substances such as structural variants of the target protein, nucleic acids, viruses or endotoxins. The purpose of the separation is to reduce these variants and trace contaminants to acceptable levels for the application. In contrast to capture steps where a fast, high capacity, step elution is most commonly used, a polishing step will therefore focus on achieving the highest possible resolution. An example of this approach is shown in Figure 74 in which Mono S 5/50 GL was used to separate a recombinant DNA binding protein, transposase TniA, from minor contaminants remaining after partial purification by anion exchange and heparin affinity chromatography. The final product was >90% pure.

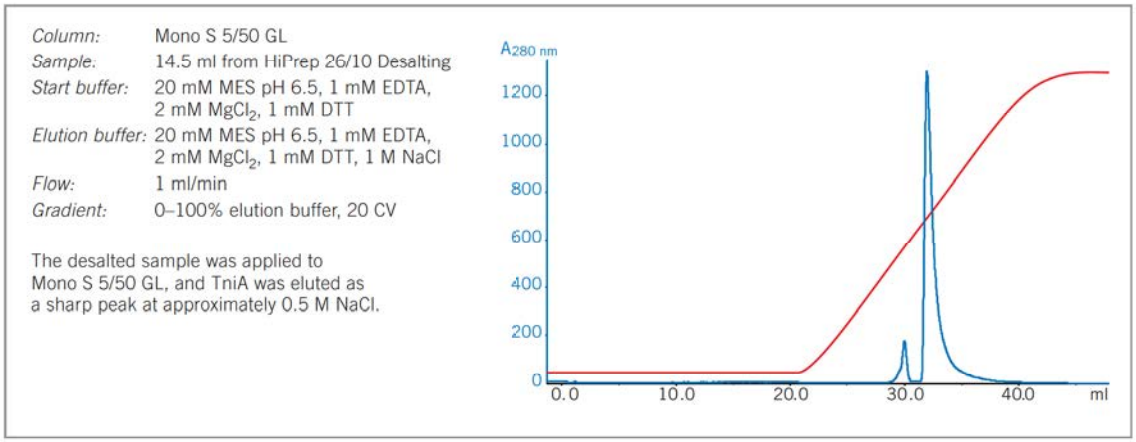


Fig. 74. High resolution cation exchange chromatography on Mono S 5/50 GL.

IEX media for **polishing** steps should offer highest resolution:

1. MiniBeads (3 µm particle size) – polishing at microscale when highest resolution is essential.
2. MonoBeads (10 µm particle size) – polishing at laboratory scale when highest resolution is essential and a higher capacity than MiniBeads is required.
3. SOURCE 15 (15 µm particle size) – rapid, high resolution polishing for laboratory or large scale applications using flows up to 1800 cm/h.
4. SOURCE 30 (30 µm particle size) – an alternative to SOURCE 15 for large scale applications when flows up to 2000 cm/h can be used.

Optimize the gradient elution to maximize selectivity. Use high efficiency media with small bead sizes to improve resolution.

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Alternative techniques for polishing steps

Most commonly, separations by charge, hydrophobicity or affinity will have been used in earlier stages of a purification strategy so that high resolution gel filtration is ideal for the final polishing step. The product can be purified and transferred into the required buffer in one step and dimers and aggregates can be removed, as shown in Figure 75.

Gel filtration is also the slowest of the chromatography techniques and the size of the column determines the volume of sample that can be applied. It is therefore most logical to use gel filtration after techniques that reduce sample volume so that smaller columns can be used. Media for polishing steps should offer highest possible resolution. Superdex is the first choice or gel filtration at laboratory scale and Superdex prep grade for large scale applications.

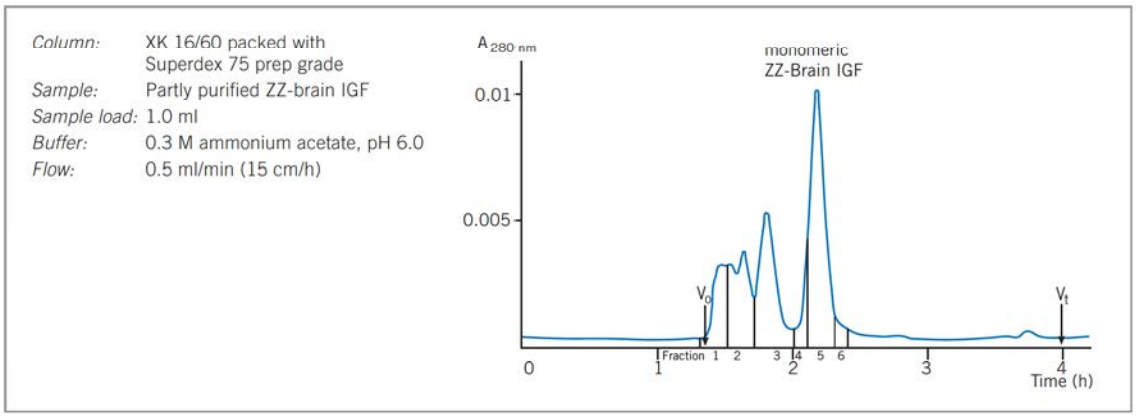


Fig. 75. Final polishing step: separation of dimers and multimers on Superdex 75 prep grade.

RPC can also be considered for a polishing step, provided that the target protein can withstand the run conditions. Reversed phase chromatography (RPC) separates proteins and peptides on the basis of hydrophobicity. RPC is a high selectivity (high resolution) technique, usually requiring the use of organic solvents. The technique is widely used for purity check analyses when recovery of activity and tertiary structure are not essential. Since many proteins are denatured by organic solvents, RPC is not generally recommended for protein purification because recovery of activity and return to a correct tertiary structure may be compromised. However, in the polishing phase, when the majority of protein impurities have been removed, RPC can be an excellent technique, particularly for small target proteins that are not often denatured by organic solvents.

CIPP does not mean that there must always be three purification steps. For example, capture and intermediate purification may be achievable in a single step, as may intermediate purification and polishing. Similarly, purity demands may be so low that a rapid capture step is sufficient to achieve the desired result. For purification of therapeutic proteins, a fourth or fifth purification step may be required to fulfill the highest purity and safety demands. The number of steps used will always depend upon the purity requirements and intended use for the protein.

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Chapter 5
Chromatofocusing: principles and methods

Introduction

This chapter introduces the principles and methods of chromatofocusing, a chromatography technique that separates proteins according to differences in their isoelectric point (pI).

Use chromatofocusing as a polishing step for partially purified samples. The fewer components in the sample, the better the chance for a well-resolved separation of individual proteins. For specific applications, chromatofocusing can resolve molecules where pI values differ by as little as 0.02 pH units. This high resolving ability can be particularly useful for the separation of very similar substances.

Use chromatofocusing for high resolution, analytical separations.

Use chromatofocusing as a complementary technique when proteins have not been resolved by other chromatography techniques such as charge (using ion exchange), size or hydrophobicity.

As explained in Chapter 1, each protein has its own unique *pH versus net charge relationship* which can be visualized as a *titration curve*. This curve reflects how the overall net charge of the protein changes according to the pH of the surroundings. *The pI of each protein is the pH at which the protein has zero surface charge.* Proteins with different pIs can be separated by being passed through a chromatofocusing column (packed with *a specifically-designed medium*) while a pH gradient is generated on the column by *specifically-designed and matched amphoteric buffers*. Proteins elute in order of their isoelectric points, as shown in Figure 76.

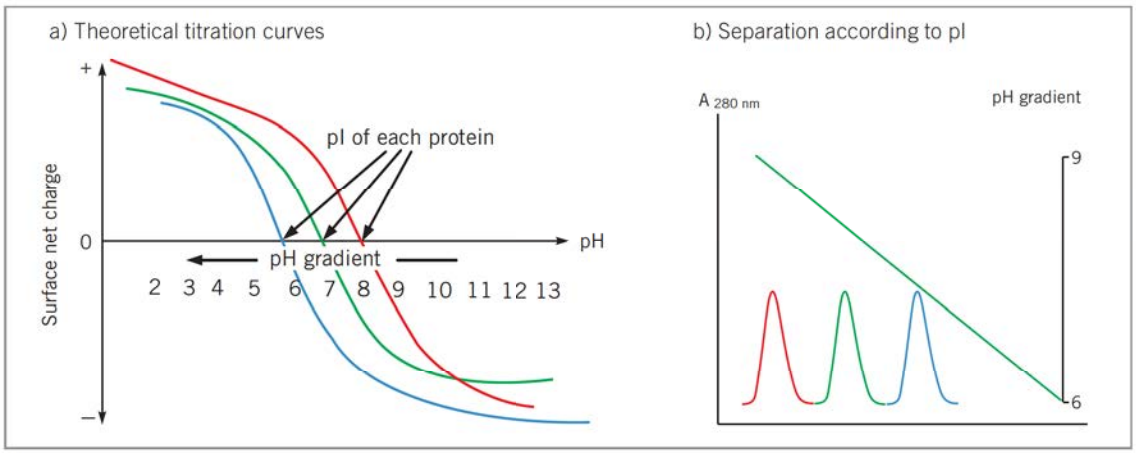


Fig. 76. Separating proteins according to their isoelectric points by chromatofocusing.

In order to achieve consistently high resolution, generation of the linear pH gradient requires an even buffering capacity over the entire pH range used for a separation. Hence the need for specifically designed buffers (Polybuffer 74, Polybuffer 96 or Pharmalyte™ 8–10.5) and media substituted with charged, buffering amines (Mono P™, PBE 118 or PBE 94).

Although chromatofocusing can be a high resolution technique, it is less suitable for the isolation of proteins that precipitate irreversibly at or near their isoelectric point since these proteins are likely to precipitate on the column if they reach a high enough concentration.

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Separation mechanism: on-column generation of pH gradients and protein behavior

In order to separate proteins according to their different pIs, the chromatofocusing medium is equilibrated with start buffer at a pH slightly above the highest pH required. An elution buffer (adjusted to the lowest pH required) is passed through the column and begins to titrate the amines on the medium and the proteins. As the buffer flows through the column, the pH is lowered and a moving, *descending* pH gradient is generated (Figure 77).

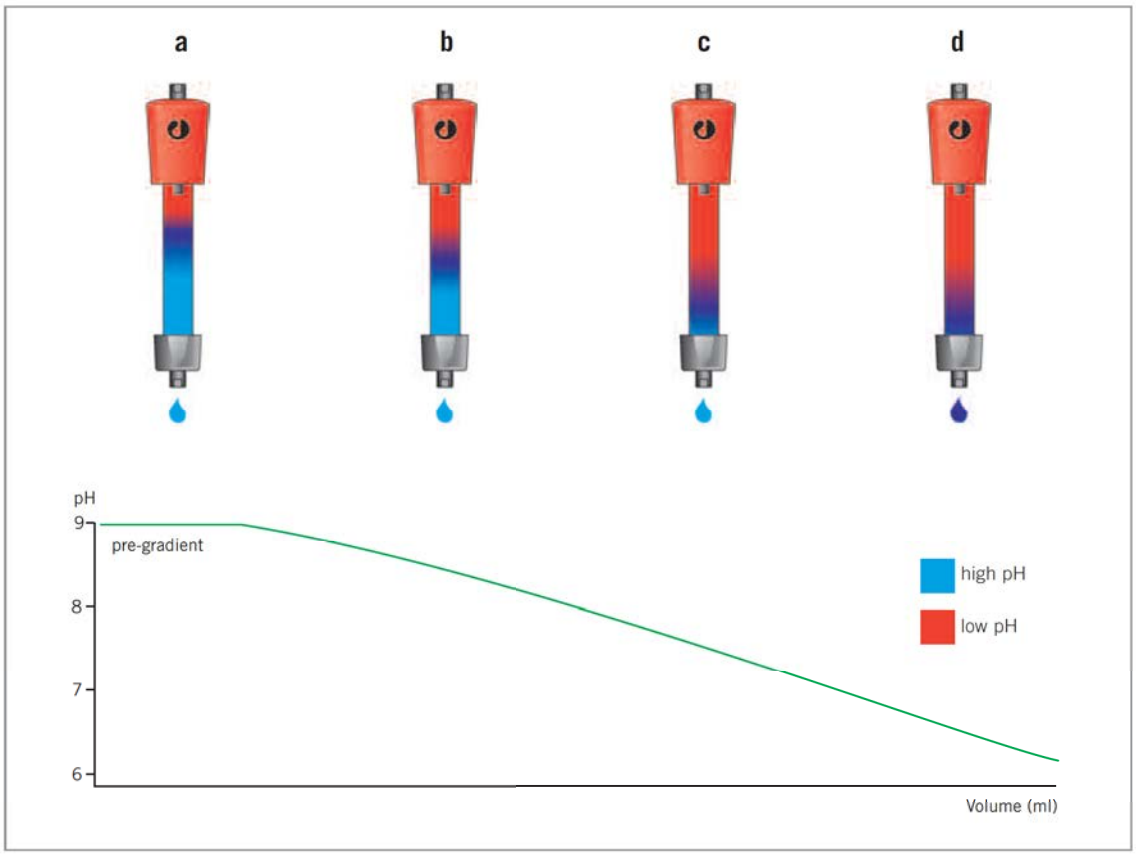


Fig. 77. Development of a pH gradient in a chromatofocusing column. The column is pre-equilibrated with start buffer at high pH (a) and elution with Polybuffer at low pH (b,c,d,) generates a descending linear pH gradient.

After a pre-gradient volume of elution buffer has passed, sample (in start buffer) is applied to the column. The proteins in the sample are titrated (pH adjusted) as soon as they are introduced into the column. Proteins in the sample that are at a pH *above* their pI are negatively-charged and retained near the top of the column (binding to the positively-charged amine groups). Any proteins that are at a pH *below* their pI begin to migrate down the column with the buffer flow and will not bind until they reach a zone where the pH is above their pI. This is the beginning of the separation process that is illustrated in Figure 78.

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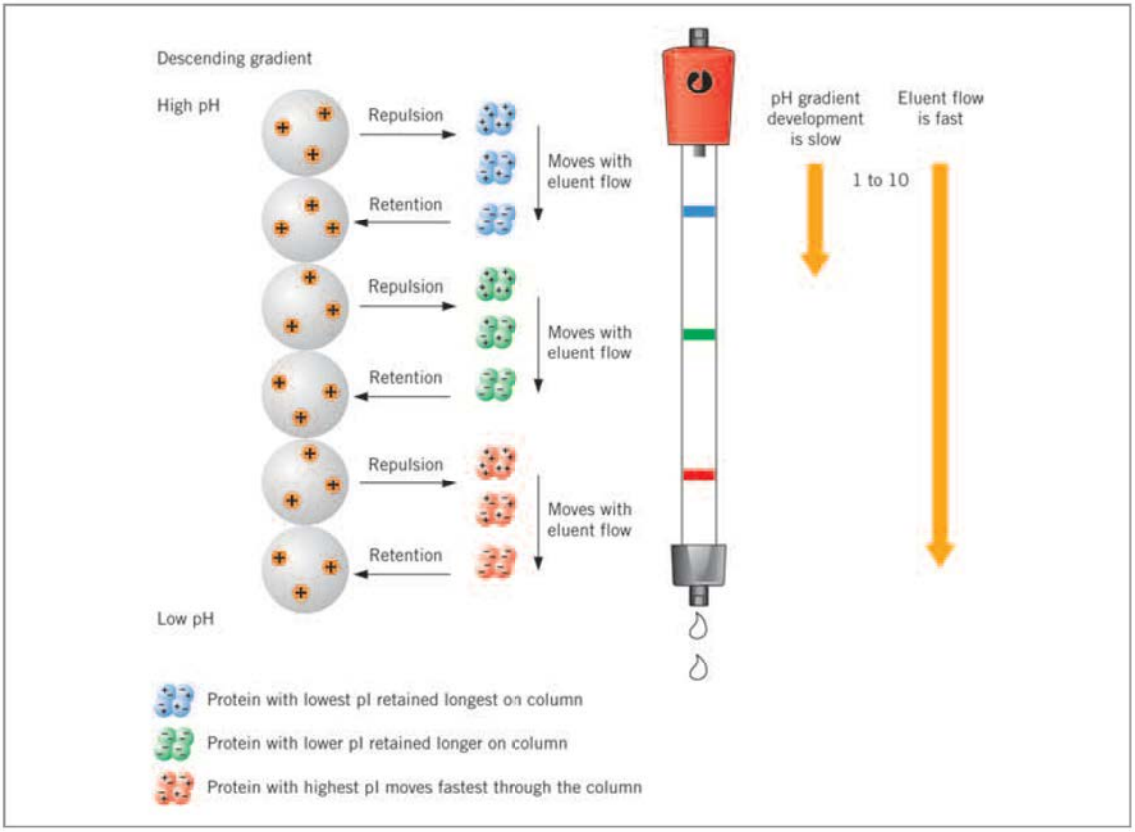


Fig. 78. Proteins with different pIs separate as they pass through the column. Molecules with the same isoelectric point are focused in narrow bands during the separation.

