instructions

nProtein A Sepharose™ 4 Fast Flow

71-5028-05 Edition AA

Amersham Biosciences

1. Introduction

nProtein A Sepharose™ 4 Fast Flow is an affinity medium, designed for the purification of monoclonal and polyclonal antibodies at both laboratory and process scale. The medium is manufactured without using any animal-derived components.

The specificity of protein A is primarily for the Fc region of IgG, through which it binds, leaving the antigen combining sites free. In addition, one molecule of immobilized protein A has the capacity to bind at least two molecules of IgG.

Purified protein A is coupled to Sepharose 4 Fast Flow by the well established and proven CNBr method.

To ensure best performance and trouble-free operation, please read these instructions before using nProtein A Sepharose 4 Fast Flow.

2. Media characteristics

Amersham Biosciences Protein A is produced by fermenting a selected strain of *Staphylococcus aureus*.

The base matrix, Sepharose 4 Fast Flow, is a highly cross-linked, 4 % agarose derivative with excellent kinetics, making it ideal for process scale applications. Purified protein A is coupled to Sepharose 4 Fast Flow by the well established CNBr method resulting in a very stable medium with very low leakage of protein A. Pressure/flow rate curves are shown in Fig. 2.

The total binding capacity of human IgG is approximately 35 mg/ml drained medium. The dynamic capacity of chromatographic adsorbents is a function of the flow rate used and it increases with decreasing flow rate. In Fig. 1 the flow rate/capacity dependence is shown for four different flow rates.

nProtein A Sepharose 4 Fast Flow has high chemical stability (Table 1). This enables it to withstand rigorous cleaning and

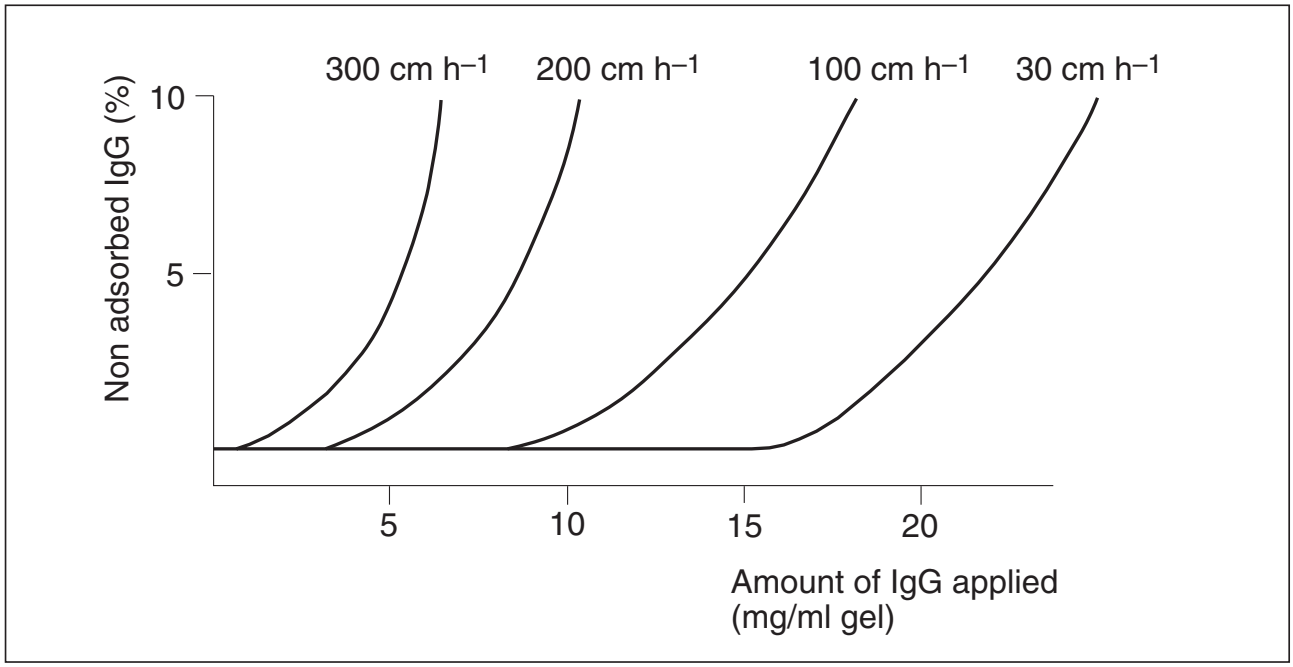

Fig. 1. One example of how the capacity for human IgG depends on the flow rate with nProtein A Sepharose 4 Fast Flow. The non-adsorbed IgG (%) was measured as a function of the amount applied to the column at 4 different flow rates, 300, 200, 100 and 30 cm/h. Concentration of the applied sample: 0.33 mg IgG/ml. Column: HR 5/5 containing 1 ml of nProtein A Sepharose 4 Fast Flow. Buffer system: 0.1 M Na_2HPO_4, pH 7.0. (Work from Amersham Biosciences AB).

sanitizing procedures, despite the relative labile nature of protein ligands. Even with strong chemical treatment, nProtein A Sepharose 4 Fast Flow performance is consistent over at least hundred process cycles.

Table 1. Characteristics of nProtein A Sepharose 4 Fast Flow

Dynamic binding capacity	min 20 mg human IgG/ml drained medium
Degree of substitution	approx. 6 mg protein A/ml drained medium
Bead form	Spherical, diameter 45 – 165 µm
Bead structure	highly cross-linked 4% agarose
Recommended pH	
working range	3-9
cleaning-in-place	2-10
Chemical stability	No significant change in chromatographic performance after 1 week storage using 8 M urea, 6 M gua-HCl, 2% benzyl alcohol or 20% ethanol
Cleaning-in-place stability	No significant chang in chromatographics performance after 100 cycles normal use at room temperature, which corresponds to a total contact time of 16.7 hours using 50 mM NaOH+1 M NaCl, or 50 mM NaOH + 1 M Na_2SO_4, or 6 M Gua-HCl
Temperature stability	4-40 °C
Recommended	
working flow velocity	30-300 cm/h