As the pH continues to decrease near the top of the column, any protein that drops *below* its pI becomes positively charged, is repelled by the positively-charged amine groups, and begins to migrate down the column with the elution buffer, travelling faster than the speed at which the pH gradient moves down the column. However, as the protein migrates down the column, the pH of the surroundings increases. When the protein reaches a zone in which the pH is *above* its pI, it becomes negatively charged and binds to the column again. The protein remains bound until the developing pH gradient reduces the local pH when, again, the protein drops below its pI, becomes positively charged, and begins to move down the column, catching up with the gradient. This process continues until the protein is eluted from the column at a pH near to its pI (when it has almost no net charge).

Figure 78 illustrates the *focusing effect* which takes place during a separation and contributes significantly to the high resolution achievable with chromatofocusing. In a descending pH gradient, a protein can exist in three charged states: positive, negative or neutral and is constantly changing its charged state as the pH gradient develops and the protein travels through different pH zones on the column. Molecules at the rear of a zone will migrate more rapidly than those at the front, gradually forming narrower bands of proteins, each band comprised of one or more proteins of the same isoelectric point.

Thus, during chromatofocusing, proteins with different pIs migrate at different rates down the column as the pH gradient develops, continually binding and dissociating while being focused into narrow bands and finally eluted. The protein with the highest pI elutes first and the protein with the lowest pI will elute last.

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Media selection

Use prepacked, Mono P for fast separations (up to ten times faster than PBE).

Use Mono P 5/200 GL for highest resolution separations.

Use Mono P 5/50 GL rapid scouting of elution conditions (pH gradients) or for separations not requiring highest resolution.

Use PBE 94 for scaling up from Mono P. Note that, under the same separation conditions, resolution may be lower due to the longer diffusion times within larger particles. Re-optimization of the gradient and flow rate should give similar resolution.

Use PBE 118 when pH gradients above pH 9 are required.

Mono P is based on the same matrix as Mono Q and Mono S (see Chapter 3). The 10 µm MonoBeads particles are substituted with tertiary and quaternary amines and the small bead size contributes significantly to the high resolution that can be achieved.

Polybuffer exchangers, PBE 118 and PBE 94, are based on Sepharose CL-6B, a cross-linked agarose matrix. The charged secondary, tertiary and quaternary amine groups are coupled to monosaccharide units in the 90 µm Sepharose CL-6B particles by ether linkages. For all chromatofocusing media the charged groups have been selected to give an even buffering capacity across a broad pH range. The buffering capacity and titration curves for PBE 118, PBE 94 and Mono P are shown in Figures 79 and 80.

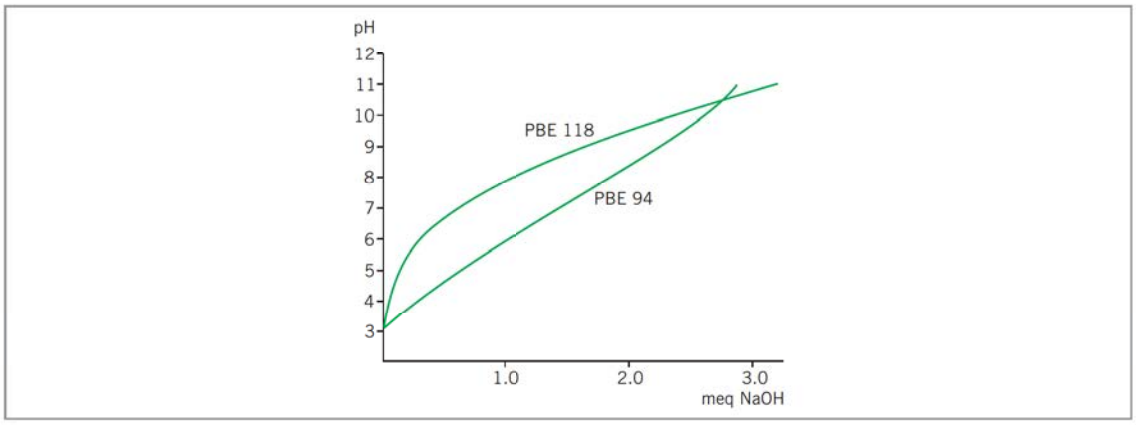


Fig. 79. Titration of 10 ml aliquots of PBE 118 and PBE 94 in 1 M KCl against NaOH shows the smooth buffering capacity of these media over a broad pH range.

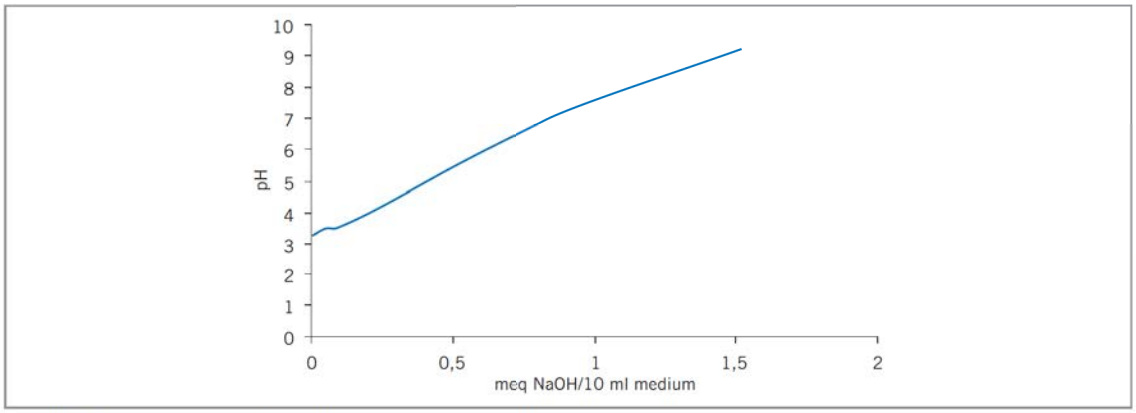


Fig. 80. Titration curve of Mono P column shows smooth buffering capacity.

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Buffer selection

Use Polybuffer 74 for any pH gradient between 7 and 4. Use a pH gradient 7–4 when starting to work with proteins of unknown pI.

Use Polybuffer 96 for pH gradients that should begin above pH 7 e.g. when the proteins of interest have a pI above or close to 7.

Use Pharmalyte pH 8–10.5 when higher pH values are required.

Refer to section 'Selection of pH gradient and buffers' for further details.

Polybuffers are specifically designed to form pH gradients by titration of the charged groups on PBE 118, PBE 94 and Mono P media. Polybuffers are mixtures of selected amphoteric buffering substances of different pI and pKa values. Each mixture is designed to give an even buffering capacity across a wide pH range, in order to generate the required linear pH gradient. The titration curves for Polybuffer 96 and Polybuffer 74 are shown in Figure 81.

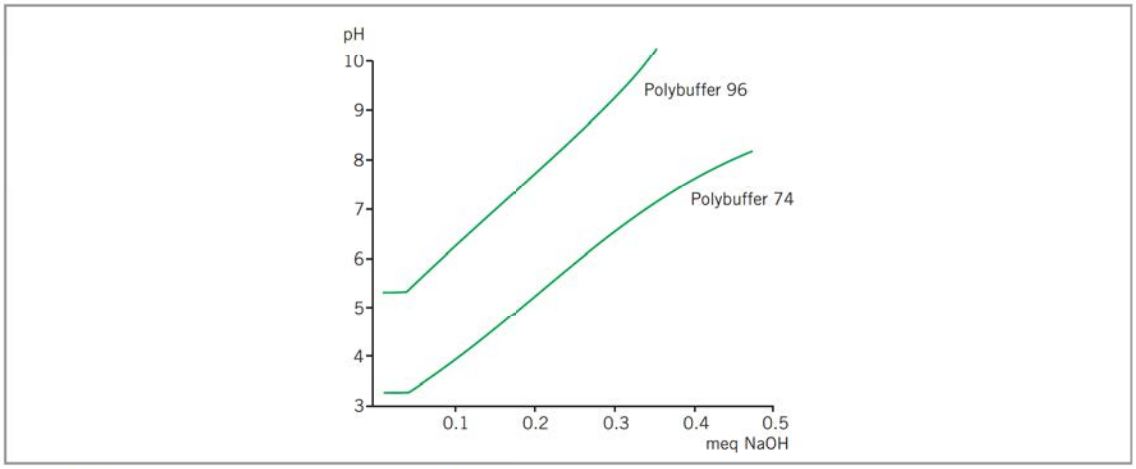


Fig. 81. Titration of 2 ml Polybuffer with 0.1 M NaOH.

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Purification options

Product	Maximum loading capacity	Recommended operating flow*	Maximum flow**	Working pH range**	Maximum operating back pressure *** (MPa/psi) 1 MPa=10 bar
Mono P 5/50 GL	10 mg/column	0.5–1.5 ml/min (305–450 cm/h)	3.0 ml/min	4–9	4/580
Mono P 5/200 GL	40 mg/column	0.5–1.5 ml/min (305–450 cm/h)	2.0 ml/min	4–9	4/580
PBE 118	1–20 mg/ml medium	30–40 cm/h	115 cm/h	8–11	0.015/2
PBE 94	1–20 mg/ml medium	30–40 cm/h	115 cm/h	4–9	0.015/2

**See Appendix 5 to convert linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa.*

***Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

****Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

Buffer options

Buffer	pH range	Compatible with medium
Polybuffer 74	7–4	PBE 94 Mono P
Polybuffer 96	9–6	PBE 94 Mono P
Pharmalyte pH 8–10.5	11–8	PBE 118

Purification examples

High resolution

The potential for high resolution separation of proteins with otherwise similar properties makes chromatofocusing an excellent technique for the study of different isoforms. For example, haemoglobins can be separated into 4 distinguishable sub-groups, each with a different isoelectric point (Figure 82). The isoelectric points of sub-groups A and F differ by only 0.05 pH units, yet the peaks are well resolved. The focusing effect of the linear pH gradient and excellent peak symmetry (achieved by using small, uniform particles in a well-packed column) combine to give a high resolution separation.

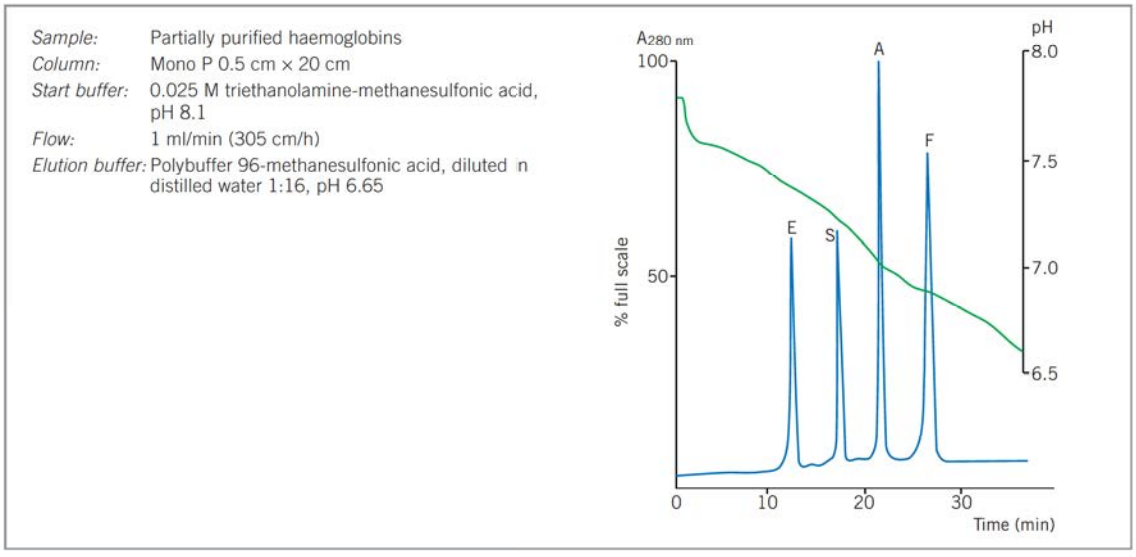


Fig. 82. Chromatofocusing of haemoglobin variants.

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Under ideal conditions with a very well packed column, Polybuffer exchangers can give almost as good resolution as Mono P. Figure 83 shows an example in which model proteins that differ by as little as 0.02 pH units in their pI values have been resolved on a Polybuffer exchanger.

Sample: Whale myoglobin (2 mg), horse myoglobin (2 mg), carboxyhaemoglobin (2 mg)
Column: PBE 94, 1 cm × 18 cm bed height
Start buffer: 0.025 M ethanolamine, pH 9.5
Flow: 15 cm/h (0.2 ml/min)
Elution buffer: Polybuffer 96, pH 7, dilution 1:10 from stock buffer

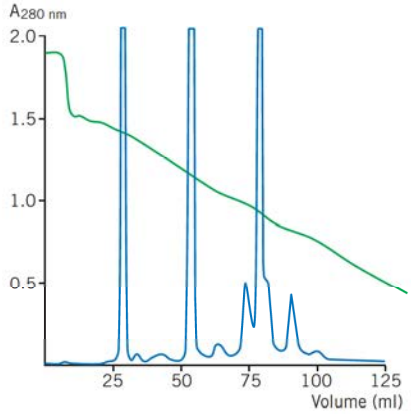


Fig. 83. Chromatofocusing of standard protein mixture.

High selectivity

Figure 84 demonstrates the high selectivity of chromatofocusing that enables detection of subtle changes in individual proteins. In this application researchers were able to follow the desialylation of transferrin as sialic acid groups were removed by neurominidase.

Sample: Iron-saturated diferric transferrin
Column: Mono P 0.5 cm × 20 cm
Start buffer: 0.025 M bis-Tris HCl, pH 6.45, 10% betaine hydrate, 3 mM $FeCl_3$
Flow: 1 ml/min (305 cm/h)
Elution buffer: Polybuffer 74 diluted 1:10 in distilled water, pH 5, 10% betaine hydrate, 3 mM $FeCl_3$

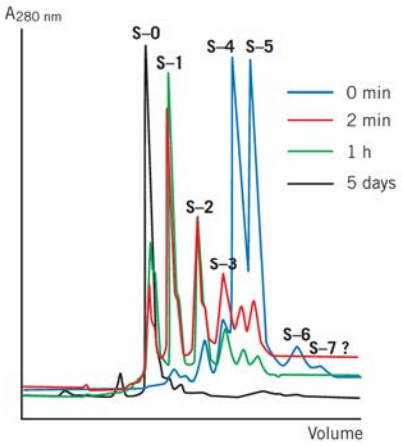


Fig. 84. Following the desialylation of transferrin.

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Polishing step

Figure 85 shows an example of chromatofocusing being used for polishing in a purification strategy (in a polishing step most impurities have been removed and the objective is to achieve final purity by removing any trace impurities or closely related substances). In this example highly pure leukotriene A4 hydrolase was required for functional and structural studies. The enzyme was expressed as $(His)_6$-tagged LTA4 hydrolase in *E. coli*. After harvesting the cells and removing nucleic acids, nickel ion affinity chromatography was used as a specific protein capture step and the eluted fraction subjected to a polishing step on Mono P.

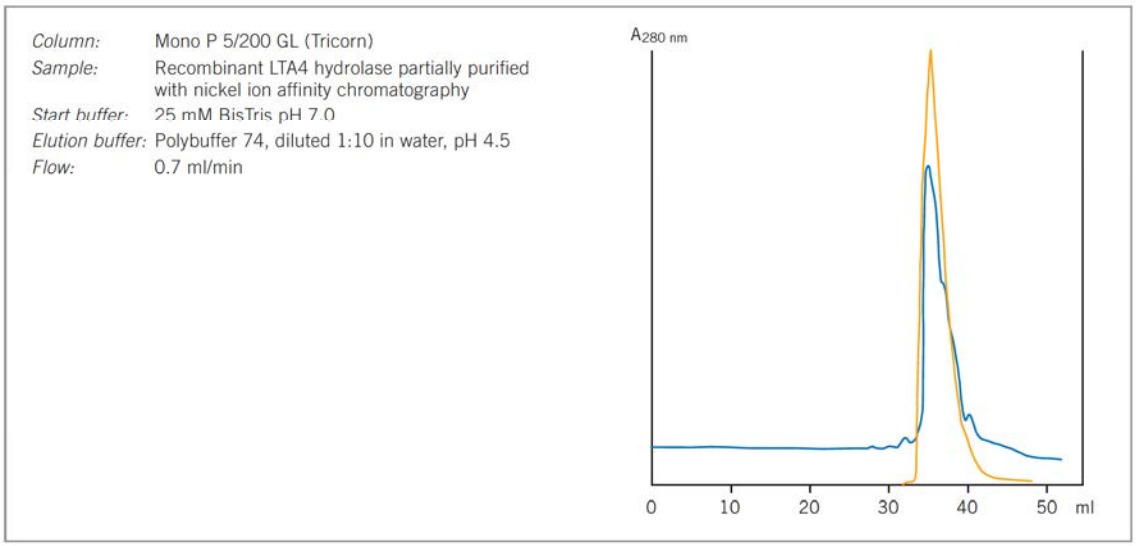


Fig. 85. Purification of leukotriene A4 hydrolase. Results courtesy of Eva Ohlson, Karolinska Institutet, Stockholm, Sweden.

Packing a column

Prepacked columns are likely to give the highest resolution and the most reproducible results, particularly with the MonoBead matrix used in Mono P 5/50 GL and Mono P 5/200 GL.

Efficient packing of a chromatofocusing column is critical to achieve the best results. Follow carefully the instructions for column packing given in Appendix 3 when packing PBE 94 or PBE 118.

Pack columns in the start buffer to be used for the separation (see Table 10, 11, 12 or 13) using long, narrow columns such as Tricorn 10/300. The amount of medium required will depend on the amount of sample, the nature of the sample and contaminants and the degree of resolution required. For most separations 20–30 ml Polybuffer exchanger will be sufficient to separate from 1–200 mg protein/pH unit in the gradient.

Degas the start buffer and the slurry in order to avoid air bubbles which can interfere with the separation.

Check column efficiency as described in Appendix 3. It may be worth checking the packing using a colored marker. Use bovine cytochrome c which is strongly repelled from the medium due to its high isoelectric point (pI=10.5). The progress of the protein band through the column can be visualized in order to check for any distortion caused by poor column packing or air bubbles.

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Selection of pH gradient and buffers

No gradient forming equipment is required for chromatofocusing since the gradient is generated on the column as buffer and medium interact. The upper limit of the gradient is defined by the pH of the start buffer and the lower limit of the gradient is defined by the pH of the elution buffer. Polybuffers perform best over pH intervals of 3 pH units or less and the narrowest pH intervals are likely to give the highest resolution.

The *gradient volume* is defined by the strength of the elution buffer (low buffer concentrations give gradual pH changes and good separations between peaks). The optimal gradient volume may need to be determined by experimentation. Recommended gradient volumes and buffers are indicated in Tables 10, 11, 12 and 13. The *pre-gradient volume* is the volume of elution buffer that passes through the column before the pH begins to decrease, hence the total volume of buffer required is always greater than the gradient volume.



Start buffers: avoid the use of polyvalent buffers. Use monovalent or amphoteric buffers with a net charge > 0 and ≤ +1and ionic strength of 25 mM. Set the pH of the start buffer to 0.4 pH units above the desired pH in order to compensate for fluctuations in pH at the start of the run. These fluctuations are caused by slight differences in the conductivity of start and elution buffers.



Avoid buffers at concentrations below 25 mM as they will require larger gradient volumes with longer elution times and give broader, less concentrated protein peaks. More concentrated buffers would give steeper gradients, but could result in loss of resolution as peaks would be eluted closer together.



Gradients ending at pH 9 are not recommended when using Mono P since this medium has low buffering capacity above pH 9.0.

Gradients for proteins of known pI

Choose a pH interval so that the protein of interest is eluted after running 30–50% of the pH gradient. Narrow pH intervals will give optimal resolution.

Gradients for proteins of unknown pI

Since most proteins have isoelectric points in the range of 7–4, start with pH interval 7–4 on PBE 94 or Mono P. Proteins with a pI above 7 will pass through the column and can be collected and re-run at a higher pH if desired. Once a suitable pH range is established, select the most suitable pH gradient and compatible medium to achieve the optimal resolution.



Save time by using the shorter Mono P 5/50 GL column to test for a suitable pH gradient before transferring to Mono P 5/200 GL to achieve the highest resolution. Routine separations on Mono P 5/50 GL may also be satisfactory if highest resolution is not essential.

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Table 10. Recommended buffer systems for broad pH intervals using Mono P 5/50 GL or Mono P 5/200 GL.

			Approx. volumes (ml)			
			Mono P 5/200 GL		Mono P 5/50 GL	
pH	Start buffer	Elution buffer (100 ml)	Total volume	Pre-gradient volume	Total volume	Pre-gradient volume
9–7	0.025 M diethanolamine, pH 9.5, HCl	1.0 ml Pharmalyte 8–10.5, 5.2 ml Polybuffer 96, pH 7.0, HCl	34	7	11	2
9–6	0.025 M diethanolamine, pH 9.5, HCl	10 ml Polybuffer 96, pH 6.0, HCl	34	9	19	2
9–6	0.075 M Tris, pH 9.3, CH_3COOH	10 ml Polybuffer 96, pH 6.0, CH_3COOH	30	3	17	2
8–6	0.025 M triethanolamine, pH 8.3, CH_3COOH	0.21 ml Pharmalyte 8–10.5, 9.0 ml Polybuffer 96, pH 6.0, CH_3COOH	37	7	15	4
8–5	0.025 M triethanolamine, pH 8.3, iminodiacetic acid*	3.0 ml Polybuffer 96, 7.0 ml Polybuffer 74, pH 5.0, iminodiacetic acid*	47	6	15	3
7–5	0.025 M bis-Tris, pH 7.1, HCl	10 ml Polybuffer 74, pH 5.0, HCl	26	3	13	3
7–4	0.025 M bis-Tris, pH 7.1, iminodiacetic acid*	10 ml Polybuffer 74, pH 4.0, iminodiacetic acid*	46	3	19	3
6–4	0.025 M bis-Tris, pH 6.3, HCl	10 ml Polybuffer 74, pH 4.0, HCl	39	3	16	3

Use a saturated solution of iminodiacetic acid to adjust the pH of the buffer.

Table 11. Recommended buffer systems for narrow pH intervals using Mono P 5/50 GL and Mono P 5/200 GL.

			Approx. volumes (ml)			
			Mono P 5/200 GL		Mono P 5/50 GL	
pH	Start buffer	Elution buffer (100 ml)	Total volume	Pre-gradient volume	Total volume	Pre-gradient volume
9–8	0.025 M diethanolamine, pH 9.4, HCl	1.0 ml Pharmalyte 8–10.5, 5.2 ml Polybuffer 96, pH 8.0, HCl	28	3	10	3
8.5–7.5	0.025 M Tris, pH 8.8, CH_3COOH	0.11 ml Pharmalyte 8–10.5, 9.5 ml Polybuffer 96, pH 7.5, CH_3COOH	29	4	10	4
8–7	0.025 M triethanolamine, pH 8.3, HCl	10 ml Polybuffer 96, pH 7.0, HCl	29	5	10	4
7.5–6.5	0.025 M methylimidazole, pH 7.6, CH_3COOH	10 ml Polybuffer 96, pH 6.5, CH_3COOH	27	9	11	6
7–6	0.025 M bis-Tris, pH 7.0, CH_3COOH	9.5 ml Polybuffer 96, 0.5 ml Polybuffer 74, pH 6.0, CH_3COOH	28	10	12	5
6.5–5.5	0.025 M bis-Tris, pH 6.7, CH_3COOH	4.0 ml Polybuffer 96, 6.0 ml Polybuffer 74, pH 5.5, CH_3COOH	23	5	9	3
6–5	0.025 M bis-Tris, pH 6.4, HCl	10 ml Polybuffer 74, pH 5.0, HCl	25	3	10	3
5.5–4.5	0.025 M piperazine, pH 6.3, HCl or iminodiacetic acid*	10 ml Polybuffer 74, pH 4.5, HCl or iminodiacetic acid*	24	3	10	3
5–4	0.025 M N-methylpiperazine, pH 5.7, HCl or iminodiacetic acid*	10 ml Polybuffer 74, pH 4.0, HCl or iminodiacetic acid*	27	7	11	3

Use a saturated solution of iminodiacetic acid to adjust the pH of the buffer.

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Table 12. Recommended buffer systems for gradients with PBE 118.

pH*	Start buffer	Elution buffer	Dilution factor	Approx. volume (in column volumes)	
				Total volume	Pre-gradient volume
10.5–9	0.025 M triethylamine, pH 11, HCl	Pharmalyte 8–10.5, pH 9	1:45	-	-
10.5–8	0.025 M triethylamine, pH 11, HCl	Pharmalyte 8–10.5, pH 8, HCl	1:45	13	1.5
10.5–7	0.025 M triethylamine, pH 11, HCl	Pharmalyte 8–10.5, pH 7, HCl	1:45	13.5	2

Table 13. Recommended buffer systems for gradients with PBE 94.

pH	Start buffer	Elution buffer	Dilution factor	Approx. volume (in column volumes)	
				Total volume	Pre-gradient volume
9–8	0.025 M ethanolamine, pH 9.4, HCl	Pharmalyte 8–10.5, pH 8.0, HCl	1:45	12	1.5
9–7	0.025 M ethanolamine, pH 9.4, HCl	Polybuffer 96, pH 7.0, HCl	1:10	14	2
9–6	0.025 M ethanolamine, pH 9.4, CH_3COOH	Polybuffer 96, pH 6.0, CH_3COOH	1:10	12	1.5
8–7	0.025 M Tris, pH 8.3, HCl	Polybuffer 96, pH 7.0, HCl	1:13	10.5	1.5
8–6	0.025 M Tris, pH 8.3, CH_3COOH	Polybuffer 96, pH 6.0, CH_3COOH	1:13	12	3
8–5	0.025 M Tris, pH 8.3, CH_3COOH	Polybuffer 74 (70%) + Polybuffer 96 (30%) pH 5.0, CH_3COOH	1:10	10.5	2
7–6	0.025 M imidazole, pH 7.4, CH_3COOH	Polybuffer 96, pH 6.0, CH_3COOH	1:13	10	3
7–5	0.025 M imidazole, pH 7.4, HCl	Polybuffer 74 pH 5.0, HCl	1:8	14	2.5
7–4	0.025 M imidazole, pH 7.4, HCl	Polybuffer 74, pH 4.0, HCl	1:8	14	2.5
6–5	0.025 M histidine, pH 6.2, HCl	Polybuffer 74, pH 5.0, HCl	1:10	10	2
6–4	0.025 M histidine, pH 6.2, HCl	Polybuffer 74, pH 4.0, HCl	1:8	9	2
5–4	0.025 M piperazine, pH 5.5, HCl	Polybuffer 74, pH 4.0, HCl	1:10	12	3

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Selection of counter-ions

As can be seen in the buffer tables, the most commonly used counter-ion is chloride. Other monovalent counter-ions can be used, but it is essential that these anions have a pKa at least two pH units below the lowest point of the chosen gradient.

Acetate is not recommended as a counter-ion for Polybuffer 74 because it has a high pKa. Multivalent counter-ions with a net charge below -1 are not recommended. Note that iminodiacetic acid is a multivalent ion.



Bicarbonate ions can result from the presence of atmospheric CO_2 or badly stored buffers. All high pH, amine-containing buffers adsorb atmospheric CO_2 and so generate bicarbonate ions which can disrupt a pH gradient by causing a fluctuation or plateau in the region of pH 5.5–6.5, depending on conditions.

These effects are most marked with Polybuffer 96 in pH gradients ending at pH 6, and can be minimized by using acetate as the counter-ion or setting the lower limit to pH 6.5. Adsorption of CO_2 can be minimized by storing solutions under nitrogen in tightly sealed bottles and at 3–8° C in the dark.

Buffer preparation



Use high quality water and chemicals. Filter buffers through 0.45 µm or 0.22 µm filters under vacuum to ensure that the solutions are thoroughly degassed. The presence of air bubbles in the column can significantly interfere with resolution.



Ensure that all buffer components are stored correctly to avoid adsorption of CO_2 (especially those containing amines). Use fresh Polybuffer from a previously unopened bottle whenever possible. Store previously opened solutions under nitrogen in tightly sealed bottles at +4 °C and in the dark to minimize adsorption of CO_2.



Prepare and use all buffers carefully at the same temperature to ensure correct pH and ionic strength. Use fresh buffers whenever possible. Buffers stored under refrigeration must reach the temperature at which they were prepared before running a separation.



The concentration of the start buffer is especially important when working at low ionic strength where microenvironments can vary by as much as 1 pH unit. Alterations in ionic strength may occur if readjustments to pH are made when buffers have been overtitrated with acid. Ensure that start and elution buffers are at the same low ionic strength in order to avoid large pH changes at the beginning or end of a pH gradient.

1. Select start and elution buffers from Tables 10, 11, 12 or 13 according to the pH gradient required and the medium to be used.
2. Calculate the required buffer volumes according to the column volume used. For the elution buffer, dilute Polybuffer and/or Pharmalyte in distilled water to approximately 95% of the final volume required.
3. Titrate buffers to the correct pH with the listed acid (e.g. 1–2 M or saturated iminodiacetic acid). Always perform the titration on a maximum volume before adding a few milliliters of water to reach the final volume.
4. When the final pH is reached, add distilled water to bring the total volume to 100 ml.



For shallower gradients within the same pH interval, prepare the elution buffer as normal, but dilute to a larger volume. Note that proteins elute with increased volumes when using diluted eluents so pre-gradient and total volumes also need to be increased.

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Sample preparation

Correct sample preparation is extremely important. Simple steps to clarify a sample before applying it to a column will avoid the risk of blockage, reduce the need for stringent washing procedures and extend the life of the packed medium. Appendix 1 contains an overview of sample preparation techniques.

Samples must be clear and free from particulate matter, particularly when working with Mono P. For small sample volumes a syringe-tip filter of cellulose acetate or PVDF can be sufficient for sample filtration.

If the pH of the sample is too low or different buffers are used, both gradient and resolution may be affected. Depending on the sample volume, use a HiTrap Desalting or HiPrep 26/10 Desalting column to remove high salt concentrations and/or transfer the sample into start buffer, see page 156. The pH of the sample is not critical if the buffer concentration of the sample is very low. It may be possible to simply dilute the sample if pH and final volume are not critical.

Since chromatofocusing is a binding technique, the volume of the sample is not significant as long as all of the sample can be applied and focused before the proteins of interest begin to elute. As a general rule, do not load a sample volume that is greater than half of the total column volume.

Performing a separation

Recommended flow: 0.5–1.5 ml/min, 305–450 cm/h (Mono P 5/50 GL, Mono P 5/200 GL), 30–40 cm/h (PBE 118, PBE 94).

Start and elution buffers: see Tables 10, 11, 12 or 13.

Monitor eluent at 280 nm since Polybuffers absorb at wavelengths below 280 nm. Monitor pH throughout the run as fluctuations can occur that may affect the separation, for example, due to the presence of CO_2 in the buffer.

Using a column for the first time or after long term storage:

1. Inject 0.5 column volumes 5 M NaOH
2. Equilibrate with start buffer until the buffer leaving the column is at the same pH as the start buffer.
3. Apply 0.5 column volumes 2 M salt solution containing the same anion as used in the acid for pH titration of the start buffer.
4. Re-equilibrate the column with start buffer until eluent leaving the column is at the start pH.

To check for pH fluctuations or other disturbances caused by incorrect buffer conditions or contaminants, consider performing a blank run using elution buffer (total volume as indicated in Tables 10, 11, 12 or 13).

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Using a column for repeated runs:

1. Equilibrate with start buffer until buffer leaving the column is at the same pH as the start buffer. See Table 10, 11, 12 or 13 for recommended pre-gradient volumes.
2. Adjust sample to pH of start buffer or exchange sample into start buffer using, for example, a HiTrap Desalting column, see page 156.
3. Apply elution buffer. Note that there is a pre-gradient volume that will be eluted before the pH gradient begins (see Tables 10, 11, 12 or 13 for typical pre-gradient volumes).
4. Apply sample.
5. Apply elution buffer using gradient volumes as indicated in Tables 10, 11, 12 or 13. Check for pH fluctuations or other disturbances during elution.
6. Wash with 2 column volumes 2 M NaCl to elute any material still bound to the column.
7. Re-equilibrate with 5 column volumes start buffer until UV absorbance and pH/conductivity values are stable.