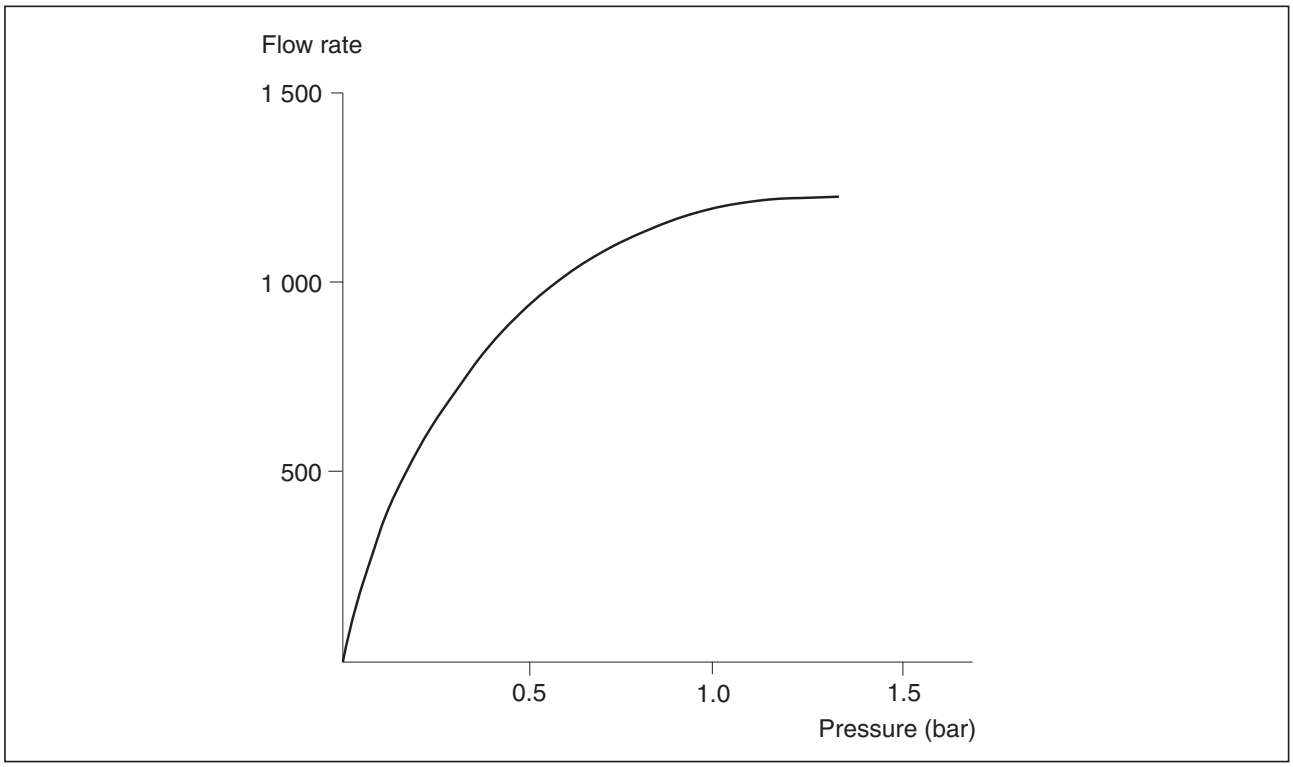

Fig. 2. Pressure /flow rate curve for nProtein A Sepharose 4 Fast Flow media (BPG™ 100; 1 bar; 5.1 cm bed height).

To maintain the same dynamic binding capacity when scaling-up the residence time must be the same as used when developing the binding conditions. Residence time is defined as:

$$\frac{\text{bed height} \quad (\text{cm})}{\text{linear flow rate} \quad (\text{cm/h})}$$

It should also be noted that individual antibodies differ in their affinity to Protein A (see section 6, Process optimization).

3. Column packing guidelines

nProtein A Sepharose 4 Fast Flow is supplied in suspension in 20% ethanol. Decant the 20% ethanol solution and replace it with starting buffer before use.

3.1 Recommended columns

Table 2. Recommended columns

Column	Inner diameter (mm)	Bed Volume	Bed Height (cm)
Lab scale:			
XK 16/40	16	8-74 ml	max. 35
XK 26/40	26	32-196 ml	max. 35
Production scale:			
BPG variable bed, glass column	100-450	2.4-131 litres	max. 83
BioProcess™ Stainless Steel (BPSS) fixed bed columns	400-1400	12-15000 litres	10-100
INdEX™ variable bed columns	70-200	Up to 24.8 litres	max. 79
CHROMAFLOW™ variable bed columns	280-2000		

3.2 General packing recommendations

Columns can be packed in different ways depending on the type of column and equipment used. Always read and follow the relevant column instruction manual carefully.

nProtein A Sepharose 4 Fast Flow is easy to pack since its rigidity allows the use of high flow rates, see Fig. 2. Four suitable types of packing methods are given:

- Pressure packing (for columns with adaptors)
- Combined pressure/suction packing (for medium sized columns with fixed bed heights)
- Suction packing (for large columns with fixed bed heights)
- Hydraulic pressure packing

How well the column is packed will have a major effect on the result of the separation. It is therefore very important to pack and test the column according to the following recommendations.

Begin the packing procedure by determining the optimal packing flow rate. Guidelines are given for determining the optimal packing flow rates for columns with adaptors and fixed bed heights.

3.3 Determining optimal packing flow rates

The optimal packing flow rate is dependent on column size and type, media volume, packing solution and temperature. The optimal packing flow rate must therefore be determined empirically for each individual system.

To determine the optimal packing flow rate, proceed as follows:

1. Calculate the exact amount of media needed for the slurry (this is especially important for columns with fixed bed heights). The quantity of media required per litre packed volume is approximately 1.15 litres sedimented media.

2. Set up the column as for packing according to the instructions in section 3.2.
3. Begin packing the media at a low flow rate (30 cm/h).
4. Increase the pressure in increments and record the flow rate when the pressure has stabilized. Do not exceed the maximum pressure of the column, or the maximum flow rate for the media.
5. The maximum flow rate is reached when the pressure/flow curve levels off or the maximum pressure of the column is reached. Stop the packing and do not exceed this flow rate. The optimal packing flow rate/pressure is 70–100% of the maximum flow rate/pressure.
6. Plot the pressure/flow rate curve as in Fig. 2 and determine the optimal packing flow rate.