To maintain a clean column, inject 0.5 column volumes of 1 M NaOH every tenth run (or more frequently if required). The injection of NaOH can be followed by 0.5 column volumes of 75% acetic acid.



If the fractions are to be analyzed by reversed phase chromatography, Polybuffer may interact with the pairing ions used during the run. If the pairing ions are non-hydrophobic Polybuffer will elute in the void volume and the retained samples can be eluted with a gradient of organic solvent. However, if the pairing ions are very hydrophobic, Polybuffer will be retained on the column and give an absorption peak at wavelengths below 280 nm during an elution.

If organic solvents must be used, note the following:

- Before running a column, check the solubility of sample and all buffers.
- The pH interval required may be altered since the pKa values of the charged groups in the buffers, Polybuffer and chromatofocusing medium will be increased.
- Perform a blank gradient to ensure maintenance of a linear pH gradient. Linearity is likely to vary more at the high and low end of a pH interval 9–4. Adjust to the highest pH in the useful buffering range of the start buffer substance and adjust the elution buffer to approximately 0.5 pH units higher than recommended in the buffer Tables 10, 11, 12 or 13.

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Optimization

- If results suggest that there may be a problem of sample solubility during the separation (see Troubleshooting) include additives such as betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) in the start and elution buffers to improve solubility. These additives should not affect the separation, but may need to be removed at a later stage thus adding an extra step to the purification strategy.
- To improve resolution:
  - –dilute Polybuffer (up to 1:20), but note that this will increase the pre-gradient and total gradient volumes so the time at which components elute will change and the volume of an eluted peak may increase
  - –decrease sample load
  - –decrease flow (flow rates as low as 0.25 ml/min have been used successfully on Mono P columns)
- To improve selectivity, use a shallower gradient (increase gradient volume), but note that this will result in broader peaks because each pH interval will occupy a larger space on the column. However, the resolution within a pH interval will increase.
- To change selectivity, use alternative buffers, salts or counter-ions.

If resolution is satisfactory, it may be possible to increase flow rate in order to speed up the separation. Flow rates up to 1.5 ml/min have been used successfully with Mono P columns without loss of resolution. Figure 86 shows the effect of increasing flow on the separation of a test mixture of proteins on PBE 118.

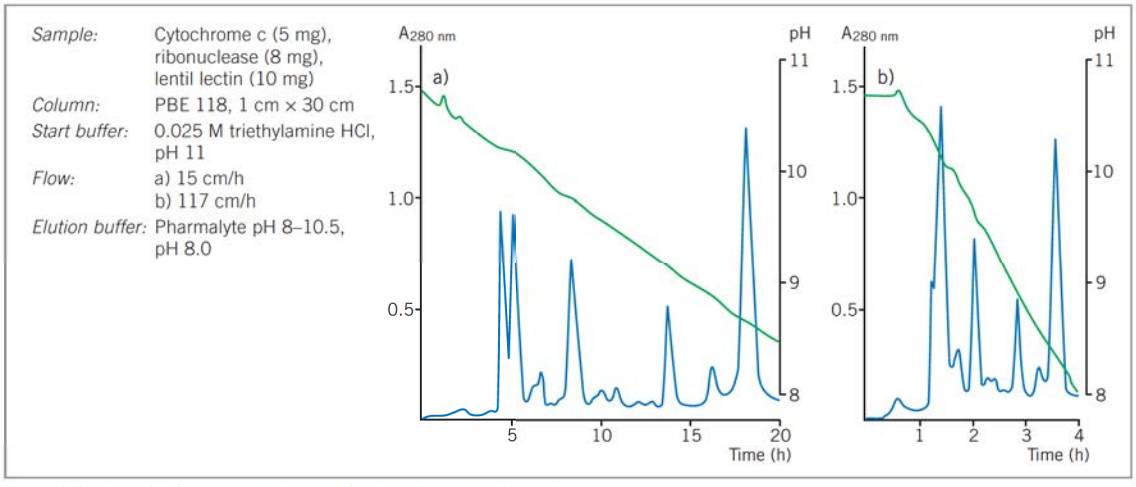


Fig. 86. Effect of flow on resolution during chromatofocusing.

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Troubleshooting

A common difficulty encountered during chromatofocusing is that some proteins precipitate when they reach high concentrations at or near their isoelectric point. On-column precipitation causes symptoms such as increased back pressure, apparent loss of sample and even blockage of the column.

To avoid precipitation it may be possible to reduce the sample load so that the proteins do not reach sufficiently high concentrations, include additives such as betaine to improve sample solubility or to alter the pH range of the separation to avoid precipitation. Alternatively, remove the proteins causing the problem by using another chromatography technique before chromatofocusing.

If the protein of interest precipitates reversibly, then it will elute later than expected. If the protein of interest precipitates irreversibly at its isoelectric point, then chromatofocusing is not a suitable technique for purification.

A second factor that can easily affect results is the linearity of the pH gradient. The presence of CO_2, especially in the start buffer, can distort the pH gradient and disrupt the separation. It is essential to monitor the pH gradient throughout the run to ensure a linear pH gradient and a satisfactory separation. Figure 87 demonstrates the significant effect that excess CO_2 can have on the formation of a pH gradient. Store buffers under nitrogen or argon (to minimize the adsorption of CO_2 which occurs with high pH solutions) and always degas before use.

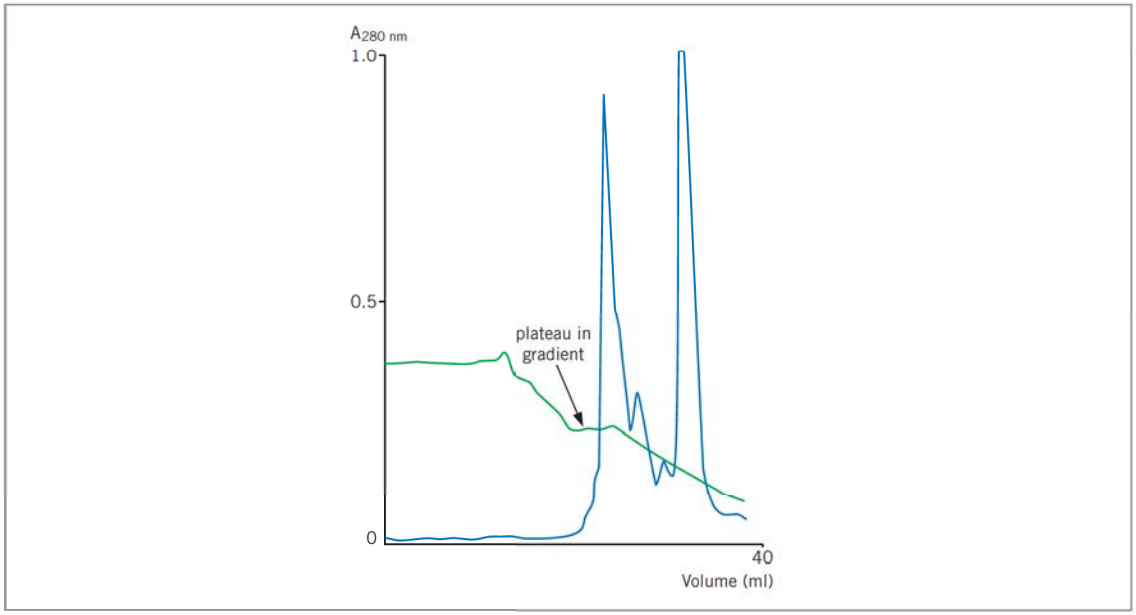


Fig. 87. A plateau in a pH gradient during chromatofocusing caused by excess CO_2 in the start buffer.

These difficulties and others that may occasionally be encountered during chromatofocusing are listed in the troubleshooting table that follows.

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Table 14. Troubleshooting.

Situation	Cause	Remedy
Reduced or no flow through the column.	Outlet closed or pumps not working.	Open outlet. Check pumps for signs of leakage (if using a peristaltic pump, check tubing also).
	Blocked filter, end-piece, adaptor or tubing.	Remove and clean or replace if possible.
	Lipoproteins or protein aggregates have precipitated.	Remove lipoproteins and aggregates during sample preparation (see Appendix 1). Follow cleaning procedures, Appendix 10.
	Proteins are precipitating at their isoelectric point.	Follow cleaning procedures, Appendix 10. Reduce sample load. Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers. Change pH range to avoid precipitation. If protein contaminants are precipitating, remove with an alternative technique before chromatofocusing.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10.
Column does not reach start pH during equilibration.	-	Inject 4 column volumes 2 M NaOH or a higher concentration of start buffer, re-equilibrate with start buffer.
Blank gradient does not reach desired pH.	Incorrect eluent composition.	Check eluent pH. Prepare fresh eluent.
	Eluent contains CO_2.	Degas all buffers, use fresh Polybuffer and restart.
Peaks poorly resolved.	Gradient fluctuates, eluent contains CO_2.	Degas all buffers, use fresh Polybuffer and restart.
	Gradient fluctuates, incorrect counterion.	Use recommended counterion.
	Protein precipitation	Follow cleaning procedures, Appendix 10. during separation. Reduce sample load. Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers. Change pH range to avoid precipitation. If protein contaminants are precipitating, remove with an alternative technique before chromatofocusing.
	Sub-optimal elution conditions.	See optimization, page 143.
	Large mixing spaces in or after column.	Reduce all post-column volumes.
	Column poorly packed.	Check column efficiency (see Appendix 1). Repack if needed. Use prepacked columns.
	Lipoproteins or protein aggregates have precipitated.	Remove lipoproteins and aggregates during sample preparation (see Appendix 3). Follow cleaning procedures, Appendix 10.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10.
Protein does not bind.	Column not equilibrated sufficiently in start buffer.	Check that pH of eluent matches pH of start buffer. Prolong equilibration step if necessary.
	Start pH too low.	Increase pH of start buffer.
	Ionic strength of eluent too high.	Check conditions against recommended buffer composition.
	Ionic strength of sample too high.	Use a desalting column e.g. HiTrap Desalting to remove excess salt (see page 156). Sample dilution may be an alternative within required limits for pH and volume.

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Situation	Cause	Remedy
	Column is contaminated with lipoproteins or proteins.	Change or clean filter. Follow cleaning procedures, Appendix 10.
Proteins do not bind or elute as expected.	Sample has changed during storage.	Prepare fresh sample.
	Sample or buffer conditions are different from previous runs.	Check sample and buffer conditions.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10.
Protein elutes at a lower than expected pH.	Sample precipitating during separation.	Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers. Change pH range to avoid precipitation.
Protein of interest binds, but does not elute.	Incorrect eluent composition.	Check pH of eluent after the run.
	Precipitation during separation.	Follow cleaning procedures, Appendix 10. pH of elution buffer is too high, use lower pH range. Reduce sample load. Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers.
Low recovery of activity, but normal recovery of protein.	Protein may be unstable or inactive in the buffer.	Determine pH and salt stability of the protein.
	Enzyme separated from co-factor or similar.	Test by pooling aliquots from the fractions and repeating the assay.
Protein yield lower than expected.	Protein may have been degraded by proteases.	Add protease inhibitors to the sample and buffers to prevent proteolytic digestion. Run sample through a medium such as Benzamidine 4 Fast Flow (high sub) to remove trypsin-like serine proteases.
	Adsorption to filter during sample preparation.	Use another type of filter.
	Sample precipitation	Follow cleaning procedures, Appendix 10. during separation. Reduce sample load. Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers. Change pH range to avoid precipitation.
	Polybuffer interfering with Lowry protein assay.	Remove Polybuffer, page 148 or use a Bradford assay.
More sample is recovered than expected.	Different assay conditions used before and after the chromatographic step.	Use same assay conditions for all assays.
	Polybuffer interfering with Lowry protein assay.	Remove Polybuffer, page 148 or use a Bradford assay.
	Protein co-eluting with other substances.	Optimize conditions to improve resolution. Check buffer conditions used for assay before and after the run.
More activity is recovered than was applied to the column.	Different assay conditions used before and after the chromatography step.	Use same assay conditions for all assays.
	Removal of inhibitors during separation.	

continues on following page

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Situation	Cause	Remedy
Back pressure increases during a run or during successive runs.	Precipitation of protein in the column filter and/or at top of the bed.	Follow cleaning procedures, Appendix 10. Reduce sample load. Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers. Change pH range to avoid precipitation. If protein contaminants are precipitating, remove with an alternative technique before chromatofocusing.
	Bed compressed.	If possible, repack column or use a new column.
	Microbial growth.	Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10.
	Turbid sample.	Improve sample preparation (see Appendix 1). Improve sample solubility: add betaine (max. 10% w/v at 25 °C), taurine (max. 4% w/v at 25 °C, below pH 8.5) or glycerol (1–2%). Or for hydrophobic samples, add ethylene glycol, urea, detergents or organic solvents*.
Air in the column.	Buffers not properly degassed. Column packed or stored at cool temperature and then warmed up.	Take special care if buffers are used after storage in a fridge or cold-room. Do not allow column to warm up due to sunlight or heating system. Degas buffers thoroughly. Flush column with distilled water and wash with 20% ethanol.
Cracks in the bed.	Large air leak in column.	Check all connections for leaks. Repack column if possible (see Appendix 3).
Unexpected peaks or spikes in chromatogram.	Buffer impurities.	Clean the buffer by running it through a precolumn. Use high quality reagents.
	Air bubble trapped in UV monitor flow cell.	Use degassed buffers.
Peaks appear on blank gradients.	Incomplete elution of previous sample.	Wash the column according to recommended methods.
Distorted peaks.	Uneven temperature distribution on the column.	Remove from any heat source e.g. sunlight through a window.

**Polar organic solvents such as methanol, ethanol and acetonitrile can be used at concentrations from 0–20%, but this may cause some proteins to lose their biological activity. The presence of organic solvents may also affect the low end of a linear pH gradient.*

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Removing Polybuffer

For most practical applications it is not necessary to remove Polybuffer since the amount that elutes with any sample is extremely low. Polybuffers do not interfere with enzyme assays or amino acid analysis, but they may interfere with certain protein assays such as Lowry.

Polybuffers can be removed from protein samples using a gel filtration medium with a suitable fractionation range. Figure 88 shows separation of Polybuffer and protein using either Sephacryl™ S-100 HR or Superdex 75. In this example Sephacryl S-100 gives the best separation between the protein myoglobin and Polybuffer. It is recommended to follow any separation by monitoring the absorbance of Polybuffer (A_{215} nm) as well as the absorbance of the eluting protein (A_{280} nm, A_{254} nm or A_{215} nm) in order to optimize the running conditions and ensure effective separation. For practical and theoretical information on gel filtration chromatography, refer to the handbook *Gel Filtration–Principles and Methods* available from Amersham Biosciences.

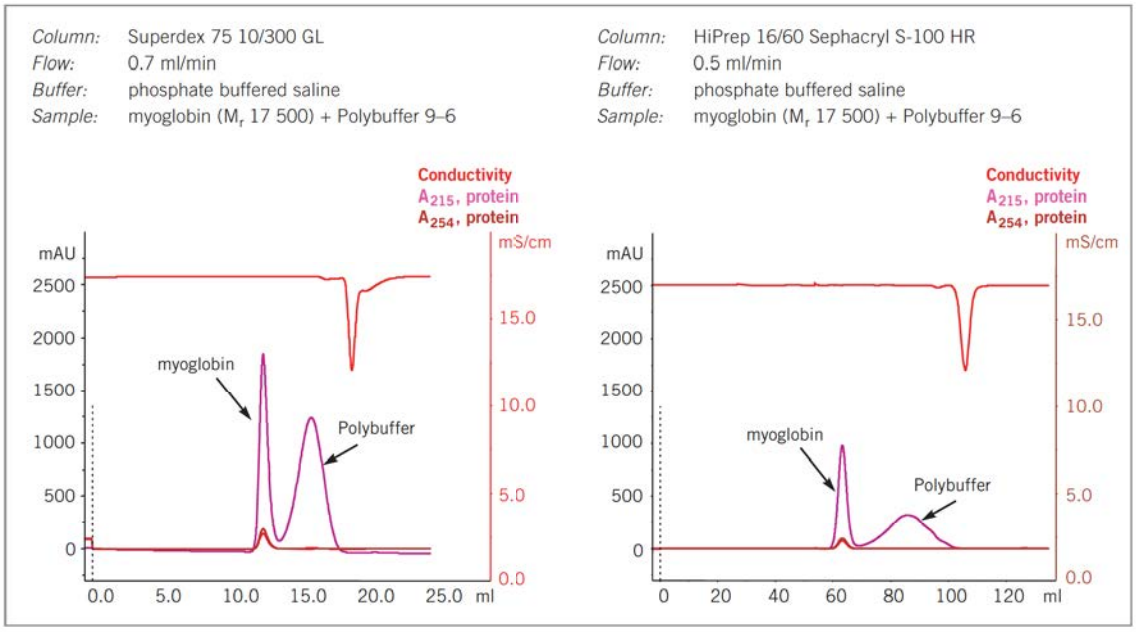


Fig. 88. Separation of Polybuffer from protein using gel filtration.

Cleaning

Mono P 5/50 GL, Mono P 5/200 GL, PBE 94, PBE 118

Since certain proteins may precipitate at or near their isoelectric point, blockage of the top filter on a chromatofocusing column is often the most common reason for an increase in back pressure. Reverse the flow direction and run through 2 column volumes of elution buffer at 0.5 ml/min (Mono P) or 30 cm/h (PBE). Return to normal flow direction, run through 5 column volumes of elution buffer.

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To remove severe contamination (often indicated by an increase in column back pressure) proceed as follows:

Reverse flow direction and run the following sequence of solutions at a flow rate of 0.25–0.50 ml/min.

1. Wash with 4 column volumes 1 M NaCl.
2. Rinse with 2 column volumes distilled water.
3. Wash with 4 column volumes 1 M NaOH.
4. Rinse with 2 column volumes distilled water.
5. Wash alternately with 0.5 column volumes 0.1 M HCl (PBE) or 1 M HCl (Mono P) and 2 column volumes distilled water until the elution profile is constant.
6. Wash with 4 column volumes 1 M NaCl.
7. Reverse flow direction and re-equilibrate column in start buffer.

If back pressure remains high, change top filter.

Save time by monitoring any cleaning procedure to check for elution of contaminants. Depending on the nature of the contaminants, the following cleaning solutions can also be used: 100% isopropanol, 20% acetonitrile, 2 M NaOH, 75% acetic acid, 20% ethanol, 100% methanol or up to 6 M guanidine hydrochloride, cationic or non-ionic detergents. Always rinse with at least 2 column volumes of distilled water after using any of these cleaning solutions. When using organic solvents, wash the column using sawtooth gradients e.g. run from 0–100% solvent in 5 column volumes then from 100–0% in 5 column volumes, including 1% trifluoroacetic acid in the water and organic solvent.

If column performance is still not restored, inject a solution of 1 mg/ml pepsin in 0.1 M acetic acid containing 0.5 M NaCl and leave overnight at room temperature or one hour at 37 °C. Depending on the contaminant, other enzymes may also be used, *e.g.* DNase. After any enzymatic treatment, repeat the steps to remove severe contamination described previously.

Media characteristics

Product	Functional group	pH stability*	Mean particle size
Mono P 5/50 GL	Tertiary and quarternary amines	Long term: 2–12 Short term: 2–14	10 µm (monosized)
Mono P 5/200 GL	Tertiary and quarternary amines	Long term: 2–12 Short term: 2–14	10 µm (monosized)
PBE 118	Tertiary and quarternary amines	Long term: 3–13 Short term: 2–14	90 µm
PBE 94	Tertiary and quarternary amines	Long term: 2–12 Short term: 1–14	90 µm

**Long term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance.*

Short term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. All ranges are estimates based on the experience and knowledge within Amersham Biosciences.

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Chemical stability

Mono P 5/50 GL, Mono P 5/200 GL

Mono P is stable in all commonly used, aqueous buffers in the range of pH 2–12, and in the presence of additives such as denaturing agents (8 M urea or 6 M guanidine hydrochloride) and non-ionic or cationic detergents.



Avoid oxidizing agents and anionic detergents.

PBE 94, PBE 118

Polybuffer exchangers are stable in all commonly used, aqueous buffers in the range of pH 3–12 and compatible with urea and other strong dissociating agents.



Avoid oxidizing agents and anionic detergents.

Storage

Mono P 5/50 GL, Mono P 5/200 GL, PBE 94, PBE pH 8–10.5

If the column is to be stored for more than two days after use, wash with 5 column volumes of distilled water and 5 column volumes of 20% ethanol.

Polybuffer 96, Polybuffer 74, Pharmalyte 118

Store at 3–8 °C in the dark, preferably under nitrogen. Avoid microbial contamination. To minimize adsorption of CO_2, store solutions under nitrogen in tightly sealed bottles after use.



Never store chromatofocusing media in 1 M HCl or 1 M NaOH. Avoid solutions containing charged groups.

Chromatofocusing and CIPP

Chromatofocusing can give high resolution of quite complex mixtures, but best results will be obtained when working with samples containing few components.

Chromatofocusing is therefore best suited as a polishing step in a purification strategy (see Figure 85) when high resolution of similar components is required and the majority of contaminants have been removed. Refer to Chapter 5 for details on CIPP, a strategic approach to purification.

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Appendix 1

Sample preparation

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

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

Sample stability

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

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



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

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

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Sample clarification

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



It is highly recommended to centrifuge and filter any sample immediately before chromatographic purification.

Centrifugation

Centrifugation removes lipids and particulate matter, such as cell debris. If the sample is still not clear after centrifugation, use filter paper or a 5 µm filter as a first step and one of the filters below as a second step filter.

- For small sample volumes or proteins that adsorb to filters, centrifuge at 10 000 g for 15 minutes.
- For cell lysates, centrifuge at 40 000–50 000 g for 30 minutes.
- Serum samples can be filtered through glass wool after centrifugation to remove any remaining lipids.

Filtration

Filtration removes particulate matter. Membrane filters that give the least amount of non-specific binding of proteins are composed of cellulose acetate or PVDF.

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

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



Check the recovery of the target protein in a test run. Some proteins may adsorb non-specifically to filter surfaces.

Desalting

Desalting columns are suitable for any sample volume and will rapidly remove low molecular weight contaminants in a single step at the same time as transferring the sample into the correct buffer conditions. Centrifugation and/or filtration of the sample before desalting is still recommended. Detailed procedures for buffer exchange and desalting are given on page 156.

At laboratory scale, when samples are reasonably clean after filtration or centrifugation, the buffer exchange and desalting step can be avoided. For affinity chromatography or hydrophobic interaction chromatography, it may be sufficient to adjust the pH of the sample and, if necessary, dilute to reduce the ionic strength of the solution.



Rapidly process small or large sample volumes. Use before and/or between purification steps, if needed (remember that each extra step can reduce yield and desalting also dilutes the sample).

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Remove salts from proteins with molecular weight M_r > 5 000.

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

Specific sample preparation steps

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

Fractional precipitation

Fractional precipitation is frequently used at laboratory scale to remove gross impurities from small sample volumes, and occasionally used in small-scale commercial production. Precipitation techniques separate fractions by the principle of differential solubility. Because protein species differ in their degree of hydrophobicity, increased salt concentrations can enhance hydrophobic interactions between the proteins and cause precipitation. Fractional precipitation can be applied to remove gross impurities in three different ways, as shown in Figure 89.

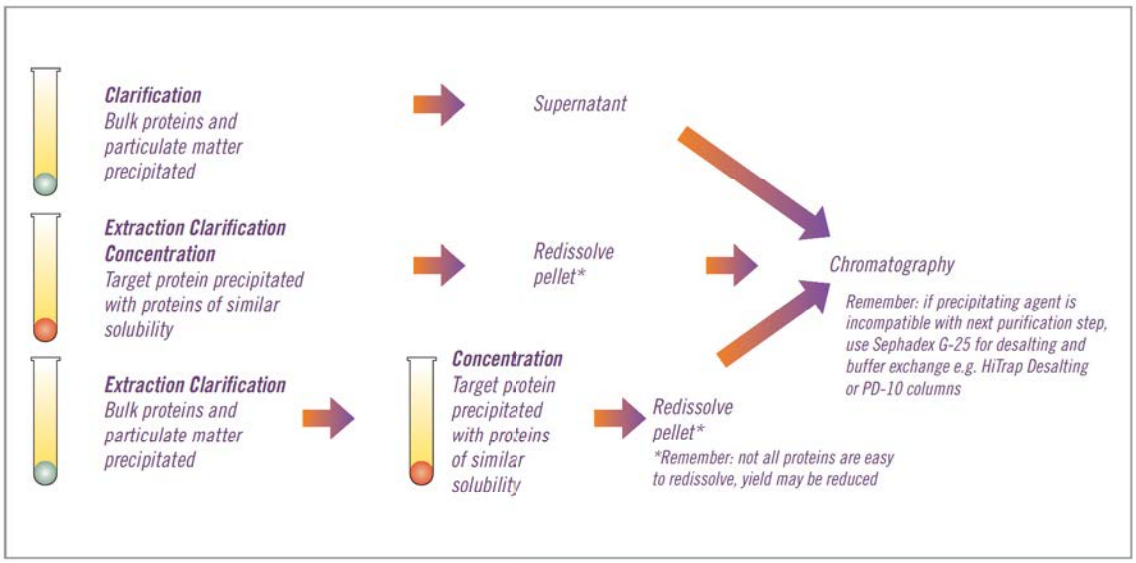


Fig. 89. Three ways to use precipitation.

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Examples of precipitation agents are reviewed in Table 15. The most common precipitation method using ammonium sulphate is described in more detail.

Table 15. Examples of precipitation techniques.

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulphate	As described below.	> 1 mg/ml proteins especially immuno-globulins.	Stabilizes proteins, no denaturation, supernatant can go directly to HIC. Helps to reduce lipid content.
Dextran sulphate	Add 0.04 ml 10% dextran sulphate and 1 ml 1 M $CaCl_2$ per ml sample, mix 15 min, centrifuge 10 000 g, discard pellet.	Samples with high levels of lipoprotein e.g ascites.	Precipitates lipoprotein.
Polyvinylpyrrolidine	Add 3% (w/v), stir 4 hours, centrifuge 17 000 g, discard pellet.	Samples with high levels of lipoprotein e.g ascites.	Alternative to dextran sulphate.
Polyethylene glycol (PEG, M_r > 4000)	Up to 20% w/vol	Plasma proteins.	No denaturation, supernatant goes directly to IEX or AC, complete removal may be difficult. Stabilizes proteins.
Acetone (cold)	Up to 80% vol/vol at ±0 °C. Collect pellet after centrifugation at full speed in an Eppendorf™ centrifuge.		May denature protein irreversibly. Useful for peptide precipitation or concentration of sample for electrophoresis.
Polyethyleneimine	0.1% w/v		Precipitates aggregated nucleoproteins.
Protamine sulphate	1% w/v		Precipitates aggregated nucleoproteins.
Streptomycin sulphate	1% w/v		Precipitation of nucleic acids.
Caprylic acid	(X/15) g where X = volume of sample.	Antibody concentration should be > 1 mg/ml.	Precipitates bulk of proteins from sera or ascites, leaving immunoglobulins in solution.

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



Ammonium sulphate precipitation

Some proteins may be damaged by ammonium sulphate. Take care when adding crystalline ammonium sulphate: high local concentrations may cause contamination of the precipitate with unwanted proteins.



For routine, reproducible purification, precipitation with ammonium sulphate should be avoided in favor of chromatography.



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

Solutions needed for precipitation:

Saturated ammonium sulphate solution (add 100 g ammonium sulphate to 100 ml distilled water, stir to dissolve).

1 M Tris-HCl, pH 8.0.

Buffer for first purification step.

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1. Filter (0.45 µm) or centrifuge the sample (10 000 g at +4 °C).
2. Add 1 part 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently. Add ammonium sulphate solution, drop by drop. Add up to 50% saturation*. Stir for 1 hour.
4. Centrifuge 20 minutes at 10 000 g.
5. Remove supernatant. Wash the pellet twice by resuspension in an equal volume of ammonium sulphate solution of the same concentration (i.e. a solution that will not redissolve the precipitated protein or cause further precipitation). Centrifuge again.
6. Dissolve pellet in a small volume of the buffer to be used for the next step.
7. Ammonium sulphate is removed during clarification/buffer exchange steps with Sephadex G-25, using desalting columns (see page 156).

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

The quantity of ammonium sulphate required to reach a given degree of saturation varies according to temperature. Table 16 shows the quantities required at +20 °C.

Table 16. Quantities of ammonium sulphate required to reach given degrees of saturation at +20 °C.

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

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Resolubilization of protein precipitates

Many proteins are easily resolubilized in a small amount of the buffer to be used in the next chromatographic step. However, a denaturing agent may be required for less soluble proteins. Specific conditions will depend upon the specific protein. These agents must always be removed to allow complete refolding of the protein and to maximize recovery of mass and activity. A chromatographic step often removes a denaturant during purification. Table 17 gives examples of common denaturing agents.

Table 17.

Denaturing agent	Typical conditions for use	Removal/comment
Urea	2 M–8 M	Remove using Sephadex G-25.
Guanidine hydrochloride	3 M–6 M	Remove using Sephadex G-25 or during IEX.
Triton X-100	2%	Remove using Sephadex G-25 or during IEX.
Sarcosyl	1.5%	Remove using Sephadex G-25 or during IEX.
N-octyl glucoside	2%	Remove using Sephadex G-25 or during IEX.
Sodium dodecyl sulphate	0.1%–0.5%	Exchange for non-ionic detergent during first chromatographic step, avoid anion exchange chromatography.
Alkaline pH	> pH 9, NaOH	May need to adjust pH during chromatography to maintain solubility.

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

Buffer exchange and desalting

Dialysis is frequently mentioned in the literature as a technique to remove salt or other small molecules and to exchange the buffer composition of a sample. However, dialysis is generally a very slow technique, requiring large volumes of buffer. During handling or as a result of proteolytic breakdown or non-specific binding to the dialysis membranes, there is a risk of losing material. A simpler and much faster technique is to use a desalting column, packed with Sephadex G-25, to perform a group separation between high and low molecular weight substances. Proteins are separated from salts and other small molecules.

In a fast, single step, the sample is desalted, transferred into a new buffer and low molecular weight materials are removed.

Desalting columns are used not only to remove low molecular weight contaminants, such as salt, but also for buffer exchange before or after different chromatographic steps and for the rapid removal of reagents to terminate a reaction.

Sample volumes up to 30% of the total volume of the desalting column can be processed. Sample concentration does not influence the separation as long as the concentration of proteins does not exceed 70 mg/ml when using normal aqueous buffers. The sample should be fully dissolved. Centrifuge or filter to remove particulate material.

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For small sample volumes it may be possible to dilute the sample with the start buffer that is to be used for chromatographic purification, but cell debris and particulate matter must still be removed.

To prevent possible ionic interactions the presence of a low salt concentration (25 mM NaCl) is recommended during desalting and in the final sample buffer.

Volatile buffers such as 100 mM ammonium acetate or 100 mM ammonium hydrogen carbonate can be used if it is necessary to avoid the presence of NaCl.

Figure 90 shows a typical buffer exchange and desalting separation. The process can be monitored by following changes in UV absorption and conductivity.

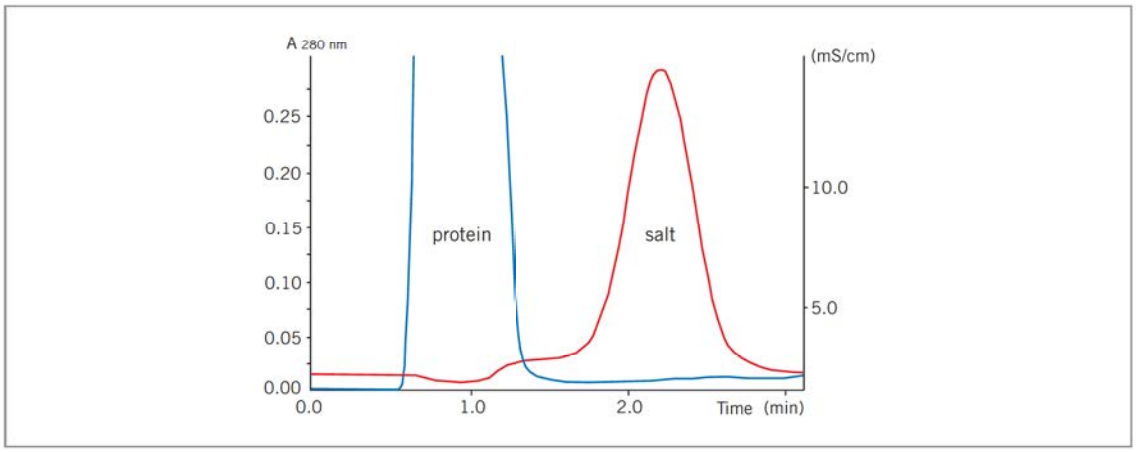


Fig. 90. Buffer exchange of mouse plasma (10 ml) on HiPrep 26/10 Desalting.