The operational flow rate/pressure should be <70% of the packing flow rate/pressure.

Note: For BPSS columns, pack the column according to instructions in section 3.6.

3.4 Pressure packing

BPG columns

BPG columns are supplied with a movable adaptor. They are packed by conventional pressure packing by pumping the packing solution through the chromatographic bed at constant flow rate (or back pressure).

1. Pour some water (or packing buffer) into the column. Make sure that there is no air trapped under the bottom net. Leave about 2 cm of liquid in the column.
2. Mix the packing buffer with the medium to form a 50–70% slurry. (Sedimented bed volume/slurry volume = 0.5–0.7.)

Pour the slurry into the column. Insert the adaptor and lower it to the surface of the slurry, making sure no air is trapped under the adaptor. Secure the adaptor in place.

3. Seal the adaptor O-ring and lower the adaptor a little into the slurry, enough to fill the adaptor inlet with packing solution.
4. Connect a pump and a pressure meter and start packing at the predetermined packing flow rate (or pressure). Keep the flow rate (or pressure) constant during packing and check the pressure at the column inlet. Never exceed the pressure limit for column or medium.
5. When the medium has settled, mark the bed height on the column tube, close the bottom valve and stop the pump. The bed starts rising in the column. Loosen the O-ring and lower the adaptor to about 0.5–1.0 cm from the medium surface.
6. Seal the O-ring, start the pump and continue packing. Repeat steps 5 and 6 until there is a maximum of 1 cm between medium surface and adaptor when the medium has stabilized.
7. Close the bottom valve, stop the pump, disconnect the column inlet and push the adaptor down to approximately 3 mm below the mark on the column tube, without loosening the adaptor O-ring. The packing solution will flush the adaptor inlet. Remove any trapped air by pumping liquid from the bottom (after the inlet tubing and the bottom valve have been properly filled).

3.5 Combined pressure/suction packing

Process Stack (PS 370) Column

The Process Stack Column is supplied with fixed end-pieces and a fixed bed height of 15 cm. It is packed by a combined pressure/suction technique.

1. Fit an extra column section on top of the column tube, for use as a packing device.

2. Pour some water (or packing buffer) into the column. Make sure that there is no trapped air under the bottom net. Leave about 2 cm of liquid in the column.
3. Pour the slurry into the column. Add buffer to within 1–2 cm of the rim of the upper section. Stir gently to give a homogeneous slurry. Add buffer until level with the upper rim and secure the lid in place.
4. Connect a pump and a pressure meter and start packing at the predetermined packing flow rate (or pressure). Keep the flow rate (or pressure) constant during packing and check the pressure at the column inlet. Never exceed the pressure limit for column or medium.
5. When the medium has stabilized, the top of the bed should be exactly level with the top of the column tube. At this point, exclude the buffer tank from the system by simultaneously switching the valve at the column outlet and the valve on the suction side of the pump, as shown in Fig. 3. Packing buffer is now recirculated through the system . If, when stabilized, the packed bed is not exactly level with the top of the column, add or remove slurry. Always stir the slurry thoroughly before packing.
6. Keeping the pump running, disconnect the column inlet from the lid and direct it to waste. The packing solution in the packing section can be removed by suction through the bed.
7. While the packing section is being emptied, loosen the bolts holding the column and the packing section together so that the packing section can be removed. During this operation, manually press down on the packing section to prevent leakage between the two sections.
8. When the packing solution is within 5–8 m of the bed surface, close the valve at column outlet, stop the pump, quickly remove the packing section and replace it with the lid. Manually press down on the lid while it is secured in place.

This final operation should be completed as quickly as possible because the bed will expand when the flow stops.

9. Start pumping buffer with upward flow through the column to remove any air bubbles trapped under the lid.

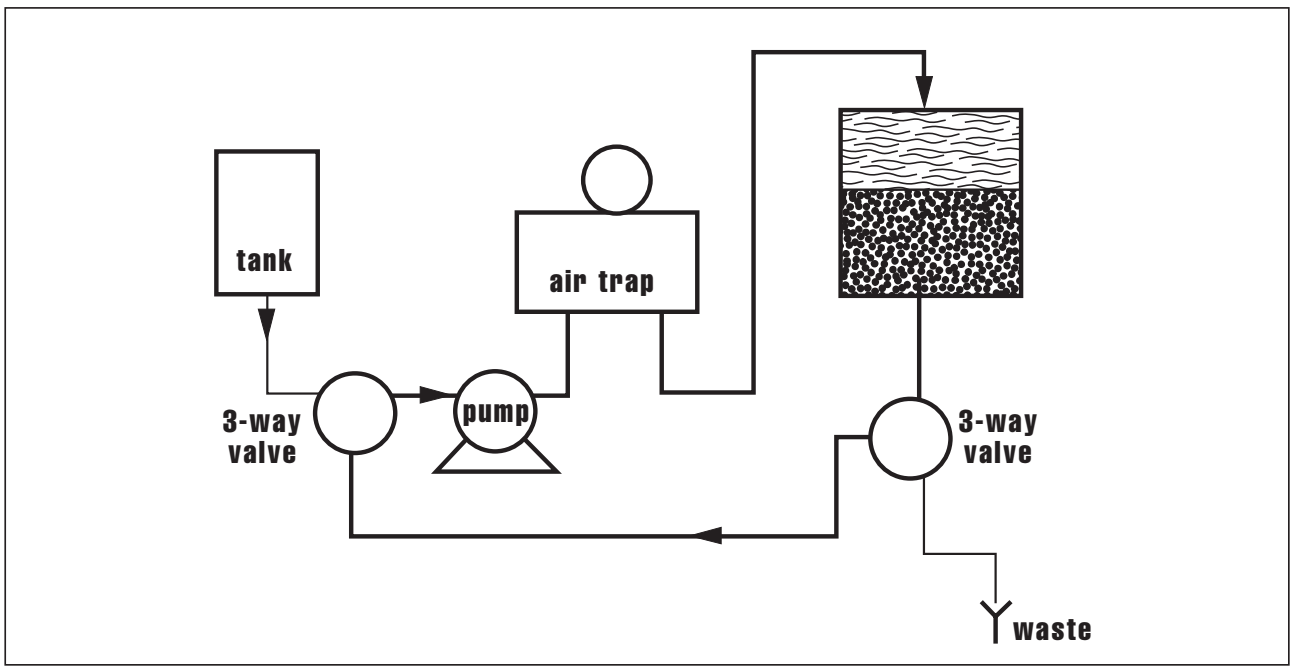

Fig. 3. Equipment set up for pressure/suction packing.

3.6 Suction packing

BioProcess Stainless Steel (BPSS) Columns

BioProcess Stainless Steel Columns are supplied with fixed end-pieces. They are packed by suction, i.e. by sucking packing solution through the chromatographic bed at a constant flow rate.

1. Fit a packing device on top of the column tube.
2. Pour some water (or packing solution) into the column. Make sure that there is no air trapped under the bottom net. Leave about 23 cm of liquid in the column.
3. Mix the packing buffer with the medium to form a 50% slurry (sedimented bed volume/slurry = 0.5). Pour the slurry into the column. Stir gently to give a homogeneous slurry.
4. Connect the column outlet valve to the suction side of a pump and start packing the bed by suction through the bed at the predetermined flow rate, see Fig. 3. Keep the flow rate constant during packing.
5. When the bed has stabilized, the top of the bed should be just below the junction of the column and the packing device.

   If, when stabilized, the level of the bed is incorrect, add or remove slurry. Always stir the slurry thoroughly before packing.
6. Just before the last of the solution has entered the packed bed (before the surface starts to dry), close the valve at the column outlet, stop the pump, quickly remove the packing device and replace it with the lid.

   This final operation should be completed as quickly as possible because the bed will expand when the flow stops.
7. Start pumping buffer with upward flow through the column to remove any air bubbles trapped under the lid.

3.7 Hydraulic packing

INdEX Columns

INdEX Columns are supplied with an hydraulic function which allows an extremely simple, rapid and reproducible packing procedure. The medium is packed at the same time as the adaptor is lowered into position at the correct pressure. The adaptor is pushed down by a constant hydraulic pressure, forcing water through the slurry and compressing it so that a packed bed is gradually built up. The hydraulic pressure can be generated using a pump and a pressure relief valve.

1. Pour some water (or packing solution) into the column. Make sure that there is no air trapped under the bottom net. Leave about 2 cm of liquid in the column.
2. Pour the slurry into the column. Fill the column with buffer solution up to the top of the glass tube and mix the slurry. Allow the medium to settle.
3. Put the adaptor in a resting position against the bevel of the glass tube. Avoid trapping air bubbles under the adaptor by slightly tilting the adaptor while mounting.
4. Lower the lid and secure it in place.
5. Connect a pump to the inlet of the hydraulic chamber, with a manometer and a pressure relief valve in-line between the pump and the hydraulic chamber. The manometer should be placed after the valve in the direction of the flow.
6. Open the hydraulic inlet (A), see Fig. 4, and the hydraulic outlet (C). Start the pump and flush the hydraulic chamber (E) free of air and any residual media.
7. Close (C) and open the elution inlet/outlet (B) to release any trapped air in the adaptor net.
8. Close (B) and open the elution inlet/outlet (D) to start the packing, applying a predefined constant hydraulic packing pressure.

9. When the bed has settled (no flow at the column outlet), stop the packing procedure by closing (A) and (D). The adaptor stops when the hydraulic force exerted downwards is balanced by the mechanical force of the bed exerted upwards.
10. To unpack the column, connect the outlet from the pump to (B) and open (C) while keeping (D) closed. This will cause the adaptor to rise from the bed surface.

Optimal hydraulic packing pressure when packing Sepharose 4 Fast Flow media in an INdEX column to a bed height of 15 cm is 1.5 bar for INdEX 100 and 0.8 bar for INdEX 200. This corresponds to approximately 12% compression of the bed during the final mechanical compression at the end of the packing. The degree of mechanical compression is critical for the flow properties of the packed bed.

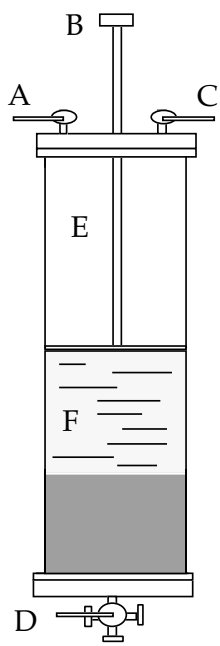

Fig. 4. Schematic representation of INdEX columns showing the hydraulic functions.

4. Evaluation of packing

To check the quality of the packing and to monitor this during the working life of the column, column efficiency should be tested directly after packing, at regular intervals afterwards, and when separation performance is seen to deteriorate.

The best method of expressing the efficiency of a packed column is in terms of the height equivalent to a theoretical plate, HETP, and the asymmetry factor, A_S. These values are easily determined by applying a sample such as 1% acetone solution to the column. Sodium chloride can also be used as a test substance. Use a concentration of 2.0 M NaCl in water with 0.5 M NaCl in water as eluent.

It is of utmost importance to realize that the calculated plate number will vary depending on the test conditions and it should therefore be used as a reference value only. It is also important that conditions and equipment are kept constant so that results are comparable. Changes in solute, solvent, eluent, sample volume, flow rate, liquid pathway, temperature, etc., will influence the results.

For optimal results, the sample volume should be at max 2.5% of the column volume and the flow rate between 15 and 30 cm/h.

If an acceptance limit is defined in relation to column performance, the column plate number can be used as part of the acceptance criteria for column use.

Method for measuring HETP and A_S

To avoid dilution of the sample, apply it as close to the column inlet as possible.