For laboratory scale operations, Table 18 shows a selection guide for prepacked, ready to use desalting and buffer exchange columns.

Table 18. Selection guide for desalting and buffer exchange.

Column	Sample volume	Sample elution volume
MicroSpin™ G-25	0.1–0.15 ml	0.1–0.15 ml
PD-10 (gravity feed column)	1.5–2.5 ml	2.5–3.5 ml
HiTrap Desalting 5 ml	0.25–1.5 ml	1.0–2.0 ml
HiPrep 26/10 Desalting	2.5–15 ml	7.5–20 ml

To desalt larger sample volumes:

- connect up to 5 HiTrap Desalting 5 ml columns in series to increase the sample volume capacity, e.g. 2 columns: sample volume 3 ml, 5 columns: sample volume 7.5 ml.
- connect up to 4 HiPrep 26/10 Desalting columns in series to increase the sample volume capacity, e.g. 2 columns: sample volume 30 ml, 4 columns: sample volume 60 ml. Even with 4 columns in series, the sample can be processed in 20 to 30 minutes, at room temperature, in aqueous buffers.

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

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Alternative 1: Manual desalting with HiTrap Desalting 5 ml using a syringe

1. Fill the syringe with buffer. Remove the stop plug. To avoid introducing air into the column, connect the column "drop to drop" to the syringe (via the adapter provided).
2. Remove the twist-off end.
3. Wash the column with 25 ml buffer at 5 ml/min to remove completely the 20% ethanol (supplied as storage buffer). If air is trapped in the column, wash with degassed buffer until the air disappears. Air bubbles introduced onto the column by accident during sample application do not influence the separation.
4. Apply the sample using a 2–5 ml syringe at a flow rate between 1–10 ml/min. Discard the liquid eluted from the column.
5. If the sample volume is less than 1.5 ml, change to buffer and proceed with the injection until a total of 1.5 ml has been eluted. Discard the eluted liquid.
6. Elute the protein with the appropriate volume selected from Table 19.

Collect the desalted protein in the volume indicated.

Note: 5 ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column. A simple peristaltic pump can also be used to apply sample and buffers.

The maximum recommended sample volume is 1.5 ml. See Table 19 for the effect of reducing the sample volume applied to the column.

Table 19. Recommended sample and elution volumes using a syringe or Multipipette™.

Sample load ml	Add buffer ml	Elute and collect ml	Yield %	Remaining salt %	Dilution factor
0.25	1.25	1.0	> 95	0.0	4.0
0.50	1.0	1.5	> 95	< 0.1	3.0
1.00	0.5	2.0	> 95	< 0.2	2.0
1.50	0	2.0	> 95	< 0.2	1.3

A simple peristaltic pump can also be used to apply sample and buffers.

Alternative 2: Simple desalting with ÄKTAprime

ÄKTAprime™ contains pre-programmed templates for individual HiTrap Desalting 5 ml and HiPrep 26/10 Desalting columns.



Buffer Preparation

Prepare at least 500 ml of the required buffer.

1. Follow the instructions supplied on the ÄKTAprime cue card to connect the column and load the system with buffer.
2. Select the Application Template.
3. Start the method.
4. Enter the sample volume and press OK.

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Figure 91 shows a typical result obtained from ÄKTAprime. The UV (protein) and conductivity (salt) traces enable pooling of the desalted fractions.

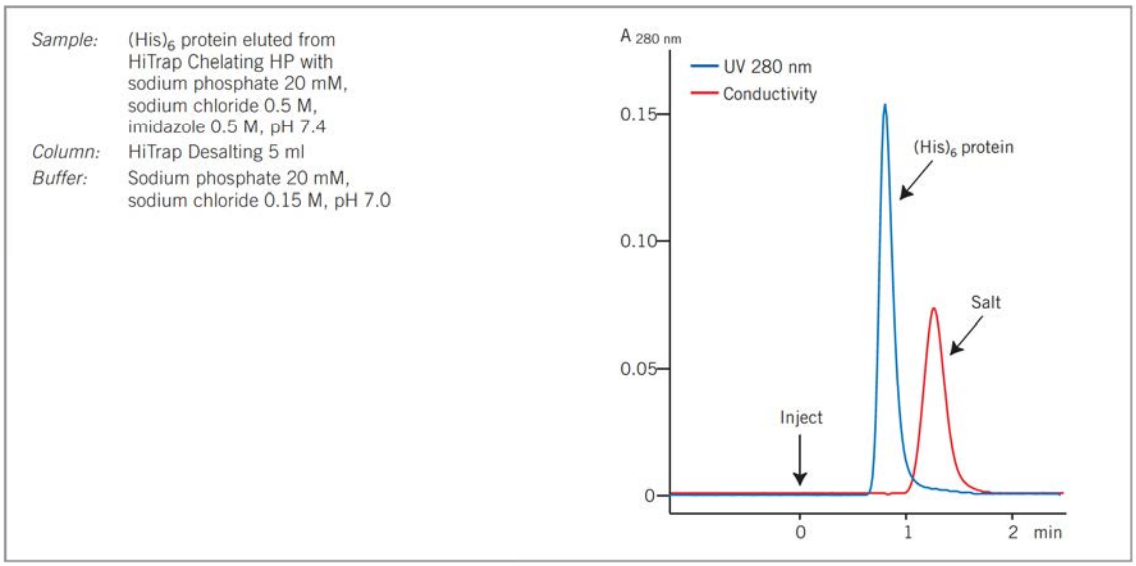


Fig. 91. Desalting of a $(His)_6$ fusion protein on ÄKTAprime.

Removal of lipoproteins

Lipoproteins and other lipid material can rapidly clog chromatography columns and it is advisable to remove them before beginning purification. Precipitation agents such as dextran sulphate and polyvinylpyrrolidine, described under Fractional precipitation, are recommended to remove high levels of lipoproteins from samples such as ascitic fluid.

Centrifuge samples to avoid the risk of non-specific binding of the target molecule to a filter.

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

Removal of phenol red

Phenol red is frequently used at laboratory scale as a pH indicator in cell culture. Although not directly interfering with purification, phenol red may bind to certain purification media and should be removed as early as possible to avoid the risk of contamination. It is known to bind to anion exchange media at pH > 7.

Use a desalting column to simultaneously remove phenol red (a low molecular weight molecule) and transfer sample to the correct buffer conditions for further purification, as described under Buffer exchange and desalting.

Removal of low molecular weight contaminants

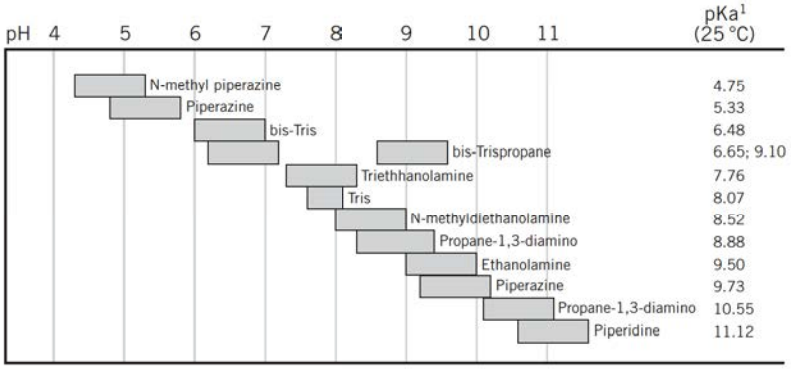
If samples contain a high level of low molecular weight contaminants, use a desalting column before the first chromatographic purification step, as described under Buffer exchange and desalting.

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Appendix 2

Non-volatile and volatile buffer systems

Non-volatile buffers for anion exchange chromatography

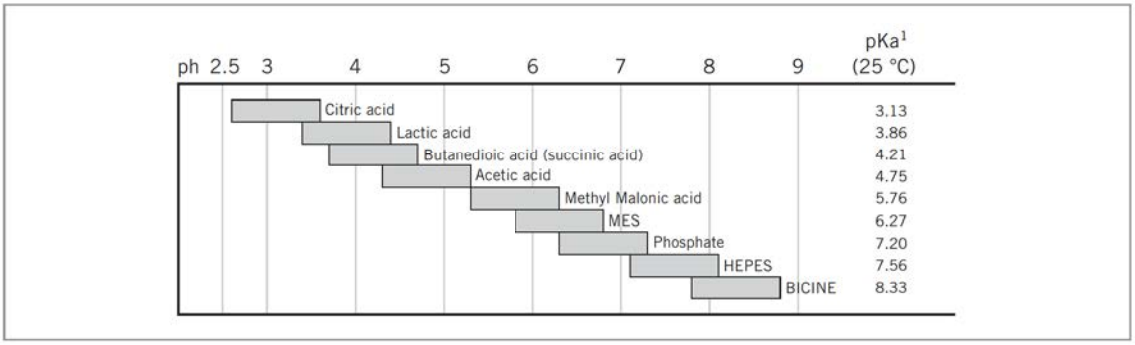


pH interval	Substance	Conc. (mM)	Counter-ion	pKa (25 °C)[1]	d(pKa)/dT (°C)
4.3–5.3	N-Methylpiperazine	20	Cl^-	4.75	-0.015
4.8–5.8	Piperazine	20	Cl^- or $HCOO^-$	5.33	-0.015
5.5–6.5	L-Histidine	20	Cl^-	6.04	
6.0–7.0	bis-Tris	20	Cl^-	6.48	-0.017
6.2–7.2; 8.6–9.6	bis-Tris propane	20	Cl^-	6.65; 9.10	
7.3–8.3	Triethanolamine	20	Cl^- or CH_3COO^-	7.76	-0.020
7.6–8.6	Tris	20	Cl^-	8.07	-0.028
8.0–9.0	N-Methyldiethanolamine	20	SO_4^{2-}	8.52	-0.028
8.0–9.0	N-Methyldiethanolamine	50	Cl^- or CH_3COO^-	8.52	-0.028
8.4–9.4	Diethanolamine	20 at pH 8.4	Cl^-	8.88	-0.025
		50 at pH 8.8			
8.4–9.4	Propane 1,3-Diamino	20	Cl^-	8.88	-0.031
9.0–10.0	Ethanolamine	20	Cl^-	9.50	-0.029
9.2–10.2	Piperazine	20	Cl^-	9.73	-0.026
10.0–11.0	Propane 1,3-Diamino	20	Cl^-	10.55	-0.026
10.6–11.6	Piperidine	20	Cl^-	11.12	-0.031

[1] *Ref: Handbook of chemistry and physics, 83rd edition, CRC, 2002–2003.*

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Non-volatile buffers for cation exchange chromatography



pH interval	Substance	Conc. (mM)	Counter-ion	pKa (25 °C)[1]	d(pKa)/dT (°C)
1.4–2.4	Maleic acid	20	Na^+	1.92	
2.6–3.6	Methyl malonic acid	20	Na^+ or Li^+	3.07	
2.6–3.6	Citric acid	20	Na^+	3.13	-0.0024
3.3–4.3	Lactic acid	50	Na^+	3.86	
3.3–4.3	Formic acid	50	Na^+ or Li^+	3.75	+0.0002
3.7–4.7; 5.1–6.1	Succinic acid	50	Na^+	4.21; 5.64	-0.0018
4.3–5.3	Acetic acid	50	Na^+ or Li^+	4.75	+0.0002
5.2–6.2	Methyl malonic acid	50	Na^+ or Li^+	5.76	
5.6–6.6	MES	50	Na^+ or Li^+	6.27	-0.0110
6.7–7.7	Phosphate	50	Na^+	7.20	-0.0028
7.0–8.0	HEPES	50	Na^+ or Li^+	7.56	-0.0140
7.8–8.8	BICINE	50	Na^+	8.33	-0.0180

[1] *Ref: Handbook of chemistry and physics, 83rd edition, CRC, 2002–2003.*

Volatile buffer systems

pH range	Buffer system	Counter-ion	pKa-values for buffering ions[1]
3.3–4.3	Formic acid	H^+	3.75
3.3–4.3; 4.8–5.8	Pyridine/formic acid	$HCOO^-$	3.75; 5.25
3.3–4.3; 9.3–10.3	Trimethylamine/formic acid	$HCOO^-$	4.75; 9.81
4.3–5.8	Pyridine/acetic acid	CH_3COO^-	4.75; 5.25
4.3–5.3; 9.3–10.3	Trimethylamine/acetic acid	CH_3COO^-	4.75; 9.81
3.3–4.3; 8.8–9.8	Ammonia/formic acid	$HCOO^-$	3.75; 9.25
4.3–5.3; 8.8–9.8	Ammonia/acetic acid	CH_3COO^-	4.75; 9.25
5.9–6.9; 9.3–10.3	Trimethylamine/carbonate	CO_3^{2-}	6.35; 9.81
5.9–6.9; 8.8–9.8	Ammonium bicarbonate	HCO_3^-	6.35; 9.25
5.9–6.9; 8.8–9.8	Ammonium carbonate/ammonia	CO_3^{2-}	6.35; 9.25
5.9–6.9; 8.8–9.8	Ammonium carbonate	CO_3^{2-}	6.35; 9.25
4.3–5.3: 7.2–8.2	N-ethylmorpholine/acetate	$HCOO^-$	4.75; 7.72

[1] *Ref: Handbook of chemistry and physics, 83rd edition, CRC, 2002–2003.*

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Appendix 3

Column packing and preparation

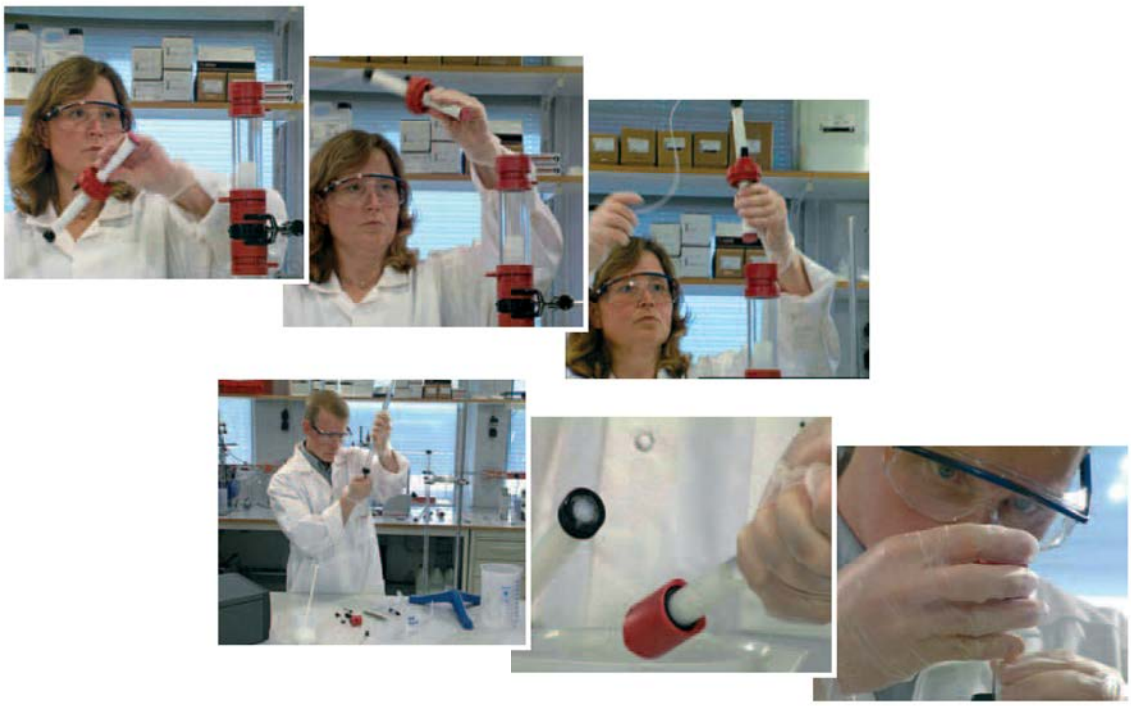
Prepacked columns from Amersham Biosciences will ensure reproducible results and the highest performance.

Use small prepacked columns for media scouting and method optimization, to increase efficiency in method development e.g. HiTrap IEX Selection Kit.

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

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

IEX media can be packed in either Tricorn or XK columns available from Amersham Biosciences. A step-by-step demonstration of column packing can be seen in "The Movie", available in CD format (see Ordering Information).



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

Note that IEX media from Amersham Biosciences are supplied ready to use. Decanting of fines that could clog the column is unnecessary.



Avoid using magnetic stirrers since they may damage the matrix.

4. Estimate the amount of slurry (resuspended medium) required on the basis of the recommendations supplied.
5. Pour the required volume of slurry into the column. Pouring down a glass rod held against the wall of the column will minimize the introduction of air bubbles.
6. Immediately fill the column with buffer.
7. Mount the column top piece and connect to a pump.
8. Open the column outlet and set the pump to the desired flow rate.



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



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



Do not exceed the maximum operating pressure of the medium or column.

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



Do not exceed 75% of the packing flow rate during any purification.

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



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



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

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Column selection

Tricorn and XK columns are fully compatible with the high flow rates achievable with modern media and a broad range of column dimensions are available. Columns most suitable for packing IEX media are listed under the column packing section for each IEX medium (Chapter 3). In most cases the capacity of the IEX medium and the amount of sample to be purified will determine the column size required. For a complete listing refer to the Amersham Biosciences BioDirectory™ or web catalog (*www.chromatography.amershambiosciences.com*) or visit *www.tricorncolumns.com* for more details on Tricorn columns.

Column packing and efficiency

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

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

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

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

H is calculated from the expression:

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

L = height of packed bed.



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

Column performance should be checked at regular intervals by injecting acetone to determine column efficiency (N) and peak symmetry (assymetry factor, A_s). Since the observed value for N depends on experimental factors such as flow rate and sample loading, comparisons must be made under identical conditions. In IEX, efficiency is measured under isocratic conditions by injecting acetone (which does not interact with the medium) and measuring the eluted peak as shown in Figure 92.

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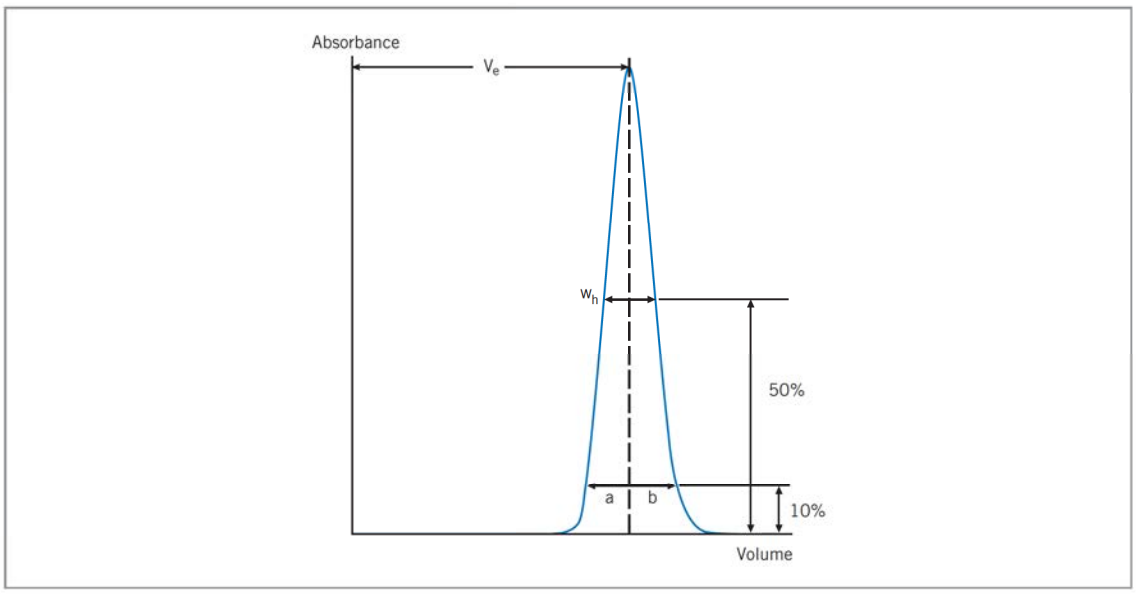


Fig. 92.

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

The symmetry factor (As) is expressed as:

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

where

a = 1st half peak width at 10% of peak height

b = 2nd half peak width at 10% of peak height

A_s should be as close as possible to 1. A reasonable A_s value for a short column as used in IEX is 0.80–1.80.

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

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

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Appendix 4

Selection of purification equipment

Simple IEX chromatography, such as elution by a step-gradient, can be performed using a syringe or peristaltic pump with prepacked HiTrap columns. A chromatography system is required in order to achieve a high resolution separation using accurately controlled linear gradient elution, to take advantage of the high flow rates of modern media, or when the same column is to be used for many runs.

Way of working	Standard ÄKTAdesign configurations					
	Pilot	Explorer 100	Purifier 10	FPLC	Prime	Syringe or peristaltic pump + HiTrap column
Simple, step-gradient elution	✓	✓	✓	✓	✓	✓
Reproducible performance for routine separation	✓	✓	✓	✓	✓	
Optimization of one step separation to increase purity	✓	✓	✓	✓	✓	
System control and data handling for regulatory requirements, e.g. GLP	✓	✓	✓	✓		
Automatic method development and optimization	✓	✓	✓	✓		
Automatic buffer preparation	✓	✓	✓			
Automatic pH scouting	✓	✓	✓			
Automatic media or column scouting	✓	✓				
Automatic multi-step purification	✓	✓				
Scale up, process development and transfer to production	✓	✓				
Proven sanitary design for cGMP with easily exchanged wetted parts	✓					



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Appendix 5

Converting from linear flow (cm/hour) to volumetric flow rates (ml/min) and *vice versa*

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

From linear flow (cm/hour) to volumetric flow rate (ml/min)

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

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

where

Y = linear flow in cm/h

d = column inner diameter in cm

Example:

What is the volumetric flow rate in an XK 16/70 column (i.d. 1.6 cm) when the linear flow is 150 cm/hour?

Y = linear flow = 150 cm/h

d = inner diameter of the column = 1.6 cm

$$\text{Volumetric flow rate} = \frac{150 \times \pi \times 1.6 \times 1.6}{60 \times 4} \text{ ml/min}$$

$$= 5.03 \text{ ml/min}$$

From volumetric flow rate (ml/min) to linear flow (cm/hour)

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

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

where

Z = volumetric flow rate in ml/min

d = column inner diameter in cm

Example:

What is the linear flow in an HR 5/5 column (i.d. 0.5 cm) when the volumetric flow rate is 1 ml/min?

Z = Volumetric flow rate = 1 ml/min

d = column inner diameter = 0.5 cm

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

$$= 305.6 \text{ cm/h}$$

From ml/min to using a syringe

1 ml/min = approximately 30 drops/min on a HiTrap 1 ml column

5 ml/min = approximately 120 drops/min on a HiTrap 5 ml column

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Appendix 6

Conversion data: proteins, column pressures

Mass (g/mol)	1 µg	1 nmol
10 000	100 pmol; 6 x 10^{13} molecules	10 µg
50 000	20 pmol; 1.2 x 10^{13} molecules	50 µg
100 000	10 pmol; 6.0 x 10^{12} molecules	100 µg
150 000	6.7 pmol; 4.0 x 10^{12} molecules	150 µg

Protein	A_{280} for 1 mg/ml
IgG	1.35
IgM	1.20
IgA	1.30
Protein A	0.17
Avidin	1.50
Streptavidin	3.40
Bovine Serum Albumin	0.70

1 kb of DNA = 333 amino acids of coding capacity
= 37 000 g/mol
270 bp DNA = 10 000 g/mol
1.35 kb DNA = 50 000 g/mol
2.70 kb DNA = 100 000 g/mol
Average molecular weight of an amino acid = 120 g/mol.

Column pressures

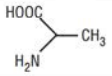
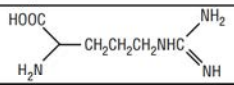
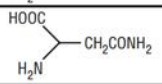
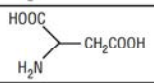
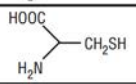
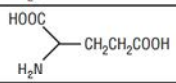
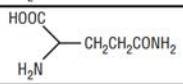
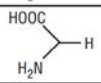
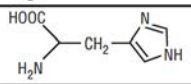
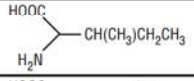
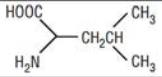
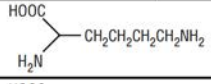
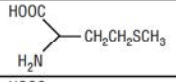
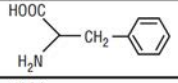
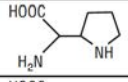
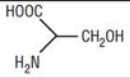
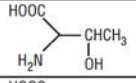
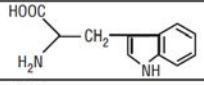
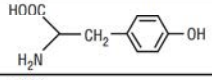
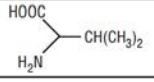
The maximum operating back pressure refers to the pressure above which the column contents may begin to compress.

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

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Appendix 7

Table of amino acids

Amino acid	Three-letter code	Single-letter code	Structure
Alanine	Ala	A	
Arginine	Arg	R	
Asparagine	Asn	N	
Aspartic Acid	Asp	D	
Cysteine	Cys	C	
Glutamic Acid	Glu	E	
Glutamine	Gln	Q	
Glycine	Gly	G	
Histidine	His	H	
Isoleucine	Ile	I	
Leucine	Leu	L	
Lysine	Lys	K	
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	
Serine	Ser	S	
Threonine	Thr	T	
Tryptophan	Trp	W	
Tyrosine	Tyr	Y	
Valine	Val	V	

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Formula	M_r	Middle unit residue (-H_2O) Formula	M_r	Charge at pH 6.0–7.0	Hydrophobic (non-polar)	Uncharged (polar)	Hydrophilic (polar)
$C_3H_7NO_2$	89.1	C_3H_5NO	71.1	Neutral	■		
$C_6H_{14}N_4O_2$	174.2	$C_6H_{12}N_4O$	156.2	Basic (+ve)			■
$C_4H_8N_2O_3$	132.1	$C_4H_6N_2O_2$	114.1	Neutral		■	
$C_4H_7NO_4$	133.1	$C_4H_5NO_3$	115.1	Acidic(-ve)			■
$C_3H_7NO_2S$	121.2	C_3H_5NOS	103.2	Neutral		■	
$C_5H_9NO_4$	147.1	$C_5H_7NO_3$	129.1	Acidic (-ve)			■
$C_5H_{10}N_2O_3$	146.1	$C_5H_8N_2O_2$	128.1	Neutral		■	
$C_2H_5NO_2$	75.1	C_2H_3NO	57.1	Neutral		■	
$C_6H_9N_3O_2$	155.2	$C6H_7N_3O$	137.2	Basic (+ve)			■
$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	■		
$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	■		
$C_6H_{14}N_2O_2$	146.2	$C_6H_{12}N_2O$	128.2	Basic(+ve)			■
$C_5H_{11}NO_2S$	149.2	C_5H_9NOS	131.2	Neutral	■		
$C_9H_{11}NO_2$	165.2	C_9H_9NO	147.2	Neutral	■		
$C_5H_9NO_2$	115.1	C_5H_7NO	97.1	Neutral	■		
$C_3H_7NO_3$	105.1	$C_3H_5NO_2$	87.1	Neutral		■	
$C_4H_9NO_3$	119.1	$C_4H_7NO_2$	101.1	Neutral		■	
$C_{11}H_{12}N_2O_2$	204.2	$C_{11}H_{10}N_2O$	186.2	Neutral	■		
$C_9H_{11}NO_3$	181.2	$C_9H_9NO_2$	163.2	Neutral		■	
$C_5H_{11}NO_2$	117.1	C_5H_9NO	99.1	Neutral	■		

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Appendix 8

Analytical assays during purification

Analytical assays are essential to follow the progress of purification. They are used to assess the effectiveness of each step in terms of yield, biological activity, recovery and to help during optimization of experimental conditions. The importance of a reliable assay for the target molecule cannot be over-emphasized.

When testing chromatographic fractions, ensure that the buffers used for purification do not interfere with the assay.

Total protein determination

Lowry or Bradford assays are used most frequently to determine the total protein content. The Bradford assay is particularly suited to samples where there is a high lipid content that may interfere with the Lowry assay.

Purity determination

Purity is most often estimated by SDS-PAGE. Alternatively, isoelectric focusing, capillary electrophoresis, reversed phase chromatography or mass spectrometry may be used.

SDS-PAGE Analysis

Reagents Required

6X SDS loading buffer: 0.35 M Tris-HCl (pH 6.8), 10.28% (w/v) SDS, 36% (v/v) glycerol, 0.6 M dithiothreitol (or 5% 2-mercaptoethanol), 0.012% (w/v) bromophenol blue. Store in 0.5 ml aliquots at -80 °C.

1. Add 2 µl of 6X SDS loading buffer to 5–10 µl of supernatant from crude extracts, cell lysates or purified fractions as appropriate.
2. Vortex briefly and heat for 5 minutes at +90 to +100 °C.
3. Load the samples onto an SDS-polyacrylamide gel.
4. Run the gel and stain with Coomassie Blue (Coomassie Blue R Tablets) or silver (PlusOne™ Silver Staining Kit, Protein).

The percentage of acrylamide in the SDS-gel should be selected according to the expected molecular weight of the protein of interest (see Table 20).

Table 20.

% Acrylamide in resolving gel		Separation size range
Single percentage:	5%	36 000–200 000
	7.5%	24 000–200 000
	10%	14 000–200 000
	12.5%	14 000–100 000
	15%	14 000–60 000*
Gradient:	5–15%	14 000–200 000
	5–20%	10 000–200 000
	10–20%	10 000–150 000

* The larger proteins fail to move significantly into the gel.

For information and advice on electrophoresis techniques, please refer to the section Additional reading and reference material.

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Functional assays

Immunospecific interactions have enabled the development of many alternative assay systems for the assessment of active concentration of target molecules.

- Western blot analysis is used when the sensitivity of SDS-PAGE with Coomassie Blue or silver staining is insufficient.

1. Separate the protein samples by SDS-PAGE.
2. Transfer the separated proteins from the gel to an appropriate membrane, such as Hybond™ ECL™ (for subsequent ECL detection) or Hybond P (for subsequent ECL Plus™ detection).
3. Develop the membrane with the appropriate specified reagents.

Electrophoresis and protein transfer may be accomplished using a variety of equipment and reagents. For further details, refer to the *Protein Electrophoresis Technical Manual and Hybond ECL instruction manual*, both from Amersham Biosciences and available on *www.amershambiosciences.com*

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

Detection and assay of tagged proteins

SDS-PAGE, Western blotting and ELISAs can also be applied to the detection and assay of genetically engineered molecules to which a specific tag has been attached. In some cases, an assay based on the properties associated with the tag itself can be developed, e.g. the GST Detection Module for enzymatic detection and quantification of GST tagged proteins. Further details on the detection and quantification of GST and $(His)_6$ tagged proteins are available in *The Recombinant Protein Handbook: Protein Amplification and Simple Purification* and the *GST Gene Fusion System Handbook* from Amersham Biosciences.

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Appendix 9

Storage of biological samples

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

General recommendations

- Add stabilizing agents, if essential. Stabilizing agents are often required for storage of purified proteins.
- Serum, culture supernatants and ascitic fluid should be kept frozen at -20 °C or -70 °C, in small aliquots.
- Avoid repeated freeze/thawing or freeze drying/re-dissolving that may reduce biological activity.
- Avoid conditions close to stability limits for example pH or salt concentrations, reducing or chelating agents.
- Keep refrigerated at +4 °C in a closed vessel to minimize bacterial growth and protease activity. Above 24 hours at +4 °C, add a preserving agent if possible (e.g. merthiolate 0.01%).

Sodium azide can interfere with many coupling methods and some biological assays and can be a health hazard. It can be removed by using a desalting column (see page 156).

General recommendations for purified proteins

- Store as a precipitate in high concentration of ammonium sulphate, for example 4.0 M.
- Freeze in 50% glycerol, especially suitable for enzymes.
- Avoid the use of preserving agents if the product is to be used for a biological assay. Preserving agents should not be added if *in vivo* experiments are to be performed. Instead store samples in small aliquots and keep frozen.
- Sterile filter to prolong storage time.
- Add stabilizing agents, e.g. glycerol (5–20%), serum albumin (10 mg/ml), ligand (concentration is selected based on concentration of active protein) to help to maintain biological activity. Remember that any additive will reduce the purity of the protein and may need to be removed at a later stage.
- Avoid repeated freeze/thawing or freeze drying/re-dissolving that may reduce biological activity.

Sodium azide can interfere with many coupling methods and some biological assays. It can be removed by using a desalting column (see page 156).

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

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Appendix 10

Column cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

Removal of common contaminants

The following procedure should be satisfactory to remove common contaminants:

1. Wash with at least 2 column volumes of 2 M NaCl (see Table 21 for recommended flow).
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 1) until pH and conductivity values have reached the required values.