Conditions	
sample volume:	2.5% of the bed volume
sample conc.:	1.0% v/v acetone
flow rate:	15 cm/h
UV:	280 nm, 1 cm, 0.1 AU

Calculate HETP and A_s from the UV curve (or conductivity curve) as follows:

$$HETP = L/N$$

and $N = 5.54(V_e/Wh)^2$

where L = Bed height (cm)

N = number of theoretical plates

V_e = Peak elution distance

W_h = Peak width at half peak height

V_e and W_h are in the same units

To facilitate comparison of column performance the concept of reduced plate height is often used.

The reduced plate height is calculated:

$$\frac{HETP}{d}$$

where d is the diameter of the bead. As a guideline, a value of <3 is normally acceptable.

The peak should be symmetrical, and the asymmetry factor as close as possible to 1 (values between 0.8–1.5 are usually acceptable). A change in the shape of the peak is usually the first indication of bed deterioration due to use.

Peak asymmetry factor calculation:

$$A_s = b/a$$

where

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

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

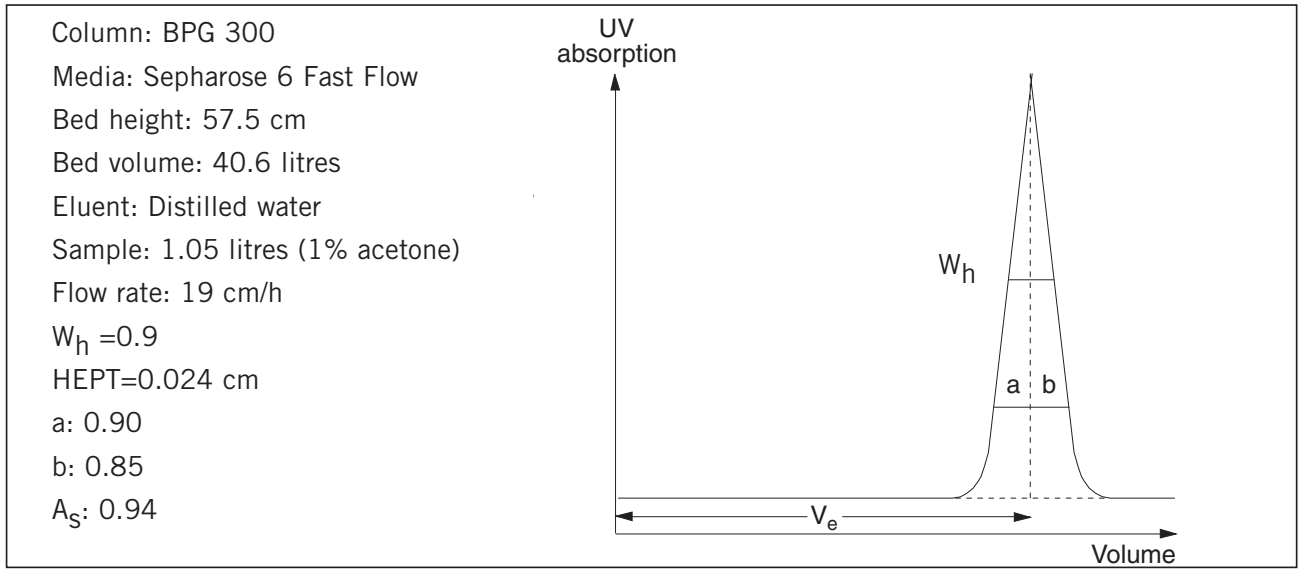

Fig. 5. UV trace for acetone in a typical test chromatogram showing the HETP and A_S value calculations.

Figure 5 shows a UV trace for acetone in a typical test chromatogram in which the HETP and A_S values are calculated.

5. Maintenance

For best performance from nProtein A Sepharose 4 Fast Flow over a long working life, follow the procedures described below.

Equilibration

After packing, and before a chromatographic run, equilibrate with working buffer by washing with at least 5 bed volumes.

Regeneration

After each separation cycle, regenerate the bed by washing with approximately 3 bed volumes of 0.1 M citrate buffer, pH 3, until the base line is stable, followed by at least 3 column volumes of binding buffer.

Cleaning-in-place

Cleaning-in-place, (CIP) is the removal of very tightly bound, precipitated or denatured substances from the purification system. If such contaminants are allowed to accumulate they may affect the chromatographic properties of the column. If the fouling is severe, it may block the column, increase back pressure and reduce flow rate. Regular CIP prevents the build up of these contaminants in the packing bed, and helps to maintain the capacity, flow properties and general performance.

CIP protocol

Precipitated or denatured substances	Wash with 2 column volumes of 6 M guanidine hydrochloride[1] 10 mM $NaOH^{2,}$ 0.1 M H_3 PO_4 or 50 mM NaOH in 1.0 M NaCl or 50 mM NaOH in 1.0 M Na_2SO_4. Wash immediately with at least 5 column volumes of sterile filtered binding buffer at pH 7–8. Reversed flow direction.
Hydrophobically bound substances	Wash the column with 2 column volumes of a non-ionic detergent[1] (e.g. conc. 0.1%). Wash immediately with at

	least 5 column volumes of sterile filtered binding buffer at pH 7–8. Reversed flow direction.
or	Wash the column with 3–4 column volumes of 70% ethanol[1] or 30% isopropanol[1]. Wash immediately with at least 5 column volumes of sterile filtered binding buffer at pH 7–8. Reversed flow direction. Apply increasing gradients to aviod air bubble formation when using high concentrations of organic solvents

[1] Apply for an approximate contact time of 10 minutes on the column.

[2] Apply for an approximate contact time of 30 minutes on the column.

[3] Apply for an approximate time of 16 minutes on the column.

6. Sanitization

Sanitization reduces microbial contamination of the bed to a minimum. Equilibrate the column with a solution consisting of 2% hibitane digluconate and 20% ethanol. Allow to stand for 6 hours, then wash with at least 5 column volumes of sterile binding buffer.

or

Equilibrate the column with a solution consisting of 0.1 M actetic acid and 20% ethanol. Allow to stand for 1 hour, then wash with at least 5 column volumes of sterile binding buffer.

or

Equilibrate the column with 70% ethanol. Allow to stand for 12 hours, then wash with at least 5 column volumes of sterile binding buffer

Note: Specific regulations may apply when using 70% ethanol since it can require the use of explosionproof areas and equipment.

7. Process optimization

nProtein A Sepharose 4 Fast Flow is designed for purification of monoclonal and polyclonal antibodies. The primary specificity of protein A is for the Fc region of IgG, but it can also bind the Fab region through secondary sites. Binding affinity for the Fc region is usually stronger, which enables Fab or F(ab)2 to be fractionated from Fc.

The degree to which protein A binds to IgG varies with respect to both origin and antibody subclass (Table 2). This is an important consideration when developing the purification protocol. To achieve efficient capture of weakly bound antibodies, it is usually necessary to alter the formulation of the binding buffer in one of the following ways:

- Increasing pH titrates opposing histidyl residues in the binding sites of protein A and IgG. This reduces electrostatic repulsion between protein A and IgG, allowing an inhibited affinity interaction.
- Increasing salt concentration reduces electrostatic repulsion, and increases hydrophobic interactions.
- Reducing temperature has been reported to improve binding.

Table 3. Affinity of protein A for selected classes of monoclonal antibodies. This Table is compiled from a variety of sources and conditions, comparisons should be approximated.

Antibody	Affinity	Binding pH	Elution pH
Human			
IgG_1	very high	6.0–7.0	3.5–4.5
IgG_2	very high	6.0–7.0	3.5–4.5
IgG_3	low-none	8.0–9.0	≤ 7.0
IgG_4	low-high	7.0–8.0	3.0–6.0
Mouse			
IgG_1	low	8.0–9.0	5.5–7.5
IgG_{2a}	moderate	7.0–8.0	4.5–5.5
IgG_{2b}	high	≈ 7.0	3.5–4.5
IgG_3	low-high	≈ 7.0	4.0–7.0
Rat			
IgG_1	very low	>9.0	7.0–8 .0
IgG_{2a}	low-none	>9.0	≤8.0
IgG_{2b}	low-none	>9.0	≤8.0
IgG_{2c}	very low	8.0–9.0	≤7.0

7.1 Operation

Pack the column as described in section 3.

Wash the packed bed with at least 3 column volumes of binding buffer to remove preservative.

Filter the sample through a 0.2–20.45 μm filter and load the sample (the sample pH should be the same as the binding buffer pH).

Wash the medium with binding buffer until the baseline is stable.

Elute the sample in normal or reversed flow direction.

7.2 Optimizing conditions

Optimizing the method for antibody fractionation with nProtein A Sepharose 4 Fast Flow is best done as follows:

- Pack and equilibrate a small column (or use a HiTrap™ Protein A) in high salt conditions.
- Load a small sample of antibody
- Elute in a linear pH gradient.

Antibodies that elute early can be eluted using linear gradients

Bed dimensions:	1x1.25 cm (1 ml)
Start buffer:	0.05 M boric acid, 0.05 M Na_2PO_4, 0.05 M Na citrate, 4.0 M NaCl, pH 9.0
Elution buffer:	0.05 M boric acid, 0.05 M Na_2PO_4, 0.05 M Na citrate, pH 3.0
Sample:	10 ml filtered cell culture supernatant, equilibrated to buffer A by addition of dry buffers salts and titration with NaOH
Flow rate:	75 cm/h (1 ml/min)
Conditions:	Equilibrate column with 5 column volumes (CV) of starting buffer, apply sample, wash 2 CV starting buffer, elute with a 10 CV linear gradient beginning at 100% start buffer and ending at 100% elution buffer, strip with 5 CV 100% elution buffer
Peak 1:	Bovine IgG
Peak 2:	Monoclonal IgG

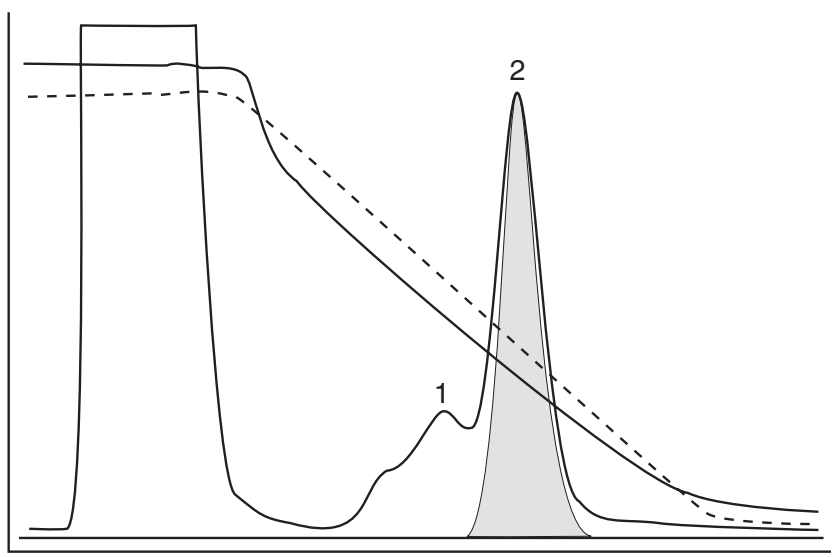

Fig. 6. Analytical scale linear pH gradient elution of mouse IgG_{2a} from nProtein A Sepharose 4 Fast Flow.

since this provides the best and most reproducible fractionation from contaminating antibodies, see Fig. 6.

For antibodies that bind more strongly to protein A, step gradient

Bed dimensions:	5.0x6.3 cm (125 ml)
Starting buffer:	0.05 M boric acid, 0.05 M Na_2PO_4, 0.05 M Na citrate, 4.0 M NaCl, pH 9.0
Elution buffer:	0.05 M boric acid, 0.05 M Na_2PO_4, 0.05 M Na citrate, pH 3.0
Sample:	125 ml filtered ascites, equilibrated to start contitions by direct addition of dry buffers salts and titration with NaOH
Flow rate:	75 cm/h (25 ml/min)
Conditions:	Equilibrate column with 5 column volumes (CV) of starting buffer, apply sample, wash 2 CV starting buffer, elute with a 10 CV linear gradient beginning at 100% start buffer and ending at 100% elution buffer, strip with 5 CV elution buffer