Removal of precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins

To remove precipitated proteins

1. Inject 1 column volume of pepsin (1 mg/ml in 0.5 M NaCl, 0.1 M acetic acid). Leave overnight at room temperature or for one hour at 37 °C.
2. Rinse with at least 2 column volumes of distilled water (see Table 21 for recommended flow) until the UV-baseline and the eluent pH are stable.
3. Wash with at least 4 column volumes of start buffer or storage buffer, same flow as step 2, until eluent pH and conductivity have reached the required values.

Alternatively,

1. Wash with 2 column volumes of 6 M guanidine hydrochloride (see Table 21 for recommended flow).
2. Wash immediately with at least 5 column volumes of buffer at pH 7–8 (see Table 21 for recommended flow).
3. Rinse with at least 2 column volumes of distilled water (same flow as step 2) until the UV-baseline and eluent pH are stable.
4. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 2) until pH and conductivity values have reached the required values.

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Table 21. Recommended flow according to medium, column dimensions and eluent.

Column (volume) or medium	Water, buffer, 2 M NaCl or 1 M NaOH*	6 M guanidine hydrochloride	70% ethanol or 30% isopropanol
MiniBeads (0.24 ml)	0.2 ml/min	0.1 ml/min	0.1 ml/min
MiniBeads (0.8 ml)	0.2 ml/min	0.1 ml/min	0.1 ml/min
MonoBeads (1.7 ml)	0.2 ml/min	0.1 ml/min	0.1 ml/min
MonoBeads (1 ml)	0.5 ml/min	0.25 ml/min	0.25 ml/min
MonoBeads (8 ml)	2 ml/min	1 ml/min	1 ml/min
MonoBeads (20 ml)	5 ml/min	2.5 ml/min	2.5 ml/min
SOURCE 15 4.6/100 PE	0.2 ml/min	0.1 ml/min	0.1 ml/min
RESOURCE 1 ml	1 ml/min	0.5 ml/min	0.5 ml/min
RESOURCE 6 ml	6 ml/min	3 ml/min	3 ml/min
SOURCE in larger columns**	40 cm/h	20 cm/h	20 cm/h
HiTrap (1 ml)	1 ml/min	0.5 ml/min	0.5 ml/min
HiTrap (5 ml)	5 ml/min	2.5 ml/min	2.5 ml/min
HiPrep (20 ml)	5 ml/min	2.5 ml/min	2.5 ml/min
HiLoad (20 ml)	3 ml/min	2.5 ml/min	2.5 ml/min
HiLoad (53 ml)	8 ml/min	5 ml/min	5 ml/min
Sepharose High Performance in larger columns**	40 cm/h	20 cm/h	20 cm/h
Sepharose Fast Flow in larger columns**	40 cm/h	20 cm/h	40 cm/h
Sepharose XL in larger columns**	40 cm/h	20 cm/h	40 cm/h
Sepharose Big Beads**	40 cm/h	40 cm/h	40 cm/h

** If contamination is thought to be significant, use a lower flow rate to increase the contact time when using 1 M NaOH.*

*** When cleaning larger columns, allow a contact time of 1–2 hours for any solution that is used as an initial cleaning step.*

To remove lipids, hydrophobically bound proteins or lipoproteins

Organic solvents or detergents may be required to completely remove contaminants of this type.

Before using organic solvents, wash the medium with at least 4 column volumes of distilled water to avoid any salts precipitating on the column.

When applying organic solvents or solutions it may be necessary to reduce the flow rate to avoid over-pressuring the column.

Use cleaning solutions such as up to 100% isopropanol, up to 100% methanol, up to 100% acetonitrile, up to 2 M NaOH, up to 75% acetic acid, up to 100% ethanol, ionic or non-ionic detergents.

Always check for solvent compatibility in the instructions supplied with the medium or column.

Avoid anionic detergents with Q, DEAE and ANX charged groups. Avoid cationic detergents with S, SP and CM charged groups.

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Examples of cleaning procedures:

1. Wash with 4 column volumes of up to 70% ethanol or 30% isopropanol (see Table 21 for recommended flow).
2. Rinse with at least 2 column volumes of distilled water (see Table 21 for recommended flow) until the UV-baseline and eluent pH are stable.
3. Wash immediately with 3 column volumes of start buffer (same flow as step 2).

Alternatively,

1. Wash with 2 column volumes of detergent in a basic or acidic solution, e.g. 0.1–0.5% non-ionic detergent in 0.1 M acetic acid (see Table 21 for recommended flow).
2. Rinse with 5 column volumes 70% ethanol to remove residual detergent (seeTable 21 for recommended flow).
3. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
4. Wash with 3 column volumes of start buffer (same flow as step 1).

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Additional reading

	Code No.
Purification	
Antibody Purification Handbook	18-1037-46
Protein Purification Handbook	18-1132-29
Recombinant Protein Handbook: Protein Amplification and Simple Purification	18-1142-75
GST Gene Fusion System Handbook	18-1157-58
Affinity Chromatography Handbook: Principles and Methods	18-1022-29
Gel filtration Handbook: Principles and Methods	18-1022-18
Hydrophobic Interaction Chromatography Handbook: Principles and Methods	18-1020-90
Reversed Phase Chromatography Handbook: Principles and Methods	18-1112-93
Expanded Bed Adsorption Handbook: Principles and Methods	18-1124-26
Protein and Peptide Purification Technique Selection	18-1128-63
Fast Desalting and Buffer Exchange of Proteins and Peptides	18-1128-62
Gel Filtration Columns and Media Selection Guide	18-1124-19
Ion Exchange Columns and Media Selection Guide	18-1127-31
HIC Columns and Media Product Profile	18-1100-98
Affinity Columns and Media Product Profile	18-1121-86
Convenient Protein Purification, HiTrap Column Guide	18-1128-81
ÄKTAdesign Brochure	18-1158-77
ÄKTA 3D Kit Brochure	18-1160-45
GST Fusion System Brochure	18-1159-30
Protein Purifier Software	18-1155-49
Protein Purification: Principles, High Resolution Methods and Applications, J-C. Jansson and L.Rydén	18-1128-68
Column Packing Video (PAL)	17-0893-01
Column Packing Video (NTSC)	17-0894-01
Analysis	
Protein analysis–using the power of 2-D electrophoresis	18-1124-82
2D Electrophoresis Handbook	80-6429-60
Protein Electrophoresis Technical Manual	80-6013-88
ECL Western and ECL Plus Western Blotting Application Note	18-1139-13

Many of these items can be downloaded from ***www.chromatography.amershambiosciences.com***.
Other useful web links include: ***www.hitrap.com*** and ***www.tricorncolumns.com***.

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References

	Code No.
Reference lists are available from ***www.chromatography.amershambiosciences.com***	
Reference list HiTrap Desalting	18-1156-70
Reference list HiPrep 26/10 Desalting	18-1156-89
Reference list HiLoad Q Sepharose HP and HiLoad Q Sepharose FF	18-1156-98
Reference list HiLoad SP Sepharose HP and HiLoad SP Sepharose FF	18-1156-99
Reference list HiPrep 16/10 CM FF	18-1156-91
Reference list HiPrep 16/10 DEAE FF	18-1156-90
Reference list HiPrep 16/10 Q FF	18-1156-92
Reference list HiTrap Q HP and HiTrap Q FF	18-1156-82
Reference list HiTrap SP HP and HiTrap SP FF	18-1156-83
Reference list HiTrap CM FF	18-1156-84
Reference list HiTrap DEAE FF	18-1156-85
Reference list Mono Q (2000–2002)	18-1166-18
Reference list Mono S (1998–2002)	18-1166-16
Reference list MiniBeads (1998–2002)	18-1166-17

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Ordering information

Ion exchange

SOURCE, Sepharose High Performance, Sepharose Fast Flow. Sepharose XL and Sepharose Big Beads are all available as BioProcess media for large scale production. Please contact your local Amersham Biosciences representative for details.

Product	Quantity	Code No.
MiniBeads		
Mini Q PC 3.2/3*	1 × 0.24 ml	17-0686-01
Mini S PC 3.2/3*	1 × 0.24 ml	17-0687-01
Mini Q 4.6/50 PE	1 × 0.8 ml	17-5177-01
Mini S 4.6/50 PE	1 × 0.8 ml	17-5178-01
MonoBeads		
Mono Q 5/50 GL	1 × 1 ml	17-5166-01
Mono Q 10/100 GL	1 × 8 ml	17-5167-01
Mono Q 4.6/100 PE	1 × 1.7 ml	17-5179-01
Mono Q HR 16/10	1 × 20 ml	17-0506-01
Mono S 5/50 GL	1 ×1 ml	17-5168-01
Mono S 10/100 GL	1 × 8 ml	17-5169-01
Mono S 4.6/100 PE	1 × 1.7 ml	17-5180-01
Mono S HR 16/10	1 × 20 ml	17-0507-01
SOURCE		
RESOURCE Q	1 x 1 ml	17-1177-01
RESOURCE Q	1 x 6 ml	17-1179-01
SOURCE 15Q 4.6/100 PE	1 x 1.7 ml	17-5181-01
SOURCE 15Q	10 ml	17-0947-20
SOURCE 15Q	50 ml	17-0947-01
SOURCE 15Q	200 ml	17-0947-05
SOURCE 30Q	10 ml	17-1275-10
SOURCE 30Q	50 ml	17-1275-01
SOURCE 30Q	200 ml	17-1275-05
RESOURCE S	1 × 1 ml	17-1178-01
RESOURCE S	1 × 6 ml	17-1180-01
SOURCE 15S 4.6/100 PE	1 × 1.7 ml	17-5182-01
SOURCE 15S	10 ml	17-0944-10
SOURCE 15S	50 ml	17-0944-01
SOURCE 15S	200 ml	17-0944-05
SOURCE 30S	10 ml	17-1273-20
SOURCE 30S	50 ml	17-1273-01
SOURCE 30S	200 ml	17-1273-02
Sepharose High Performance		
HiTrap Q HP	5 × 1 ml	17-1153-01
HiTrap Q HP	5 × 5 ml	17-1154-01
HiLoad 16/10 Q Sepharose High Performance	1 × 20 ml	17-1064-01
HiLoad 26/10 Q Sepharose High Performance	1 × 53 ml	17-1066-01
Q Sepharose High Performance	75 ml	17-1014-01

Product	Quantity	Code No.
Sepharose High Performance (continued)		
HiTrap SP HP	5 × 1 ml	17-1151-01
HiTrap SP HP	5 × 5 ml	17-1152-01
HiLoad 16/10 SP Sepharose High Performance	1 × 20 ml	17-1137-01
HiLoad 26/10 SP Sepharose High Performance	1 × 53 ml	17-1138-01
SP Sepharose High Performance	75 ml	17-1087-01
HiTrap IEX Selection Kit**	7 × 1 ml	17-6002-33
** Q Sepharose Fast Flow, DEAE Sepharose Fast Flow, SP Sepharose Fast Flow, CM Sepharose Fast Flow, ANX Sepharose 4 Fast Flow (high sub), Q Sepharose XL and SP Sepharose XL		
Sepharose Fast Flow		
HiTrap Q FF	5 × 1 ml	17-5053-01
HiTrap Q FF	5 × 5 ml	17-5156-01
HiPrep 16/10 Q FF	1 × 20 ml	17-5190-01
Q Sepharose Fast Flow	25 ml	17-0510-10
Q Sepharose Fast Flow	300 ml	17-0510-01
HiTrap SP FF	5 × 1 ml	17-5054-01
HiTrap SP FF	5 × 5 ml	17-5157-01
HiPrep 16/10 SP FF	1 × 20 ml	17-5192-01
SP Sepharose Fast Flow	25 ml	17-0729-10
SP Sepharose Fast Flow	300 ml	17-0729-01
HiTrap DEAE FF	5 × 1 ml	17-5055-01
HiTrap DEAE FF	5 × 5 ml	17-5154-01
HiPrep 16/10 DEAE FF	1 × 20 ml	17-5090-01
DEAE Sepharose Fast Flow	25 ml	17-0709-10
DEAE Sepharose Fast Flow	500 ml	17-0709-01
HiTrap CM FF	5 × 1 ml	17-5056-01
HiTrap CM FF	5 × 5 ml	17-5155-01
HiPrep 16/10 CM FF	1 × 20 ml	17-5091-01
CM Sepharose Fast Flow	25 ml	17-0719-10
CM Sepharose Fast Flow	500 ml	17-0719-01
HiTrap ANX FF (high sub)	5 × 1 ml	17-5162-01
HiTrap ANX FF (high sub)	5 × 5 ml	17-5163-01
HiPrep 16/10 ANX FF (high sub)	1 × 20 ml	17-5191-01
ANX Sepharose 4 Fast Flow (high sub)	25 ml	17-1287-10
ANX Sepharose 4 Fast Flow (high sub)	500 ml	17-1287-01
Sepharose XL		
HiTrap Q XL	5 × 1 ml	17-5158-01
HiTrap Q XL	5 × 5 ml	17-5159-01
HiPrep 16/10 Q XL	1 × 20 ml	17-5092-01
Q Sepharose XL	300 ml	17-5072-01
Q Sepharose XL virus licensed	25 ml	17-5437-10
HiTrap SP XL	5 × 1 ml	17-5160-01
HiTrap SP XL	5 × 5 ml	17-5161-01
HiPrep 16/10 SP XL	1 × 20 ml	17-5093-01
SP Sepharose XL	300 ml	17-5073-01

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Product	Quantity	Code No.
Sepharose Big Beads		
Q Sepharose Big Beads	1 l	17-0989-03
SP Sepharose Big Beads	1 l	17-0657-03
Desalting columns		
HiTrap Desalting	5 × 5 ml	17-1408-01
HiTrap Desalting	100 × 5 ml	11-0003-29
HiPrep 26/10 Desalting	1 × 20 ml	17-5087-01
HiPrep 26/10 Desalting	4 × 20 ml	17-5087-02
PD-10 Desalting columns	30	17-0857-01
PD-10 Desalting columns	50	17-0735-01
Column Packing CD		
The Movie	1	18-1165-33
Empty Columns		
Complete information on the range of Tricorn columns is available at ***www.tricorncolumns.com***		
Tricorn 10/100 column Tricorn columns are delivered with a column tube, adaptor unit, end cap, a filter kit containing adaptor and bottom filters and O-rings, two stop plugs, adaptor lock and filter holder, and two M6 connectors for connection to FPLC systems if required.	1	18-1163-15
XK 16/20 column	1	18-8773-01
XK 26/20 column	1	18-1000-72
XK 50/20 column XK columns are delivered with one AK adaptor, TEFZEL tubing (0.8 mm i.d. for XK 16 and XK 26 columns, 1.2 mm i.d. for XK 50 columns, with M6 connectors, thermostatic jacket, support snap-on net rings, dismantling tool (XK 16 and XK 26 only), and instructions.	1	18-1000-71
Empty Disposable PD-10 Desalting columns	50/pk	17-0435-01
LabMate PD-10 Buffer Reservoir	1	18-3216-03
Accessories and spare parts		
For a complete listing refer to Amersham Biosciences Biodirectory or ***www.chromatography.amershambiosciences.com***		
Packing Connector XK 16	1	18-1153-44
Packing Connector XK 26	1	18-1153-45
Tricorn packing equipment 10/100 includes Tricorn packing connector 10–10, Tricorn 10/100 glass tube, bottom unit and stop plug.	1	18-1153-25
Tricorn packing connector 10–10* Connects extra glass column to a Tricorn 10 column to act as a packing reservoir for efficient packing.	1	18-1153-23

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Chromatofocusing

Product	Quantity	Code No.
Mono P 5/50 GL	1 × 1 ml	17-5170-01
Mono P 5/200 GL	1 × 4 ml	17-5171-01
PBE 118 Polybuffer exchanger	200 ml	17-0711-01
PBE 94 Polybuffer exchanger	200 ml	17-0712-01
Polybuffer 74	250 ml	17-0713-01
Polybuffer 96	250 ml	17-0714-01
Pharmalyte pH 8–10.5	25 ml	17-0455-01

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Mini Q, Mini S, Mono Q, Mono S, Mono P, RESOURCE, SOURCE, Sepharose, BioProcess, HiTrap, HiLoad, HiPrep, Tricorn, FPLC, MiniBeads, MonoBeads, ÄKTA, ÄKTAexplorer, ÄKTApurifier, Sephadex, Sephacel, Sephacryl, BPG, Pharmalyte, ÄKTAprime, ÄKTApilot, STREAMLINE, Hybond, ECL, ECL Plus, Superdex, PhastGel, PlusOne, PhastSystem, BioDirectory, FineLINE and Drop Design are trademarks of Amersham Biosciences Limited.

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