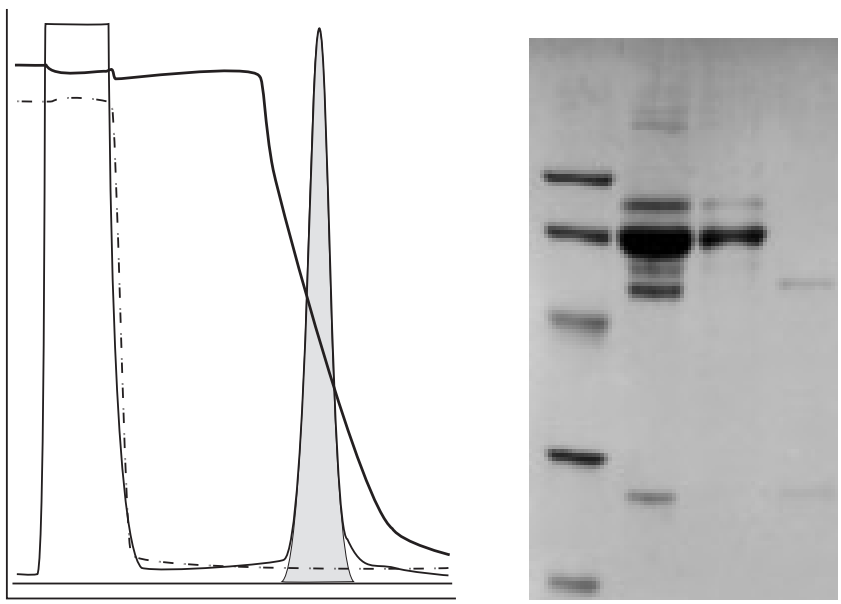

Fig .7. Preparative scale step pH gradient elution of a mouse IgG, from nProtein A Sepharose 4 Fast Flow. Lane 1: LMW stds. Line 2: ascites. Line 3: flow-through. Line 4: purified IgG.

elution is recommended since this shortens the time that the antibody is exposed to the elution conditions, see Fig. 7.

Be sure that the antibody is stable under the elution conditions selected.

If there is any doubt, titrate the antibody fraction to neutrality immediately upon elution in order not to lose biological activity.

To accomplish this, prefill the fraction vessels to 5–10% of the intended fraction volume with a buffer concentrate at about pH 7.5 (for example 1 M Tris-HCl or 1 M sodium phosphate).

Another frequent practice to reduce exposure of the antibody to harsh conditions is to reverse the flow direction during elution. This also elutes the antibody in a more concentrated fraction.

Removal of leached protein A

Leakage of protein A from nProtein A Sepharose 4 Fast Flow is generally low. When such a low level of leakage is of concern e.g. in purification of monoclonal antibodies for clinical applications, a step has to be added to eliminate the possibility of contamination by leached protein. Leached protein A can be removed from the final product in several ways:

- Size exclusion chromatography on Superdex™ 200 prep grade can be performed in low pH conditions in which the particular monoclonal is known to be fully dissociated from protein A. However, exposure of the antibody to low pH conditions increases the risk of loss of activity and aggregation.

Aggregates of protein A-IgG can be removed by chromatography under moderate pH and conductivity conditions (although it may be difficult to achieve quantitative fractionation.

Both examples are illustrated in Fig. 8.

Column: XK 16/70, bed height 60 cm (120 ml)
Sample: 5 ml protein A purified antibody 0.5 mg/ml
Flow rate: 50 cm/h (1.7 ml/min)
Buffer (run A): 0.1 M Na_2PO_4, 0.3 M NaCl, pH 7.0
Buffer (run B): 0.1 M Na acetate, 0.3 M NaCl, pH 4.1

Protein A is indicated by the black bar

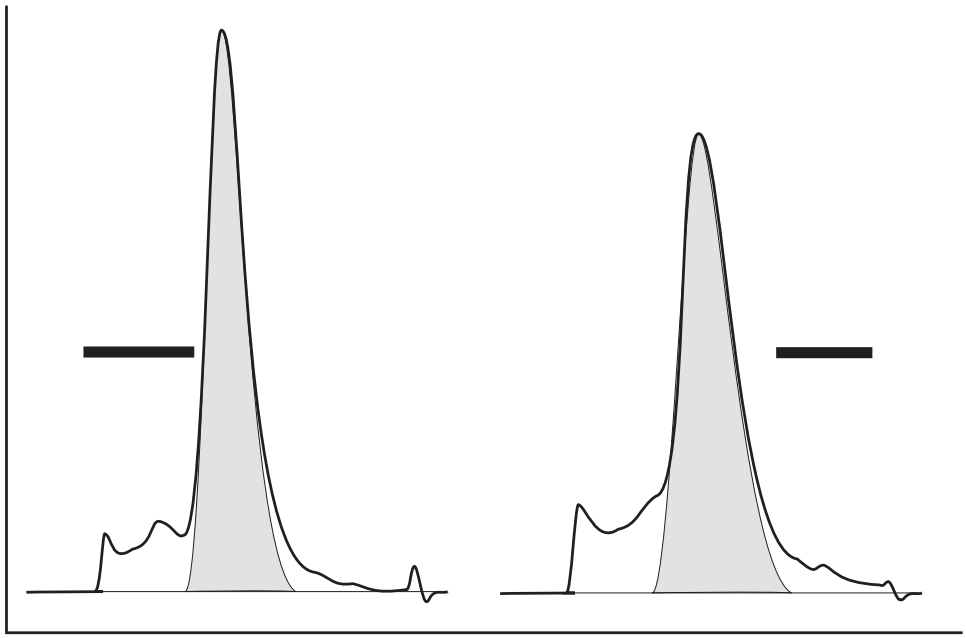

Fig. 8. Removal of leached protein A from mouse IgG2b monoclonal by size exclusion chromatography on Superdex 200 prep grade. Note the higher proportion of aggregates and the broader peak in the sample run at low pH.

- Cation exchange chromatography is an effective tool for removing residual protein A, especially when the particular monoclonal has strong cation exchange binding characteristics. Perform the run at a pH in which the antibody is known to dissociate from protein A, see Fig. 9. Protein A binds poorly to cation exchangers and elutes early in the gradient. The relative speed, capacity and scalability of this technique makes it preferable to size exclusion chromatography at low pH.

Bed dimensions:	1x8 cm (8.5 ml)
Start buffer:	0.05 M MES, pH 5.6
Elution buffer:	0.05 M MES, 1.0 M NaCl, pH 5.6
Sample:	6 ml protein A purified antibody 0.5 mg/ml
Flow rate:	200 cm/h (4.2 ml/min)
Conditions:	Equilibrate column with CV of start buffer, apply sample, wash 5 CV starting buffer, elute with a 10 CV linear gradient beginning at 100% start buffer and ending at 25% elution buffer, strip with 5 CV 100% elution buffer

Protein A is indicated by the black bar

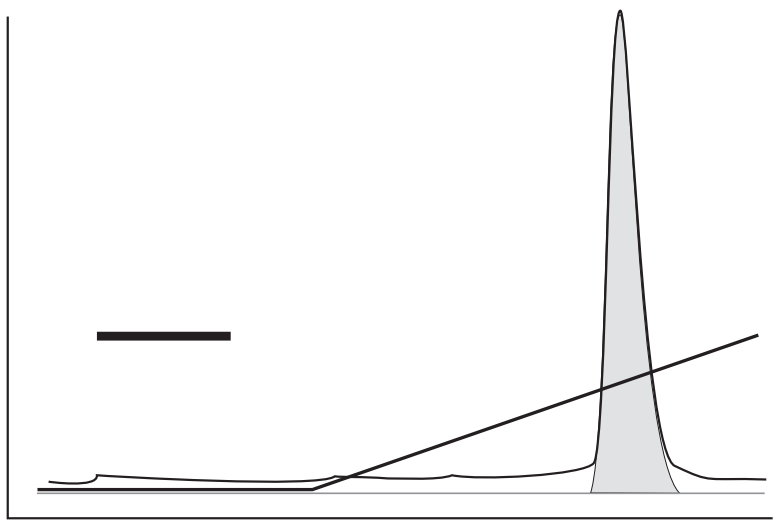

Fig. 9. Removal of leached protein A from mouse IgG, monoclonal by cation exchange chromatography on S Sepharose High Performance.

- Anion exchange works best with antibodies that are retained weakly on anion exchangers. Since protein A binds strongly to anion exchangers, complexes of IgG-protein A are retained more strongly than non-complex antibodies. These complexes do not form separate peaks, but often exhibit a trailing shoulder. To determine the ability of anion exchange chromatography to remove complex protein A, equilibrate the column to 0.05 M Tris, pH 8.6, apply sample and elute in a linear gradient ending at 0.25 M NaCl (0.05 M Tris, pH 8.6). Collect fractions across the antibody peak and screen for protein A.

6.3 Scaling-up

After optimizing the antibody fractionation at laboratory scale, the process can be scaled up. For this, some parameters will change while others remain constant.

- First consider the residence time. This should not be shorter than the time established in the small scale experiments.
- Select bed volume according to required binding capacity.
- Select column diameter to obtain a bed height of 5–30 cm so that high flow rates can be used. (See Fig. 2, pressure/flow rate curve. Max. flow rate is approx. inversely proportional to the bed height. Expect to operate at no more then 70% of the max. flow rate.)
- Keep sample concentration and the ratio of gradient volume/media volume constant.

The larger equipment needed when scaling-up may cause some deviations from the optimized method at small scale. In such cases, check the buffer delivery system and monitoring system for time delays or volume changes. Different lengths and diameters of outlet pipes can cause zone spreading on larger systems.

8. Trouble-shooting

High back-pressure

1. Check that all valves between the pump and the collection vessel are fully open.
2. Check that all valves are clean and free from blockage.
3. Check if equipment in use up to and after the column is generating any back-pressure. (For example valves and flow cells of incorrect dimensions.)
4. Perform CIP to remove tightly bound material from the media.
5. Check column parts such as filters, nets etc., according to the column instruction manual.

Unexpected chromatographic results

1. Check the recorder speed/signal.
2. Check the flow rate.
3. Check the buffers.
4. Check that there are no gaps between the adaptor and the bed, or back-mixing of the sample before application.
5. Check the efficiency of the column packing, see section 4.
6. Check if there have been any changes in the pretreatment of the sample.

Infections

1. Check the connections and prefilters.
2. Check the in-going components such as buffers, sample components, etc.
3. Check that the column has been properly sanitized.

Trapped air

1. Check that the buffers are equilibrated to the same temperature as the packed column.
2. Check that there are no loose connections or leaking valves.

If air has entered the column, the column should be repacked. However, if only a small amount of air has been trapped on top of the bed, or between the adaptor net and head, it can be removed by pumping eluent in the opposite direction. After this, check the efficiency of the packed bed, see section 4, and compare the result with the original efficiency values.

9. Ordering information

Product	Pack size	Code No
nProtein A Sepharose 4 Fast Flow	5 ml	17-5280-01
	25 ml	17-5280-04
	200 ml	17-5280-02
	1 L	17-5280-03
	5 L	17-5280-05
	10 L	17-5280-06
Pre-packed columns:		
HiTrap Protein A	5 x 1 ml	17-0402-01
HiTrap Protein A	1 x 5 ml	17-0403-01

All bulk media products are supplied in suspension in 20% ethanol.

For additional information, including Data File, application references and Regulatory Support File, please contact your local Amersham Biosciences representative.

Reference literature

Adner, N., & Sofer, G. *(1993) BioPharm vol 7 (3), 44–48*

Füglistaller, P. *(1989) J. Immunol. Methods 124, 171–177.*

Griffiths, H., Crisp, F., Henwood, & Rowland, D. *(1993) J. Cellul. Biochem.*

Protein Purification and Engineering, 22nd Keystone Symposia, Wiley-Liss.

For general advice on optimization, scaling-up and other aspects relating to process chromatography we recommend:

Process chromatography - A practical guide. Eds., G.K. Sofer & L.E. Nyström, Academic Press, London, 1989.

Further information

Please read these instructions carefully before using nProtein A Sepharose 4 Fast Flow. For further informations visit www.amershambiosciences.com or contact your local Amersham Biosciences representative.

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